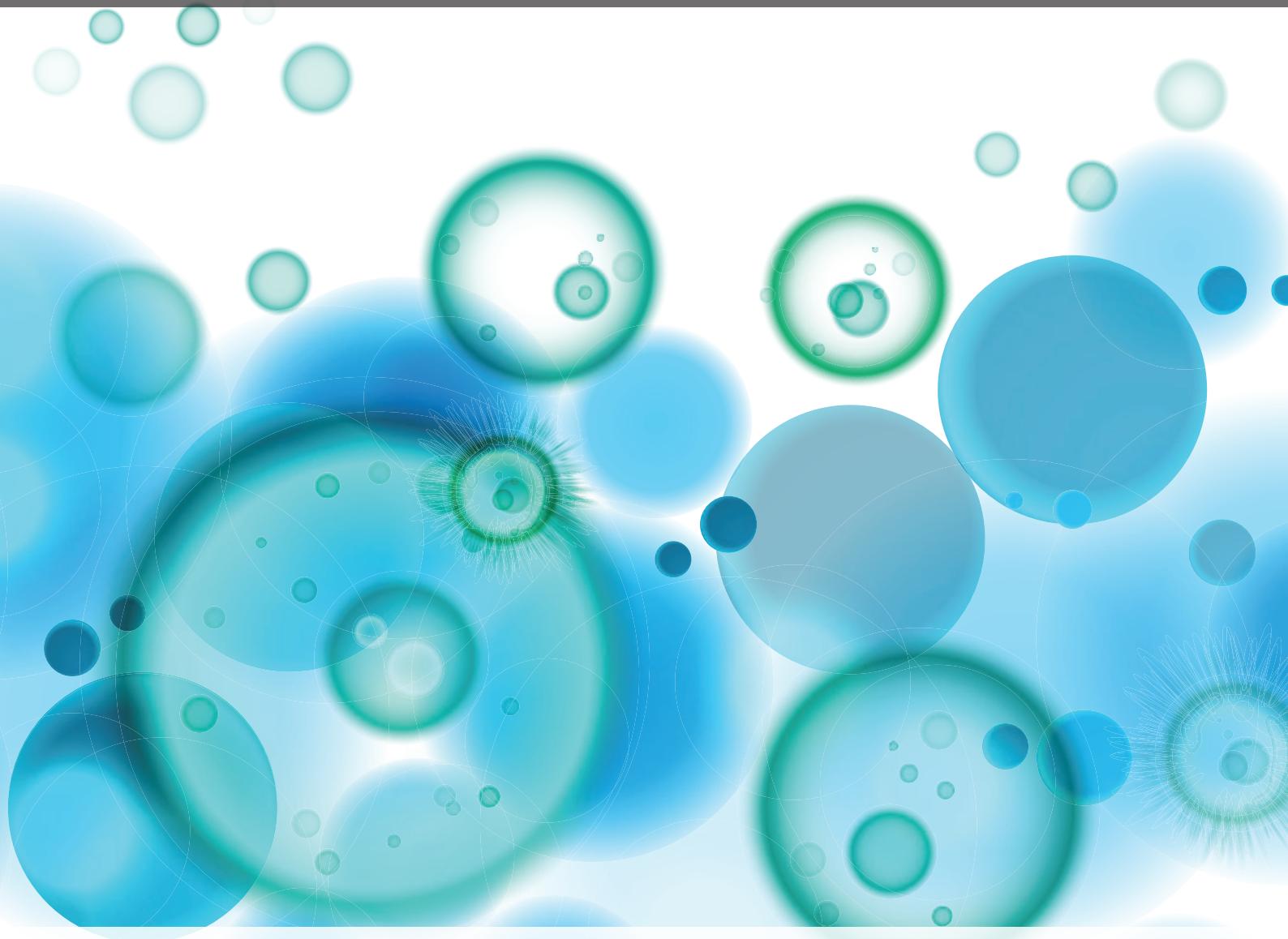


PATTERN RECOGNITION RECEPTORS AND CANCER

EDITED BY: Anton G. Kutikhin and Arseniy E. Yuzhalin
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PATTERN RECOGNITION RECEPTORS AND CANCER

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The group of pattern recognition receptors (PRRs) includes families of Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), and AIM-2-like receptors (ALRs). Conceptually, receptors constituting these families are united by two general features. Firstly, they directly recognize common antigen determinants of virtually all classes of pathogens (so-called pathogen-associated molecular patterns, or simply PAMPs) and initiate immune response against them via specific intracellular signaling pathways. Secondly, they recognize endogenous ligands (since they are usually released during cell stress, they are called damage-associated molecular patterns, DAMPs), and, hence, PRR-mediated immune response can be activated without an influence of infectious agents. So, pattern recognition receptors play the key role performing the innate and adaptive immune response. In addition, many PRRs have a number of other vital functions apart from participation in immune response realization. The fundamental character and diversity of PRR functions have led to amazingly rapid research in this field. Such investigations are very promising for medicine as immune system plays a key role in vast majority if not all human diseases, and the process of discovering the new aspects of the immune system functioning is rapidly ongoing. The role of Toll-like receptors in cancer was analyzed in certain reviews but the data are still scattered. This collection of reviews systematizes the key information in the field.

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Editorial: Pattern recognition receptors and cancer

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Keywords: toll-like receptors, NOD-like receptors, C-type lectin receptors, RIG-I-like receptors, pattern recognition receptors, innate immunity, inflammation, cancer

The problem of cancer remains one of the most immense challenges to current biomedical research. Affecting populations in all countries and all regions, this disease is responsible for millions of deaths annually (1). Evasion of the immune system is an ominous feature of cancers, which often leads to tumor outgrowth, epithelial–mesenchymal transition (EMT), and consequently, metastatic disease. The need to understand basic mechanisms governing immune response to tumors is increasingly acute, since contemporary cancer research gradually progresses toward highly specialized personalized medicine. In this respect, oncoimmunology of pattern recognition receptors (PRRs) is a promising area of research which requires more attention and broader interpretation.

The group of PRRs includes families of toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), and AIM-2-like receptors (ALRs). United by two general features, these receptors are the key players in human immunity. First, they directly recognize antigen determinants of nearly all classes of pathogens [pathogen-associated molecular patterns (PAMPs)] and promote their elimination by triggering innate and adaptive immune response. Second, they recognize endogenous ligands released during cell stress [damage-associated molecular patterns (DAMPs)], and therefore can activate immune response in the absence of an infectious agent. In addition, PRRs are known to possess a number of other vital functions, regulating the processes of apoptosis, DNA repair, autophagy, and angiogenesis. Remarkable functional significance and diversity of biological functions are the reasons why PRRs today are an actively growing area of research.

During the last decade, much research has been done to investigate the role of PRRs in tumor immunity. Accumulating evidence demonstrate that anti-tumor immunity can be stimulated through the activation of PRRs (2, 3). It has been repeatedly shown that reinforced PRR activation may protect the host from infectious agents and prevent, inhibit, or block carcinogenesis whereas disrupted or deregulated functioning of PRRs may promote cancer through weakening the immune system (2, 3). At the same time, PRR activation may stimulate cancer by creating a proinflammatory microenvironment which is favorable for tumor progression and chemoresistance development (4). Furthermore, it may also result in immunosuppression caused by chronic inflammation (2), which is known to promote the development of breast carcinoma, colorectal cancer, pancreatic adenocarcinoma, and possibly several other cancer types (5, 6). In this case, on the contrary, lower PRR activity should minimize effects of chronic inflammation such as enhancement of cancer initiation and promotion/progression and, consequently, decrease probability of tumor development (4). Therefore, the situation resembles a double-edged sword, where both sides can cut unless golden mean is maintained. In this respect, it is clear that a subtle balance of low and high PRR activity is required for proper functioning of the immune system. This hypothesis, initially developed for PRRs (3), may also be successfully projected on PRR intracellular signaling pathways – if their elements are overexpressed/constantly activated, it may lead to consequences similar to that

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of enhanced PRR activation (7, 8). On the other hand, if downstream members of PRR pathways are underexpressed, inactivated, or unable to work properly, it may result in the same effects that of diminished PRR activity, and therefore a balance in functioning of all genes encoding proteins constituting PRR signaling pathways should be preserved for optimal immune system function (7, 8).

Three years ago, four milestone reviews on PRR biology were published in *Immunity* (9–12); we now think that *Frontiers in Immunology* can be an excellent platform for the constellation of review articles systematizing key information in the field with regard to the recent discoveries. With this aim in mind, we invited a number of recognized experts in the field to submit review papers on various aspects of PRR biology and their role in cancer. We sincerely thank all researchers who have agreed to contribute to our Research Topic.

This collection is divided into three sections. The first section describes basic functions of PRRs along with their signaling pathways, and was established with the participation of Taro Kawai and colleagues, Mansi Saxena and Garabet Yeretssian, Huimin Yan and colleagues, together with Stephanie Reikine, Jennifer Nguyen, and Yorgo Modis. We also sought to solicit a number of additional review articles on TLR and NLR biology, since we believe these topics deserve a particular attention. Regarding TLRs, Ajay Jain, Sabina Kaczanowska, and Eduardo Davila depict the newest schemes of the IL-1 receptor-associated kinase signaling, whereas Asif Amin Dar, Rushikesh Sudam Patil, and Shubhada Vivek Chiplunkar provide the insights into the relationship

between TLRs and $\gamma\delta$ T cell response. Readers interested in NLR structure and functioning will definitely appreciate elegant papers by Irving Coy Allen, Julie Magarian Blander, and Andrew Kent together with Silvia Lucena Lage and colleagues. In addition, a brilliant review by Nelson Di Paolo fills a substantial gap in the understanding of the recognition of human oncogenic viruses by PRRs. Finally, Raunaq Singh Nagi, Ashish Bhat, and Himanshu Kumar close up the first section with the description of the general conception on the role of PRRs in cancer development.

The second section is devoted to the role of PRRs in various vital cellular processes, including apoptosis, DNA repair, autophagy, and angiogenesis. It is contributed by Gustavo Amarante-Mendes and colleagues, Anton Kutikhin and colleagues, Ji Eun Oh, and Heung Kyu Lee along with Sheeba Murad.

Finally, the last piece of the collection consists of reviews that comprehensively analyze the impact of PRRs on the development of malignant tumors (esophageal cancer, gastric cancer, colorectal cancer, lung cancer, prostate cancer, breast cancer, ovarian cancer, and lymphoma). Furthermore, Simon Heidegger and colleagues discuss the role of PRRs in graft-versus-host disease and graft-versus-leukemia following allogeneic stem cell transplantation. As a final point, Shanjana Awasthi underlines the importance of TLR agonists in cancer immunotherapy.

We created this Research Topic with the hope that it will be useful for a wide audience, particularly cancer researchers, immunologists, microbiologists, graduate, and undergraduate students of biomedical faculties as well as for their lecturers.

References

1. Stewart BW, Wild CP. *World Cancer Report 2014*. Lyon: International Agency for Research on Cancer (2014). 512 p.
2. Tsan MF. Toll-like receptors, inflammation, and cancer. *Semin Cancer Biol* (2006) **16**:32–7. doi:10.1016/j.semcaner.2005.07.004
3. Killeen SD, Wang JH, Andrews EJ, Redmond HP. Exploitation of the toll-like receptor system in cancer: a doubled-edged sword? *Br J Cancer* (2006) **95**:247–52. doi:10.1038/sj.bjc.6603275
4. Chen R, Alvero AB, Silasi DA, Mor G. Inflammation, cancer and chemoresistance: taking advantage of the toll-like receptor signaling pathway. *Am J Reprod Immunol* (2007) **57**:93–107. doi:10.1111/j.1600-0897.2006.00441.x
5. Okamoto M, Sato M. Toll-like receptor signaling in anti-cancer immunity. *J Med Invest* (2003) **50**:9–24.
6. Kinlen L. Infections and immune factors in cancer: the role of epidemiology. *Oncogene* (2004) **23**:6341–8. doi:10.1038/sj.onc.1207898
7. Kutikhin AG, Yuzhalin AE. C-type lectin receptors and RIG-I-like receptors: new points on the oncogenomics map. *Cancer Manag Res* (2012) **4**:39–53. doi:10.2147/CMAR.S28983
8. Kutikhin AG, Yuzhalin AE. Inherited variation in pattern recognition receptors and cancer: dangerous liaisons? *Cancer Manag Res* (2012) **4**:31–8. doi:10.2147/CMAR.S28688
9. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* (2011) **34**:637–50. doi:10.1016/j.immuni.2011.05.006
10. Elinav E, Strowig T, Henao-Mejia J, Flavell RA. Regulation of the antimicrobial response by NLR proteins. *Immunity* (2011) **34**:665–79. doi:10.1016/j.immuni.2011.05.007
11. Osorio F, Reis e Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity* (2011) **34**:651–64. doi:10.1016/j.immuni.2011.05.001
12. Loo YM, Gale M Jr. Immune signaling by RIG-I-like receptors. *Immunity* (2011) **34**:680–92. doi:10.1016/j.immuni.2011.05.003

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Toll-like receptor signaling pathways

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Toll-like receptors (TLRs) play crucial roles in the innate immune system by recognizing pathogen-associated molecular patterns derived from various microbes. TLRs signal through the recruitment of specific adaptor molecules, leading to activation of the transcription factors NF- κ B and IRFs, which dictate the outcome of innate immune responses. During the past decade, the precise mechanisms underlying TLR signaling have been clarified by various approaches involving genetic, biochemical, structural, cell biological, and bioinformatics studies. TLR signaling appears to be divergent and to play important roles in many aspects of the innate immune responses to given pathogens. In this review, we describe recent progress in our understanding of TLR signaling regulation and its contributions to host defense.

Keywords: TLRs, signal transduction, NF- κ B, IRFs, adaptors

INTRODUCTION

The innate immune system employs germline-encoded pattern-recognition receptors (PRRs) for the initial detection of microbes. PRRs recognize microbe-specific molecular signatures known as pathogen-associated molecular patterns (PAMPs) and self-derived molecules derived from damaged cells, referred as damage-associated molecules patterns (DAMPs). PRRs activate downstream signaling pathways that lead to the induction of innate immune responses by producing inflammatory cytokines, type I interferon (IFN), and other mediators. These processes not only trigger immediate host defensive responses such as inflammation, but also prime and orchestrate antigen-specific adaptive immune responses (1). These responses are essential for the clearance of infecting microbes as well as crucial for the consequent instruction of antigen-specific adaptive immune responses.

Mammals have several distinct classes of PRRs including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), AIM2-like receptors (ALRs), C-type lectin receptors (CLRs), and intracellular DNA sensors such as cGAS (2, 3). Among these, TLRs were the first to be identified, and are the best characterized. The TLR family comprises 10 members (TLR1–TLR10) in human and 12 (TLR1–TLR9, TLR11–TLR13) in mouse. TLRs localize to the cell surface or to intracellular compartments such as the ER, endosome, lysosome, or endolysosome, and they recognize distinct or overlapping PAMPs such as lipid, lipoprotein, protein, and nucleic acid. Each TLR is composed of an ectodomain with leucine-rich repeats (LRRs) that mediate PAMPs recognition, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain that initiates downstream signaling. The ectodomain displays a horseshoe-like structure, and TLRs interact with their respective PAMPs or DAMPs as a homo- or heterodimer along with a co-receptor or accessory molecule (4). Upon PAMPs and DAMPs recognition, TLRs

recruit TIR domain-containing adaptor proteins such as MyD88 and TRIF, which initiate signal transduction pathways that culminate in the activation of NF- κ B, IRFs, or MAP kinases to regulate the expression of cytokines, chemokines, and type I IFNs that ultimately protect the host from microbial infection. Recent studies have revealed that proper cellular localization of TLRs is important in the regulation of the signaling, and that cell type-specific signaling downstream of TLRs determines particular innate immune responses. Here, we summarize recent progress on TLR signaling pathways and their contributions to host defense responses.

PAMP RECOGNITION BY TLRs

TLRs are expressed in innate immune cells such as dendritic cells (DCs) and macrophages as well as non-immune cells such as fibroblast cells and epithelial cells. TLRs are largely classified into two subfamilies based on their localization, cell surface TLRs and intracellular TLRs. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, whereas intracellular TLRs are localized in the endosome and include TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 (5, 6).

Cell surface TLRs mainly recognize microbial membrane components such as lipids, lipoproteins, and proteins. TLR4 recognizes bacterial lipopolysaccharide (LPS). TLR2 along with TLR1 or TLR6 recognizes a wide variety of PAMPs including lipoproteins, peptidoglycans, lipoteichoic acids, zymosan, mannan, and tGPI-mucin (5). TLR5 recognizes bacterial flagellin (2). TLR10 is pseudogene in mouse due to an insertion of a stop codon, but human TLR10 collaborates with TLR2 to recognize ligands from listeria (7). TLR10 can also sense influenza A virus infection (8).

Intracellular TLRs recognize nucleic acids derived from bacteria and viruses, and also recognize self-nucleic acids in disease

conditions such as autoimmunity (9). TLR3 recognizes viral double-stranded RNA (dsRNA), small interfering RNAs, and self-RNAs derived from damaged cells (10–12). TLR7 is predominantly expressed in plasmacytoid DCs (pDCs) and recognizes single-stranded (ss)RNA from viruses. It also recognizes RNA from streptococcus B bacteria in conventional DCs (cDCs) (13). Human TLR8 responds to viral and bacterial RNA (14). Structural analysis revealed that unstimulated human TLR8 exists as a preformed dimer, and although the Z-loop between LRR14 and LRR15 is cleaved, the N- and C-terminal halves remain associated with each other and participate in ligand recognition and dimerization. Ligand binding induces reorganization of the dimer to bring the two C termini into close proximity (15). TLR13 recognizes bacterial 23S rRNA (16–18) and unknown components of vesicular stomatitis virus (19). TLR9 recognizes bacterial and viral DNA that is rich in unmethylated CpG-DNA motifs; it also recognizes hemozoin, an insoluble crystalline byproduct generated by *Plasmodium falciparum* during the process of detoxification after host hemoglobin is digested (20). TLR11 is localized in the endolysosome and recognizes flagellin (21) or an unknown proteinaceous component of uropathogenic *Escherichia coli* (UPEC) as well as a profilin-like molecule derived from *Toxoplasma gondii* (22). TLR12 is predominantly expressed in myeloid cells and is highly similar to TLR11 and recognizes profilin from *T. gondii* (23). TLR12 functions either as a homodimer or a heterodimer with TLR11 (24, 25).

TRAFFICKING OF TLRs

All TLRs are synthesized in the ER, traffic to the Golgi, and are recruited to the cell surface or to intracellular compartments such as endosomes. Intracellular localization of TLRs is thought to be critical for ligand recognition as well as for preventing TLRs from coming into contact with self-nucleic acids, which could cause autoimmunity (26–29). The multi-pass transmembrane protein UNC93B1 controls the trafficking of intracellular TLRs from the ER to endosomes. Interestingly, UNC93B1 regulates excessive TLR7 activation by employing TLR9 to counteract TLR7. This was demonstrated by experiments in mice harboring an amino acid substitution (D34A) in UNC93B1, which exhibit a TLR7-hyperreactive and TLR9-hyporeactive phenotype associated with TLR7-dependent systemic lethal inflammation. Thus, optimizing the balance between TLR7 and TLR9 is a potential mechanism for regulating autoimmunity (30). TLR trafficking is also controlled by the ER-resident protein PRAT4A, which regulates the exit of TLR1, TLR2, TLR4, TLR7, and TLR9 from the ER and their trafficking to the plasma membrane and endosomes (31). gp96, a member of the ER-resident heat-shock protein 90 family, functions as a general chaperone for most TLRs, including cell surface TLR1, TLR2, TLR4, and TLR5 and intracellular TLR7 and TLR9 (32).

In the endosome, nucleic acid-sensing TLRs undergo proteolytic cleavage by cathepsins B, S, L, H, and K and asparginyl endopeptidase to attain a functional form that mediates ligand recognition and initiates signaling (33–35). However, the N-terminal region of TLR9 is required for CpG-DNA recognition and binding (36). Interestingly, a recent study suggests that the N-terminal cleaved fragment (TLR9N) remains associated with truncated TLR9 (TLR9C) to form a complex, which acts as a functional DNA sensor (37).

CONTRIBUTION OF TIR DOMAIN-CONTAINING ADAPTORS TO TLR SIGNALING

Individual TLRs differentially recruit members of a set of TIR domain-containing adaptors such as MyD88, TRIF, TIRAP/MAL, or TRAM. MyD88 is utilized by all TLRs and activates NF- κ B and MAPKs for the induction of inflammatory cytokine genes. TIRAP is a sorting adaptor that recruits MyD88 to cell surface TLRs such as TLR2 and TLR4 (Figure 1). However, a recent study demonstrated that TIRAP also participates in signaling through endosomal TLRs such as TLR9. The lipid-binding domain of TIRAP binds to PI(4,5)P₂ at the plasma membrane and to PI(3)P on endosomes, which mediates the formation of functional TLR4 and TLR9 signaling complexes at their respective sites. Thus, TIRAP associates with both cell surface and endosomal TLRs by binding to different lipids (38). However, a high concentration of TLR9 agonists activates cells in the absence of TIRAP, suggesting that TIRAP is required for TLR9 signaling in natural situations such as HSV-1 infection (39).

TRIF is recruited to TLR3 and TLR4 and promotes an alternative pathway that leads to the activation of IRF3, NF- κ B, and MAPKs for induction of type I IFN and inflammatory cytokine genes. TRAM is selectively recruited to TLR4 but not TLR3 to link between TRIF and TLR4. TLR3 directly interacts with TRIF, and this interaction requires phosphorylation of the two tyrosine residues in the cytoplasmic domain of TLR3 by the epidermal growth factor ErbB1 and Btk (40, 41). Collectively, depending on the adaptor usage, TLR signaling is largely divided into two pathways: the MyD88-dependent and TRIF-dependent pathways.

MyD88-DEPENDENT PATHWAY

After TLR engagement, MyD88 forms a complex with IRAK kinase family members, referred to as the Myddosome (Figure 1) (42). During Myddosome formation, IRAK4 activates IRAK1, which is then autophosphorylated at several sites (43) and released from MyD88 (44). IRAK1 associates with the RING-domain E3 ubiquitin ligase TRAF6. TRAF6, along with ubiquitin-conjugating enzyme UBC13 and UEV1A, promotes K63-linked polyubiquitination of both TRAF6 itself and the TAK1 protein kinase complex. TAK1 is a member of the MAPKKK family and forms a complex with the regulatory subunits TAB1, TAB2, and TAB3, which interact with polyubiquitin chains generated by TRAF6 to drive TAK1 activation (45, 46). Although the mechanisms of TAK1 activation within this complex remain unclear, K63-linked ubiquitination or close proximity-dependent transphosphorylation may be responsible for TAK1 activation. TAK1 then activates two different pathways that lead to activation of the IKK complex-NF- κ B pathway and -MAPK pathway. The IKK complex is composed of the catalytic subunits IKK α and IKK β and the regulatory subunit NEMO (also called IKK γ). TAK1 binds to the IKK complex through ubiquitin chains, which allows it to phosphorylate and activate IKK β . The IKK complex phosphorylates the NF- κ B inhibitory protein I κ B α , which undergoes proteasome degradation, allowing NF- κ B to translocate into the nucleus to induce proinflammatory gene expression. TAK1 activation also results in activation of MAPK family members such as ERK1/2, p38 and JNK, which mediates activation of AP-1

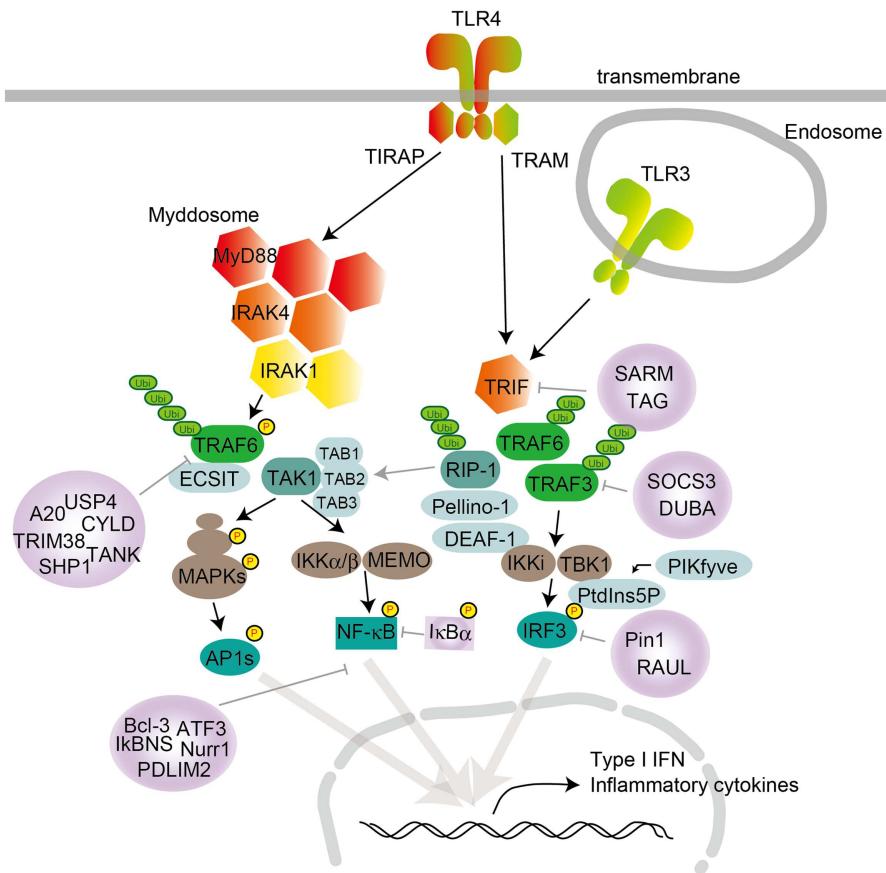


FIGURE 1 | TLR signaling in cDCs, macrophages, and MEFs. TLR4 localize to the cell surface, and TLR3 localize in the endosome compartment. Homodimer or heterodimer formation initiates signaling to the two major downstream adaptor proteins, MyD88 and TRIF. TIRAP conducts the signal from TLR4 to MyD88, and TRAM mediates the signal from TLR4 to TRIF. TLR engagement induces formation of the Myddosome, which is based on MyD88 and also contains IRAK1 and IRAK4. IRAK1 activation induces TRAF6 activation following K63-linked polyubiquitination on TRAF6 itself and TAK1. TAK1 activation leads to the activation of IKK complex-NF- κ B and MAPKs. MAPK activation leads to AP1s transcription factor activation. TRAF6 promotes ECSIT ubiquitination, resulting in increased mitochondrial and cellular ROS generation. TLR engagement also induces TRIF activation following TRAF6

and TRAF3 recruitment. TRAF6 recruits RIP-1, which activates the TAK1 complex following MAPK activation. RIP-1 activation regulates ubiquitination by Pellino-1. Pellino-1 regulates IRF3 activation by binding to DEAF-1. TRAF3 recruits TBK1 and IKK α for IRF3 phosphorylation. PtdIns5P from PIKfyve facilitates complex formation between TBK1 and IRF3. Several negative regulators modulate TLR signaling, by inhibiting either signaling complex formation or ubiquitination. MyD88 is suppressed by ST2825, NRDP-1, SOCS3, and Cbl-b; TRIF is suppressed by SARM and TAG; TRAF3 is suppressed by SOCS3 and DUBA; and TRAF6 is suppressed by A20, USP4, CYLD, TANK, TRIM38, and SHP. NF- κ B is suppressed by Bcl-3, I κ BNS, Nurr1, ATF3, and PDLIM2, while IRF3 activation is negatively regulated by Pin1 and RAUL.

family transcription factors or stabilization of mRNA to regulate inflammatory responses (2, 5).

TAK1 deficiency in mouse embryonic fibroblast cells (MEFs) reduces phosphorylation of IKKs, p38, and JNK after LPS stimulation. However, TLR4-mediated IKK, p38, and JNK activation and cytokine induction are increased in neutrophils derived from TAK1-deficient mice, suggesting a cell type-specific role for TAK1 in TLR signaling (47). Furthermore, the physiological roles of TAB proteins in TLR signaling also remain controversial: TAB1- or TAB2-deficient mice do not show any abnormality in TLR signaling pathways (48), and mice doubly deficient for TAB2 and TAB3 also exhibit normal cytokine production after TLR simulation in MEFs and macrophages (49). TAB family proteins may therefore compensate for each other in TLR signaling.

TLR2 and TLR4 ligations in macrophages increase the production of mitochondrial ROS for bactericidal action and recruit mitochondria to phagosomes (50). TRAF6 is translocated to mitochondria following bacterial infection, where it interacts with ECSIT. TRAF6 promotes ECSIT ubiquitination, resulting in increased mitochondrial and cellular ROS generation.

TRIF-DEPENDENT PATHWAY

TRIF interacts with TRAF6 and TRAF3. TRAF6 recruits the kinase RIP-1, which in turn interacts with and activates the TAK1 complex, leading to activation of NF- κ B and MAPKs and induction of inflammatory cytokines (Figure 1). In contrast, TRAF3 recruits the IKK-related kinases TBK1 and IKK α along with NEMO for IRF3 phosphorylation. Subsequently, IRF3 forms a dimer and translocates into the nucleus from

the cytoplasm, where it induces the expression of type I IFN genes (2, 5).

The Pellino family E3 ubiquitin ligases are implicated in TLR signaling (51). Pellino-1-deficient mice display impaired TRIF-dependent NF-κB activation and cytokine production (52). Pellino-1 is phosphorylated by TBK1/IKK α and thereby facilitates ubiquitination of RIP-1, suggesting that Pellino-1 mediates TRIF-dependent NF-κB activation by recruiting RIP-1. Furthermore, Pellino-1 regulates IRF3 activation by binding to DEAF-1, a transcription factor that facilitates binding of IRF3 to the IFN β promoter (51).

Recently, IRF3 activation was demonstrated to be regulated by an inositol lipid, PtdIns5P. PtdIns5P binds to both IRF3 and TBK1, and thus facilitates complex formation between TBK1 and IRF3. The accessibility of TBK1 to IRF3 mediated by PtdIns5P likely causes IRF3 phosphorylation in a closely proximal manner. Furthermore, PIKfyve was identified as a kinase responsible for production of PtdIns5P during virus infection (53).

BALANCED ACTIVATION BETWEEN MyD88- AND TRIF-DEPENDENT PATHWAYS

TLR4 activates both the MyD88-dependent and TRIF-dependent pathways. Activation of these pathways is controlled by several molecules to induce appropriate responses. Balanced production

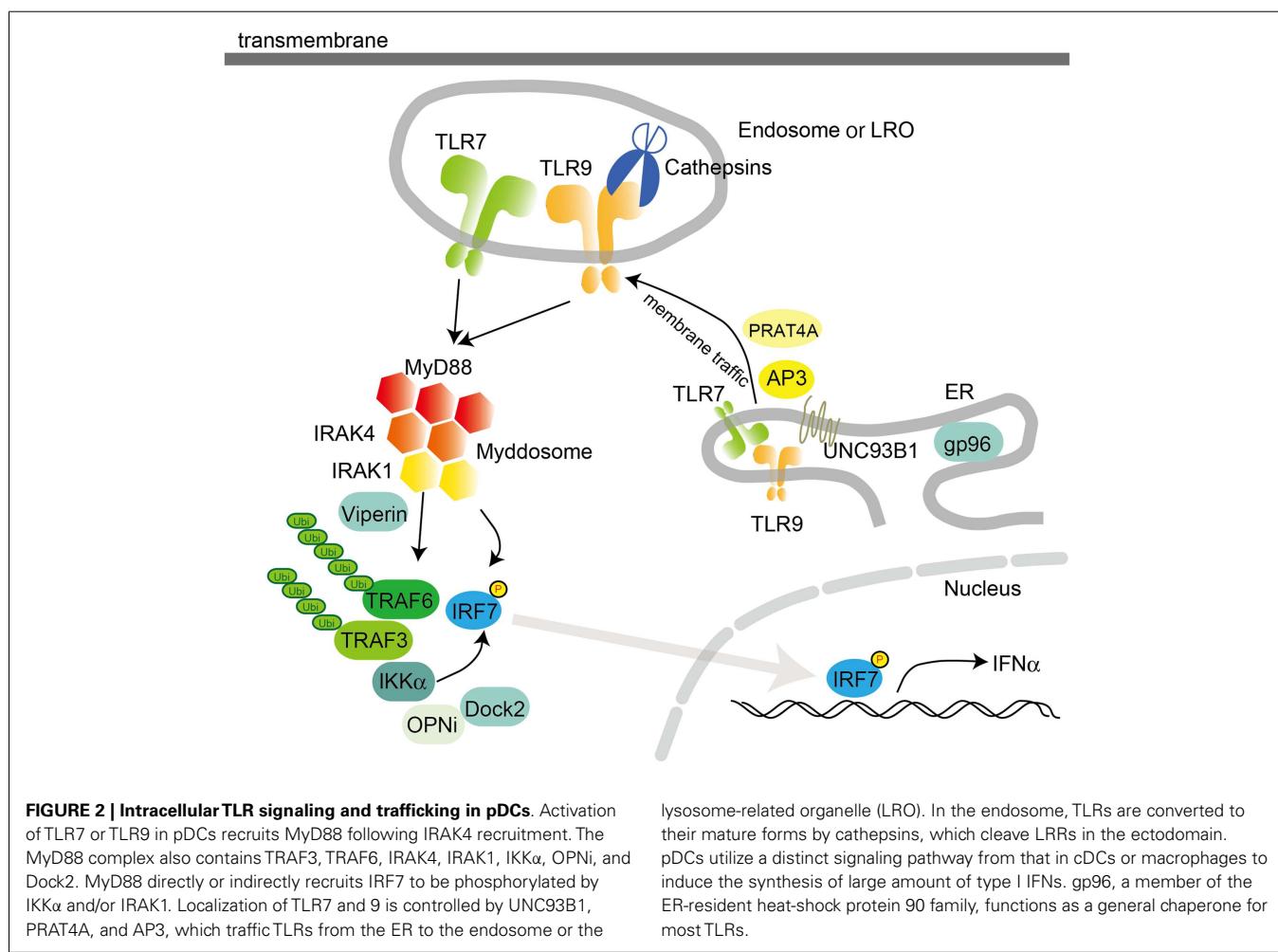
of inflammatory cytokines and type I IFN may be important for controlling tumor cell growth and autoimmune diseases.

TRAF3 was shown to be incorporated into the MyD88 complex as well as the TRIF complex in TLR4 signaling. TRAF3 within the MyD88 complex is then degraded, which causes TAK1 activation. Thus, in addition its role in promoting TRIF-dependent pathway activation, TRAF3 has a role in inhibiting the MyD88-dependent pathway. NRD β -1, an E3 ubiquitin ligase, binds and ubiquitinates MyD88 and TBK1, inducing the degradation of MyD88 and augmenting the activation of TBK1, which attenuates inflammatory cytokine production and induces preferential type I IFN production, respectively (54).

MHC class II molecules that are localized in endosomes in antigen-presenting cells interact with the tyrosine kinase Btk via the costimulatory molecule CD40 and maintain Btk activation. Activated Btk interacts with MyD88 and TRIF to promote the activation of the MyD88-dependent and TRIF-dependent pathways and thus to enhance production of inflammatory cytokines and type I IFNs, respectively (55).

TLR7 AND TLR9 SIGNALING IN PLASMACYTOID DCs

Plasmacytoid DCs are a subset of DCs with the capacity to secrete vast amounts of type I IFN in response to viral infection (Figure 2) (2, 5). In pDCs, TLR7 and TLR9 serve as primary sensors for



RNA and DNA viruses, respectively. Interestingly, the production of type I IFN by pDCs relies on a complex containing MyD88 and IRF7. This complex also contains TRAF3, TRAF6, IRAK4, IRAK1, IKK α , OPNi, and Dock2 (56, 57). Within this complex, IRF7 is phosphorylated by IRAK1 and/or IKK α and translocates into the nucleus to regulate the expression of type I IFN. Moreover, MyD88-IRAK4-TRAF6 complex drives NF- κ B-dependent inflammatory cytokine induction. The signaling complex containing MyD88-IRAK1-TRAF6-IRF7 is formed within lipid bodies by the IFN-inducible Viperin, which activates IRAK1 by lysine 63-linked ubiquitination (58). It is notable that TLR9 signals through different cellular compartments that induce either MyD88-IRF7-dependent type I IFN or MyD88- NF- κ B -dependent inflammatory cytokines (59). TLR9 initially traffics to VAMP3-positive early endosomes after CpG-DNA stimulation, where it triggers MyD88-IRAK4-TRAF6-dependent NF- κ B activation. TLR9 then traffics to LAMP2-positive lysosome-related organelles (LROs), where it incorporates TRAF3 to activate IRF7 and induce type I IFN (Figure 2). AP3 has been shown to bind to TLR9 and control the trafficking of TLR9 to LROs, and is required for type I IFN induction (28). However, AP3 is not required for TLR9-dependent type I IFN induction triggered by DNA-antibody immune complexes (ICs) in pDCs. The intracellular compartment initiating type I IFN induction by DNA-antibody ICs is regulated by the autophagy pathway (60). Thus, pDCs have diverse cargoes for ligand recognition and triggering downstream signaling pathways.

OTHER IRFs IN TLR SIGNALING

In addition to IRF3 and IRF7, several other IRFs participate in TLR signaling. IRF1 interacts with MyD88 and contributes to TLR9-mediated cytokine production in the presence of IFN γ (61), while IRF5 is involved in the MyD88-dependent signaling pathway for inducing inflammatory cytokine production (62). IRF8 was proposed to be essential for TLR9-MyD88-dependent activation of NF- κ B in pDCs (63). However, a subsequent analysis of IRF8-deficient mice demonstrated that IRF8 is involved in the second phase of feedback type I IFN production after treatment of DCs with TLR agonists (64).

ACTIVATION OF TLR SIGNALING BY CO-RECEPTORS

Recent studies have identified several transmembrane molecules that modulate TLR signaling pathways. CD14, a glycophosphatidylinositol-anchored protein, is a co-receptor with TLR4 and MD-2 for LPS recognition. It induces ITAM-mediated Syk- and PLC γ 2-dependent endocytosis to promote TLR4 internalization into endosomes for activation of TRIF-dependent signaling (65). CD14 is also required for TLR7- and TLR9-dependent induction of proinflammatory cytokines (66).

CD36, a protein in the class B scavenger receptor family, acts as a co-receptor for oxidized low-density lipoprotein (LDL) and amyloid- β peptide. Ligand recognition induces the assembly of TLR4/TLR6 heterodimers through Src kinases and consequent sterile inflammation, by inducing inflammatory cytokines and ROS and priming NLRP3 inflammasome activation (67, 68).

NEGATIVE REGULATORS

TLR signaling is negatively regulated by a number of molecules through various mechanisms to prevent or terminate the excessive immune responses that lead to detrimental consequences associated with autoimmunity and inflammatory diseases. Negative regulators target each of the key molecules in TLR signaling (Figure 1). Activation of the MyD88-dependent pathway is suppressed by ST2825, SOCS1, and Cbl-b, and activation of the TRIF-dependent pathway is suppressed by SARM and TAG (69, 70). These molecules associate with MyD88 or TRIF to prevent them from binding to TLRs or downstream molecules. TRAF3 activation is negatively regulated by SOCS3 and DUBA (71). TRAF6 is targeted by a number of inhibitory molecules such as A20, USP4, CYLD, TANK, TRIM38, and SHP (72–74). TAK1 activation is inhibited by TRIM30 α and A20 (75). In addition to these signaling molecules, the transcription factor NF- κ B is suppressed by Bcl-3, I κ BNS, Nurr1, ATF3, and PDLIM2, while IRF3 activation is negatively regulated by Pin1 and RAUL (76). The stability of mRNAs encoding signaling molecules is regulated by miRNAs such as miR-146a, miR-199a, miR-155, miR-126, miR-21, miR-29, miR-148/152, and miR-466l (74). In addition to the stability of mRNAs for signaling molecules, stability of mRNA for cytokines is regulated by Regnase-1 and TTP (5, 74).

CONCLUDING REMARKS

During the past decade, tremendous progress has been made in our understanding of TLR signaling pathways. After genetic studies revealed the contribution of TIR domain-containing adaptor usage, cell biological and biochemical approaches have highlighted the importance of cellular localization of these adaptors in the regulation of downstream signaling. Moreover, numerous reports have demonstrated that TLR trafficking, TLR cleavage, and protein modification of signaling molecules such as ubiquitination and phosphorylation play important roles in the activation of TLR signaling. On the other hand, negative regulators of TLR signaling have been discovered, and their importance in preventing autoimmune and inflammatory diseases is recognized. More recently, much effort has been focused on identifying molecules that are involved in innate immunity through an integrated approach. Indeed, by combining transcriptomics, genetic/chemical perturbations and phosphoproteomics, Polo-like kinases (Plks) 2 and 4 have been found to regulate antiviral responses downstream of TRIF and MyD88 signaling (77). mRNA stability has also attracted attention because it is an important mechanism to regulate TLR-dependent inflammation. For example, the RNase Regnase-1 interacts with IL-6 and IL-12p40 mRNA and degrades them. Regnase-1-deficient macrophages produce large amounts of cytokines after treatment with various TLR ligands, and Regnase-1-deficient mice show elevated autoantibody production (78). Furthermore, it is notable that PAMP variants may activate distinct signaling pathways although they are recognized by the same PRRs. For example, LPS variant such as smooth or rough type activates either MyD88-dependent or TRIF-dependent pathway. These findings suggest that host makes a distinction between different types of LPS-containing bacteria by activating distinct signaling pathways (79).

Although PAMP recognition by TLRs is crucial for host defense responses to pathogen infection, aberrant activation of TLR signaling by PAMPs, mutations of TLR signaling molecules, and DAMPs-mediated TLRs signaling activation are responsible for the development of several diseases such as autoimmune, chronic inflammatory, and allergic diseases. Moreover, a link between cancer and TLRs has been proposed. The innate immune activation that caused after anti-cancer drug treatment is reportedly critical for cancer elimination through TLR-mediated recognition of endogenous molecules released from dying cancer cells (80). On the contrary, mutations in molecules involved in TLR signaling are associated with cancer development. Certain types of diffuse large B-cell lymphoma acquire oncogenic ability through MyD88 mutation and show aberrant activation of NF- κ B, JAK and STAT3 (81). A mutation in A20, which is a negative regulator of TLR signaling, is also associated with B-cell lymphoma development (82, 83). Furthermore, it has been suggested that TBK1 functions as a negative regulator of cell growth in lung cancer (84). In summary, further elucidation of TLR signaling pathways should eventually allow us to manipulate them in strategies to treat various infectious and autoimmune diseases that are intimately associated with innate immune signaling, as well as cancer.

REFERENCES

- Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* (2002) **20**:197–216. doi:10.1146/annurev.immunol.20.083001.084359
- Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* (2006) **124**:783–801. doi:10.1016/j.cell.2006.02.015
- Cai X, Chiu YH, Chen ZJ. The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling. *Mol Cell* (2014) **54**:289–96. doi:10.1016/j.molcel.2014.03.040
- Botos I, Segal DM, Davies DR. The structural biology of toll-like receptors. *Structure* (2011) **19**:447–59. doi:10.1016/j.str.2011.02.004
- Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. *Nat Immunol* (2010) **11**:373–84. doi:10.1038/ni.1863
- Celhar T, Magalhaes R, Fairhurst AM. TLR7 and TLR9 in SLE: when sensing self goes wrong. *Immunol Res* (2012) **53**:58–77. doi:10.1007/s12026-012-8270-1
- Regan T, Nally K, Carmody R, Houston A, Shanahan F, Macsharry J, et al. Identification of TLR10 as a key mediator of the inflammatory response to listeria monocytogenes in intestinal epithelial cells and macrophages. *J Immunol* (2013) **191**:6084–92. doi:10.4049/jimmunol.1203245
- Lee SM, Kok KH, Jaume M, Cheung TK, Yip TF, Lai JC, et al. Toll-like receptor 10 is involved in induction of innate immune responses to influenza virus infection. *Proc Natl Acad Sci USA* (2014) **111**:3793–8. doi:10.1073/pnas.1324266111
- Blasius AL, Beutler B. Intracellular toll-like receptors. *Immunity* (2010) **32**:305–15. doi:10.1016/j.immuni.2010.03.012
- Zhang SY, Jouanguy E, Ugolini S, Smahi A, Elain G, Romero P, et al. TLR3 deficiency in patients with herpes simplex encephalitis. *Science* (2007) **317**:1522–7. doi:10.1126/science.1139522
- Bernard JJ, Cowing-Zitron C, Nakatsui T, Muehleisen B, Muto J, Borkowski AW, et al. Ultraviolet radiation damages self noncoding RNA and is detected by TLR3. *Nat Med* (2012) **18**:1286–90. doi:10.1038/nm.2861
- Takemura N, Kawasaki T, Kunisawa J, Sato S, Lamichhane A, Kobiyama K, et al. Blockade of TLR3 protects mice from lethal radiation-induced gastrointestinal syndrome. *Nat Commun* (2014) **5**:3492. doi:10.1038/ncomms4492
- Mancuso G, Gambuzza M, Midiri A, Biondo C, Papasergi S, Akira S, et al. Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells. *Nat Immunol* (2009) **10**:587–94. doi:10.1038/ni.1733
- Guiducci C, Gong M, Cepika AM, Xu Z, Tripodo C, Bennett L, et al. RNA recognition by human TLR8 can lead to autoimmune inflammation. *J Exp Med* (2013) **210**:2903–19. doi:10.1084/jem.20131044
- Tanji H, Ohto U, Shibata T, Miyake K, Shimizu T. Structural reorganization of the toll-like receptor 8 dimer induced by agonistic ligands. *Science* (2013) **339**:1426–9. doi:10.1126/science.1229159
- Hidmark A, Paul A, Dalpke AH. Cutting edge: TLR13 is a receptor for bacterial RNA. *J Immunol* (2012) **189**:2717–21. doi:10.4049/jimmunol.1200898
- Li XD, Chen ZJ. Sequence specific detection of bacterial 23S ribosomal RNA by TLR13. *eLife* (2012) **1**:e00102. doi:10.7554/eLife.00102
- Oldenburg M, Kruger A, Ferstl R, Kaufmann A, Nees G, Sigmund A, et al. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. *Science* (2012) **337**:1111–5. doi:10.1126/science.1220363
- Shi Z, Cai Z, Sanchez A, Zhang T, Wen S, Wang J, et al. A novel toll-like receptor that recognizes vesicular stomatitis virus. *J Biol Chem* (2011) **286**:4517–24. doi:10.1074/jbc.M110.159590
- Coban C, Igari Y, Yagi M, Reimer T, Koyama S, Aoshi T, et al. Immunogenicity of whole-parasite vaccines against *Plasmodium falciparum* involves malarial hemozoin and host TLR9. *Cell Host Microbe* (2010) **7**:50–61. doi:10.1016/j.chom.2009.12.003
- Mathur R, Oh H, Zhang D, Park SG, Seo J, Koblansky A, et al. A mouse model of *Salmonella typhi* infection. *Cell* (2012) **151**:590–602. doi:10.1016/j.cell.2012.08.042
- Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, Hayden MS, et al. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* (2005) **308**:1626–9. doi:10.1126/science.1109893
- Koblansky AA, Jankovic D, Oh H, Hiensy S, Sungnak W, Mathur R, et al. Recognition of profilin by toll-like receptor 12 is critical for host resistance to *Toxoplasma gondii*. *Immunity* (2013) **38**:119–30. doi:10.1016/j.jimmuni.2012.09.016
- Andrade WA, Souza Mdo C, Ramos-Martinez E, Nagpal K, Dutra MS, Melo MB, et al. Combined action of nucleic acid-sensing toll-like receptors and TLR11/TLR12 heterodimers imparts resistance to *Toxoplasma gondii* in mice. *Cell Host Microbe* (2013) **13**:42–53. doi:10.1016/j.chom.2012.12.003
- Broz P, Monack DM. Newly described pattern recognition receptors team up against intracellular pathogens. *Nat Rev Immunol* (2013) **13**:551–65. doi:10.1038/nri3479
- Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozet K, et al. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via toll-like receptors 3, 7 and 9. *Nat Immunol* (2006) **7**:156–64. doi:10.1038/ni1297
- Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature* (2008) **452**:234–8. doi:10.1038/nature06726
- Sasai M, Linehan MM, Iwasaki A. Bifurcation of toll-like receptor 9 signaling by adaptor protein 3. *Science* (2010) **329**:1530–4. doi:10.1126/science.1187029
- Lee BL, Moon JE, Shu JH, Yuan L, Newman ZR, Schekman R, et al. UNC93B1 mediates differential trafficking of endosomal TLRs. *eLife* (2013) **2**:e00291.
- Fukui R, Saitoh S, Matsumoto F, Kozuka-Hata H, Oyama M, Tabeta K, et al. Unc93B1 biases toll-like receptor responses to nucleic acid in dendritic cells toward DNA- but against RNA-sensing. *J Exp Med* (2009) **206**:1339–50. doi:10.1084/jem.20082316
- Takahashi K, Shibata T, Akashi-Takamura S, Kiyokawa T, Wakabayashi Y, Tanimura N, et al. A protein associated with toll-like receptor (TLR) 4 (PRAT4A) is required for TLR-dependent immune responses. *J Exp Med* (2007) **204**:2963–76. doi:10.1084/jem.20071132
- Yang Y, Liu B, Dai J, Srivastava PK, Zammit DJ, Lefrancois L, et al. Heat shock protein gp96 is a master chaperone for toll-like receptors and is important in the innate function of macrophages. *Immunity* (2007) **26**:215–26. doi:10.1016/j.immuni.2006.12.005
- Park B, Brinkmann MM, Spooner E, Lee CC, Kim YM, Ploegh HL. Proteolytic cleavage in an endolysosomal compartment is required for activation of toll-like receptor 9. *Nat Immunol* (2008) **9**:1407–14. doi:10.1038/ni.1669
- Ewald SE, Engel A, Lee J, Wang M, Bogyo M, Barton GM. Nucleic acid recognition by toll-like receptors is coupled to stepwise processing by cathepsins and asparagine endopeptidase. *J Exp Med* (2011) **208**:643–51. doi:10.1084/jem.20100682
- Garcia-Cattaneo A, Gobert FX, Muller M, Toscano F, Flores M, Lescure A, et al. Cleavage of toll-like receptor 3 by cathepsins B and H is essential for signaling. *Proc Natl Acad Sci USA* (2012) **109**:9053–8. doi:10.1073/pnas.1115091109
- Peter ME, Kubarenko AV, Weber AN, Dalpke AH. Identification of an N-terminal recognition site in TLR9 that contributes to CpG-DNA-mediated receptor activation. *J Immunol* (2009) **182**:7690–7. doi:10.4049/jimmunol.0900819

37. Onji M, Kanno A, Saitoh S, Fukui R, Motoi Y, Shibata T, et al. An essential role for the N-terminal fragment of toll-like receptor 9 in DNA sensing. *Nat Commun* (2013) **4**:1949. doi:10.1038/ncomms2949
38. Kagan JC, Medzhitov R. Phosphoinositide-mediated adaptor recruitment controls toll-like receptor signaling. *Cell* (2006) **125**:943–55. doi:10.1016/j.cell.2006.03.047
39. Bonham KS, Orzalli MH, Hayashi K, Wolf AI, Glanemann C, Weninger W, et al. A promiscuous lipid-binding protein diversifies the subcellular sites of toll-like receptor signal transduction. *Cell* (2014) **156**:705–16. doi:10.1016/j.cell.2014.01.019
40. Lee KG, Xu S, Kang ZH, Huo J, Huang M, Liu D, et al. Bruton's tyrosine kinase phosphorylates toll-like receptor 3 to initiate antiviral response. *Proc Natl Acad Sci USA* (2012) **109**:5791–6. doi:10.1073/pnas.1119238109
41. Yamashita M, Chattopadhyay S, Fensterl V, Saikia P, Wetzel JL, Sen GC. Epidermal growth factor receptor is essential for toll-like receptor 3 signaling. *Sci Signal* (2012) **5**:ra50. doi:10.1126/scisignal.2002581
42. Lin SC, Lo YC, Wu H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature* (2010) **465**:885–90. doi:10.1038/nature09121
43. Kollewe C, Mackensen AC, Neumann D, Knop J, Cao P, Li S, et al. Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. *J Biol Chem* (2004) **279**:5227–36. doi:10.1074/jbc.M309251200
44. Jiang Z, Ninomiya-Tsuji J, Qian Y, Matsumoto K, Li X. Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. *Mol Cell Biol* (2002) **22**:7158–67. doi:10.1128/MCB.22.20.7158-7167.2002
45. Chen ZJ. Ubiquitination in signaling to and activation of IKK. *Immunol Rev* (2012) **246**:95–106. doi:10.1111/j.1600-065X.2012.01108.x
46. Ajibade AA, Wang HY, Wang RF. Cell type-specific function of TAK1 in innate immune signaling. *Trends Immunol* (2013) **34**:307–16. doi:10.1016/j.it.2013.03.007
47. Ajibade AA, Wang Q, Cui J, Zou J, Xia X, Wang M, et al. TAK1 negatively regulates NF-kappaB and p38 MAP kinase activation in Gr-1+CD11b+ neutrophils. *Immunity* (2012) **36**:43–54. doi:10.1016/j.jimmuni.2011.12.010
48. Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, et al. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes Dev* (2005) **19**:2668–81. doi:10.1101/gad.1360605
49. Ori D, Kato H, Sanjo H, Tartey S, Mino T, Akira S, et al. Essential roles of K63-linked polyubiquitin-binding proteins TAB2 and TAB3 in B cell activation via MAPKs. *J Immunol* (2013) **190**:4037–45. doi:10.4049/jimmunol.1300173
50. West XZ, Malinin NL, Merkulova AA, Tischenko M, Kerri BA, Borden EC, et al. Oxidative stress induces angiogenesis by activating TLR2 with novel endogenous ligands. *Nature* (2010) **467**:972–6. doi:10.1038/nature09421
51. Jiang X, Chen ZJ. The role of ubiquitylation in immune defence and pathogen evasion. *Nat Rev Immunol* (2012) **12**:35–48. doi:10.1038/nri3111
52. Chang M, Jin W, Sun SC. Peli1 facilitates TRIF-dependent toll-like receptor signaling and proinflammatory cytokine production. *Nat Immunol* (2009) **10**:1089–95. doi:10.1038/ni.1777
53. Kawasaki T, Takemura N, Standley DM, Akira S, Kawai T. The second messenger phosphatidylinositol-5-phosphate facilitates antiviral innate immune signaling. *Cell Host Microbe* (2013) **14**:148–58. doi:10.1016/j.chom.2013.07.011
54. Wang C, Chen T, Zhang J, Yang M, Li N, Xu X, et al. The E3 ubiquitin ligase Nrdp1 ‘preferentially’ promotes TLR-mediated production of type I interferon. *Nat Immunol* (2009) **10**:744–52. doi:10.1038/ni.1742
55. Liu X, Zhan Z, Li D, Xu L, Ma F, Zhang P, et al. Intracellular MHC class II molecules promote TLR-triggered innate immune responses by maintaining activation of the kinase Btk. *Nat Immunol* (2011) **12**:416–24. doi:10.1038/ni.2015
56. Shinohara ML, Lu L, Bu J, Werneck MB, Kobayashi KS, Glimcher LH, et al. Osteopontin expression is essential for interferon-alpha production by plasmacytoid dendritic cells. *Nat Immunol* (2006) **7**:498–506. doi:10.1038/ni.1327
57. Gotoh K, Tanaka Y, Nishikimi A, Inayoshi A, Enjoji M, Takayanagi R, et al. Differential requirement for DOCK2 in migration of plasmacytoid dendritic cells versus myeloid dendritic cells. *Blood* (2008) **111**:2973–6. doi:10.1182/blood-2007-09-112169
58. Saitoh T, Satoh T, Yamamoto N, Uematsu S, Takeuchi O, Kawai T, et al. Antiviral protein viperin promotes toll-like receptor 7- and toll-like receptor 9-mediated type I interferon production in plasmacytoid dendritic cells. *Immunity* (2011) **34**:352–63. doi:10.1016/j.immuni.2011.03.010
59. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* (2005) **434**:772–7. doi:10.1038/nature03464
60. Henault J, Martinez J, Riggs JM, Tian J, Mehta P, Clarke L, et al. Noncanonical autophagy is required for type I interferon secretion in response to DNA-immune complexes. *Immunity* (2012) **37**:986–97. doi:10.1016/j.immuni.2012.09.014
61. Negishi H, Fujita Y, Yanai H, Sakaguchi S, Ouyang X, Shinohara M, et al. Evidence for licensing of IFN-gamma-induced IFN regulatory factor 1 transcription factor by MyD88 in toll-like receptor-dependent gene induction program. *Proc Natl Acad Sci USA* (2006) **103**:15136–41. doi:10.1073/pnas.0607181103
62. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by toll-like receptors. *Nature* (2005) **434**:243–9. doi:10.1038/nature03308
63. Tsujimura H, Tamura T, Kong HJ, Nishiyama A, Ishii KJ, Klinman DM, et al. Toll-like receptor 9 signaling activates NF-kappaB through IFN regulatory factor-8/IFN consensus sequence binding protein in dendritic cells. *J Immunol* (2004) **172**:6820–7. doi:10.4049/jimmunol.172.11.6820
64. Tailor P, Tamura T, Kong HJ, Kubota T, Kubota M, Borghi P, et al. The feedback phase of type I interferon induction in dendritic cells requires interferon regulatory factor 8. *Immunity* (2007) **27**:228–39. doi:10.1016/j.jimmuni.2007.06.009
65. Zanoni I, Ostuni R, Marek LR, Barresi S, Barbalat R, Barton GM, et al. CD14 controls the LPS-induced endocytosis of toll-like receptor 4. *Cell* (2011) **147**:868–80. doi:10.1016/j.cell.2011.09.051
66. Baumann CL, Aspalter IM, Sharif O, Pichlmair A, Bluml S, Grebien F, et al. CD14 is a coreceptor of toll-like receptors 7 and 9. *J Exp Med* (2010) **207**:2689–701. doi:10.1084/jem.20101111
67. Stewart CR, Stuart LM, Wilkinson K, Van Gils JM, Deng J, Halle A, et al. CD36 ligands promote sterile inflammation through assembly of a toll-like receptor 4 and 6 heterodimer. *Nat Immunol* (2010) **11**:155–61. doi:10.1038/ni.1836
68. Sheedy FJ, Grebe A, Rayner KJ, Kalantari P, Ramkhalawon B, Carpenter SB, et al. CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nat Immunol* (2013) **14**:812–20. doi:10.1038/ni.2639
69. Palsson-McDermott EM, Doyle SL, Mcgettrick AF, Hardy M, Husebye H, Banahan K, et al. TAG, a splice variant of the adaptor TRAM, negatively regulates the adaptor MyD88-independent TLR4 pathway. *Nat Immunol* (2009) **10**:579–86. doi:10.1038/ni.1727
70. Han C, Jin J, Xu S, Liu H, Li N, Cao X. Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Cbl-b. *Nat Immunol* (2010) **11**:734–42. doi:10.1038/ni.1908
71. Kayagaki N, Phung Q, Chan S, Chaudhari R, Quan C, O'rourke KM, et al. DUBA: a deubiquitinase that regulates type I interferon production. *Science* (2007) **318**:1628–32. doi:10.1126/science.1145918
72. Skaug B, Chen J, Du F, He J, Ma A, Chen ZJ. Direct, noncatalytic mechanism of IKK inhibition by A20. *Mol Cell* (2011) **44**:559–71. doi:10.1016/j.molcel.2011.09.015
73. Yuk JM, Shin DM, Lee HM, Kim JJ, Kim SW, Jin HS, et al. The orphan nuclear receptor SHP acts as a negative regulator in inflammatory signaling triggered by toll-like receptors. *Nat Immunol* (2011) **12**:742–51. doi:10.1038/ni.2064
74. Kondo T, Kawai T, Akira S. Dissecting negative regulation of toll-like receptor signaling. *Trends Immunol* (2012) **33**:449–58. doi:10.1016/j.it.2012.05.002
75. Shi M, Deng W, Bi E, Mao K, Ji Y, Lin G, et al. TRIM30 alpha negatively regulates TLR-mediated NF-kappa B activation by targeting TAB2 and TAB3 for degradation. *Nat Immunol* (2008) **9**:369–77. doi:10.1038/ni1577
76. Saitoh T, Tun-Kyi A, Ryo A, Yamamoto M, Finn G, Fujita T, et al. Negative regulation of interferon-regulatory factor 3-dependent innate antiviral response by the prolyl isomerase Pin1. *Nat Immunol* (2006) **7**:598–605. doi:10.1038/ni.1347
77. Chevrier N, Mertins P, Artyomov MN, Shalek AK, Iannacone M, Ciaccio MF, et al. Systematic discovery of TLR signaling components delineates viral-sensing circuits. *Cell* (2011) **147**:853–67. doi:10.1016/j.cell.2011.10.022
78. Matsushita K, Takeuchi O, Standley DM, Kumagai Y, Kawagoe T, Miyake T, et al. Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. *Nature* (2009) **458**:1185–90. doi:10.1038/nature07924

79. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* (2011) **34**:637–50. doi:10.1016/j.immuni.2011.05.006
80. Rakoff-Nahoum S, Medzhitov R. Toll-like receptors and cancer. *Nat Rev Cancer* (2009) **9**:57–63. doi:10.1038/nrc2541
81. Ngo VN, Young RM, Schmitz R, Jhavar S, Xiao W, Lim KH, et al. Oncogenically active MYD88 mutations in human lymphoma. *Nature* (2011) **470**:115–9. doi:10.1038/nature09671
82. Compagno M, Lim WK, Grunn A, Nandula SV, Brahmachary M, Shen Q, et al. Mutations of multiple genes cause deregulation of NF-kappaB in diffuse large B-cell lymphoma. *Nature* (2009) **459**:717–21. doi:10.1038/nature07968
83. Kato M, Sanada M, Kato I, Sato Y, Takita J, Takeuchi K, et al. Frequent inactivation of A20 in B-cell lymphomas. *Nature* (2009) **459**:712–6. doi:10.1038/nature07969
84. Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature* (2009) **462**:108–12. doi:10.1038/nature08460

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Insights into the relationship between toll like receptors and gamma delta T cell responses

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The tumor microenvironment is an important aspect of cancer biology that contributes to tumor initiation, tumor progression and responses to therapy. The composition and characteristics of the tumor microenvironment vary widely and are important in determining the anti-tumor immune response. Successful immunization requires activation of both innate and adaptive immunity. Generally, immune system is compromised in patients with cancer due to immune suppression, loss of tumor antigen expression and dysfunction of antigen presenting cells (APC). Thus, therapeutic immunization leading to cancer regression remains a significant challenge. Certain cells of the immune system, including dendritic cells (DCs) and gamma delta ($\gamma\delta$) T cells are capable of driving potent anti-tumor responses. The property of MHC-unrestricted cytotoxicity, high potential of cytokine release, tissue tropism and early activation in infections and malignant disease makes $\gamma\delta$ T cells as an emerging candidate for immunotherapy. Various strategies are being developed to enhance anti-tumor immune responses of $\gamma\delta$ T cells and DCs one of them is the use of novel adjuvants like toll like receptors (TLR) agonists, which enhance $\gamma\delta$ T cell function directly or through DC activation, which has ability to prime $\gamma\delta$ T cells. TLR agonists are being used clinically either alone or in combination with tumor antigens and has shown initial success in both enhancing immune responses and eliciting anti-tumor activity. TLR activated $\gamma\delta$ T cells and DCs nurture each other's activation. This provides a potent base for first line of defense and manipulation of the adaptive response against pathogens and cancer. The available data provides a strong rationale for initiating combinatorial therapy for the treatment of diseases and this review will summarize the application of adjuvants (TLRs) for boosting immune response of $\gamma\delta$ T cells to treat cancer and infectious diseases and their use in combinatorial therapy.

Keywords: immunotherapy, $\gamma\delta$ T cells, toll like receptors, tumors, dendritic cells

INTRODUCTION

Innate and adaptive immune responses are sentinels of host against the diverse repertoire of infectious agents (viruses and bacteria) and cancer. Both components of immune system identify invading microorganisms or damaged tissues as non-self and activate immune responses to eliminate them. Efficient immune responses depend upon how close an interaction is between the innate and adaptive immune system. $\gamma\delta$ T cells and toll like receptors (TLR) serve as an important link between the innate and adaptive immune responses (1–3). Extensive studies have suggested that $\gamma\delta$ T cells play important roles in host defense against microbial infections, tumorigenesis, immunoregulation and development of autoimmunity. $\gamma\delta$ T cells also have several innate cell-like characters that allow their early and rapid activation following recognition of cellular stress and infection (4, 5). However to accomplish these functions, $\gamma\delta$ T cells use both the T cell receptor (TCR) and additional activating receptors (notably NKG2D, NOTCH, and TLR) to respond to stress-induced ligands and infection. $\gamma\delta$ T cells express TLRs and modulate early immune responses against different pathogens (6). In this review, we summarize and discuss some of the recent advances of the $\gamma\delta$ T cell biology and how direct control of $\gamma\delta$ T lymphocyte function

and activation is monitored by TLR receptors and ligands. The review highlights involvement of TLR signaling in $\gamma\delta$ T cell functions and their implications in harnessing $\gamma\delta$ T cells for cancer immunotherapy.

$\gamma\delta$ T CELLS, ANATOMICAL DISTRIBUTION AND ANTIGENIC DIVERSITY

Based on the type of TCR they express, T lymphocytes can be divided into two major subsets, $\alpha\beta$ and $\gamma\delta$ T cells. $\gamma\delta$ T cell represents a small subset of T lymphocytes (1–10%) in peripheral blood. While in anatomical locations like small intestine, $\gamma\delta$ T cells comprise a major bulk of T cells (25–60% in human gut) (7). $\gamma\delta$ T cells are the first T cells to appear in thymus during T cell ontogeny in every vertebrate (8), which suggests that their primary contribution could be neonatal protection because at this point conventional $\alpha\beta$ T cell responses are severely functionally impaired and DCs are immature (9). In neonates, the V δ 2 $^+$ cells derived from human cord blood showed early signs of activation. These cells secrete IFN- γ and express perforin after short-term *in vitro* stimulation (10). In comparison to the neonate derived $\alpha\beta$ T cells of peripheral blood, $\gamma\delta$ T cell subset produces copious amount of IFN- γ and are precociously active (11). Hence, $\gamma\delta$

T cells are well engaged in newborns to contribute to immune-protection, immune-regulation and compensate for impaired $\alpha\beta$ T cell compartment.

$\gamma\delta$ T cells are unconventional CD3⁺ T cells and differ from the conventional $\alpha\beta$ T cells in their biology and function (**Table 1**). Although a sizeable fraction of $\gamma\delta$ T cells in the intraepithelial lymphocyte compartments of human and mice are CD8 $\alpha\alpha^+$ but the peripheral blood $\gamma\delta$ T cells are predominantly double negative (CD4⁻CD8⁻) T cells. The absence of CD4 or CD8 expression on majority of the circulating $\gamma\delta$ T cells is well in line with the fact that antigen recognition is not MHC restricted (12, 13). Crystal structure analysis of the $\gamma\delta$ TCR revealed that $\gamma\delta$ TCR is highly variable in length resembling immuno-globulins (Ig) more than the $\alpha\beta$ TCR. The antigen recognition property of $\gamma\delta$ T cells is fundamentally different from $\alpha\beta$ T cells but similar to antigen-antibody binding, which is more likely to occur independent of MHC cross presentation (14). However, recently butyrophilin BTN3A1, a non-polymorphic ubiquitously expressed molecule was identified as an antigen presenting molecule of V γ 9V δ 2 T cells. Soluble BTN3A1 binds (Isopentenyl diphosphate) IPP and (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) with different affinities in 1:1 ratio to stimulate $\gamma\delta$ T cells (15).

The important feature of $\gamma\delta$ T cells is their tropism to epithelial tissues. With respect to anatomical localization, $\gamma\delta$ T cell population can be divided into two groups: lymphoid-homing $\gamma\delta$ T cells that can be primed in the circulation and clonally expand in a conventional “adaptive” manner; and innate-like cells that respond rapidly and at a relatively high frequency in many tissue sites. Migration and anatomical localization of T lymphocytes is crucial for their antigen specificity and maintaining homeostasis in the mammalian immune system. Although $\gamma\delta$ T cells are well represented among peripheral blood mononuclear cells (PBMC) and in afferent and efferent lymph, they are rarely found in lymph node parenchyma, spleen, Peyer's patches and thymus. Moreover, unlike $\alpha\beta$ T cells, splenic $\gamma\delta$ T cells, if present, are not confined to the lymphoid areas (the white pulp) but are also found throughout the red pulp of spleen and marginal zones of cell trafficking (16). $\gamma\delta$ T cells are abundantly present in the epithelia of skin, genital and intestinal tract (17). In the small intestines of humans, mice, chickens and cattle, $\gamma\delta$ T cells comprise a substantial fraction of intestinal intraepithelial lymphocytes (IELs); in mice $\gamma\delta^+$

IELs constitute 50–60% of the IEL pool (18–20). The epidermal $\gamma\delta^+$ IELs of mice and cattle (but not humans) have a marked dendritic morphology and are hence known as dendritic epidermal T cells (DETCs) (21). DETCs are maintained at steady state in normal adult murine skin but on activation execute specialized functions like tissue repair (22). DETCs also maintain keratinocyte homeostasis, which along with Langerhan cells forms its neighborhood (23). Under pathological conditions, $\gamma\delta$ T cells quickly expand and infiltrate into lymphoid compartments and other tissues.

Another striking difference between $\alpha\beta$ and $\gamma\delta$ T cells is the range of antigens or ligands that are recognized by the respective TCRs. Unlike $\alpha\beta$ T cells, which recognize protein antigen processed inside the cell and presented by MHC molecules, $\gamma\delta$ T cells recognize antigens like B cells as revealed by structural and functional studies (24). $\gamma\delta$ T cells can respond to a variety of stimuli irrespective of their molecular or genetic nature. In mice, the non-classical MHC class I molecules T10 and T22 are recognized by $\gamma\delta$ T cells (25–28). Similar to T10 and T20, murine class II MHC (IA) antigens IE and IA are identified to act as ligands for $\gamma\delta$ T cell clones (29, 30). In addition, herpes glycoprotein G1-reactive $\gamma\delta$ T cell clones protect mice from herpes simplex virus (HSV) induced lethal encephalitis (31, 32). $\gamma\delta$ TCRs can also bind to an algal molecule, phycoerythrin inducing upregulation of CD44 and downregulation of CD62L in $\gamma\delta$ T cells (33). B6 murine splenic and hepatic $\gamma\delta$ T cells respond to cardiolipin (bacterial cell-wall phospholipid and endogenous component of mitochondria) presented by CD1d molecules (34). Insulin derived peptide B:9–23 is also recognized by the $\gamma\delta$ T cell clones derived from non-obese diabetic mice (NOD mice) (35). SKINT1, a mouse immunoglobulin superfamily member, bears structural similarity to human CD277 (butyrophilin 3A1) and is expressed by medullary thymic epithelial cells (mTECs) and keratinocytes that is crucial for the development of V γ 5V δ 1⁺ DETCs (36).

In humans, majority of $\gamma\delta$ T cells express a rearranged T cell receptor (TCR) composed of V γ 9 and V δ 2 domains; thus, this population is referred to as V γ 9V δ 2. The V γ 9V δ 2 T cells recognize self and microbial phosphorylated metabolites generated in eukaryotic mevalonate pathway and in the microbial 2-C-methyl-derythritol 4-phosphate (MEP) pathway (37). Initially, it was reported that the non-peptidic ligands isolated from mycobacterial cell lysates were

Table 1 | Comparison between $\alpha\beta$ and $\gamma\delta$ T cells.

S.No.	$\alpha\beta$ T cells	$\gamma\delta$ T cells
1	Constitutes about 65–70% of total PBMCs	Constitutes about 1–10% of total PBMCs
2	Recognize the processed peptide antigen with the help of antigen presenting molecule MHC1 and MHC II	Do not show MHC restriction but may require the antigen presenting molecule Butyrophilin 3A1 molecule
3	Express either CD8 $^+$ or CD4 $^+$	Mostly double negative, murine intestinal IELs may be CD8 $\alpha\alpha^+$
4	TCR junctional diversity is very diverse	TCR junctional diversity is small
5	Do not show tissue tropism	Show tissue tropism
6	$\alpha\beta$ T Cells response is late	$\gamma\delta$ T cells respond earlier
7	Regulatory phenotype is attributed to CD4 $^+$ CD25 $^+$ T cells	Regulatory phenotype is attributable to various subsets, including murine V γ 5 δ DETCs and human V γ 1 $^+$ peripheral cells

stimulatory for V γ 9V δ 2 T cell clones. Later, IPP, an intermediate metabolite of the mevalonate pathway, was isolated and identified as a stimulatory molecule. Characterization of the microbial antigens recognized by human $\gamma\delta$ T cells predicted that these are non-proteinaceous in nature and have critical phosphate residues (37, 38). Subsequent studies, conducted with *M. tuberculosis*, identified HMBPP, an intermediate metabolite of the MEP pathway, as a strong agonist of $\gamma\delta$ TCR. The measured potencies of IPP and HMBPP show an enormous difference. The ED₅₀ of IPP is \sim 20 μ M, whereas that of HMBPP is \sim 70 pM, i.e., more than 105 times lower (38).

Another stimulatory molecule is *Staphylococcus aureus* enterotoxin A (SEA) that directly interacts with the TCR V γ 9 chain independently of the paired V δ chain. The mechanism of recognition of this superantigen is different from that of phosphorylated metabolites and requires the interaction with MHC class II molecules. $\gamma\delta$ T cells kill target cells and release cytokines upon interaction with SEA but do not proliferate (39).

Recently, the TCR from a $\gamma\delta$ T cell clone derived from a cytomegalovirus (CMV)-infected transplant patient was shown to directly bind to endothelial protein C receptor (EPCR), which is a lipid carrier with a similar structure to CD1, showing again that $\gamma\delta$ TCR engagement is cargo independent (40). ATP F1 synthase has been identified as stimulatory ligand of the TCR V γ 9V δ 2. ATP F1 synthase is an intracellular protein complex involved in ATP generation. However, optimal responses of V γ 9V δ 2 T cells by tumor target cell lines expressing F1-ATPase requires apolipoprotein A1. A monoclonal antibody interacting with apolipoprotein A1 was shown to inhibit TCR $\gamma\delta$ activation as it disrupted the trimolecular complex of ApoA1, ATP F1 synthase, and $\gamma\delta$ TCR required for optimal response (41).

The second major population of human $\gamma\delta$ T cells utilizes the V δ 1 chain, which pairs with a variety of V γ chains. This subset of V δ 1 $^+$ T cells is mainly found in tissues and is activated by CD1c and CD1d-expressing cells. The group 1 CD1 molecules have ability to present lipid A to human $\gamma\delta$ T cells. The human $\gamma\delta$ T cells also recognize the related group 2 CD1 molecule as CD1d/lipid complex. Phosphatidyl ethanol amine (PE), a phospholipid, activates $\gamma\delta$ T cells in a CD1d manner dependent suggesting its CD1d restricted recognition (42). In addition, some populations of $\gamma\delta$ T cells in normal human PBMCs also recognize lipid molecules such as cardiolipin (a marker of damaged mitochondria), sulfatide (a myelin glycosphingolipid), or α -galactosylceramide (α -GalCer) in association with CD1d, which are noted ligands of natural killer T (NKT) cells (34, 43–45). Human $\gamma\delta$ T cells also recognize the stress-induced MHC class I-related MICA/MICB molecules and the UL16-binding proteins that are upregulated on malignant or stressed cells (46–48). Heat shock proteins (HSPs) expressed on the cell membrane play an important role in cancer immunity. Hsp60 expressed on oral tumors act as ligand for V γ 9V δ 2 T cells (49, 50). Hsp60 and Hsp70 expressing human oral and esophageal tumors are lysed by V γ 9V δ 2 T cells (49–51). Hsp72 expressing neutrophils were rapidly killed by $\gamma\delta$ T cells through direct cell to cell contact, indicating that hsp72 expression on cell surface pre-disposes inflamed neutrophils to killing by $\gamma\delta$ T cells (52). In another study, hsp90 expression on EBV infected B cells rapidly promoted $\gamma\delta$ T cell proliferation (53). This confirms that $\gamma\delta$ T cells recognize

qualitatively distinct antigens, which are profoundly regulated by their anatomical localization.

CO-RECEPTORS AND $\gamma\delta$ T CELL ACTIVATION

Most $\gamma\delta$ T cells respond to non-peptidic antigens even in the absence of antigen presenting cells (APCs). However, the presence of APCs can greatly enhance the $\gamma\delta$ T cell response (54). This suggests that accessory molecules/receptors may be involved in effector functions of these cells. Some of important co-receptors used by $\gamma\delta$ T cells include NOTCH, NKG2D, and TLR (55).

Our study has identified Notch as an additional signal contributing to antigen specific effector functions of $\gamma\delta$ T cells. We have shown that $\gamma\delta$ T cells express Notch1 and Notch2 at both mRNA and protein level. Inhibition of Notch signaling in anti-CD3 MAbs stimulated $\gamma\delta$ T cells resulted in marked decrease in proliferation, cytotoxic potential, and cytokine production by $\gamma\delta$ T cells confirming the involvement of Notch signaling in regulating antigen specific responses of $\gamma\delta$ T cells (55).

$\gamma\delta$ T cells express NKG2D on their cell surface resulting in their activation. Treatment of PBMC with immobilized NKG2D-specific mAb or NKG2D ligand MHC class I related protein A (MICA) resulted in the up-regulation of CD69 and CD25 on V γ 9V δ 2. Furthermore, NKG2D increased the production of TNF-alpha and release of cytolytic granules by V γ 9V δ 2 T cells (56). Later, it was shown that the protein kinase C transduction pathway as a main regulator of the NKG2D-mediated costimulation of anti-tumor V γ 9V δ 2 T cell cytolytic response (57).

TLR agonists are also known to trigger the early activation and the IFN- γ secretion by V γ 9V δ 2 T cells (58). TLR ligands indirectly increase the anti-tumocidal activity of V γ 9V δ 2 T cells (59). In this review, we will focus on TLR as an additional co-receptor modulating the function of immune cells with special focus on $\gamma\delta$ T cells.

TOLL LIKE RECEPTOR AND IMMUNE CELLS

The immune system functions in anti-microbial defense by recognizing groups of molecules unique to microorganisms (60). These unique microbial molecules are called pathogen-associated molecular patterns (PAMPs) and are recognized by a family of cellular receptors called pattern recognition receptors (PRRs) (61). TLRs along with retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptor (NLRs) are prototype PRRs, which recognize pathogen-associated molecular patterns (PAMPs) from microorganisms or danger-associated molecular patterns (DAMPs) from damaged tissues (62). Recognition of PAMPs by TLRs trigger release of inflammatory cytokines and type 1 interferon's (IFN) for host defense (60, 63–65). The adaptive immune system, on the other hand, is responsible for elimination of pathogens in the late phase of infection and in the generation of immunological memory mediated by B and T cells (66).

TLRs derived their name from *Drosophila melanogaster* Toll protein based on their homology (67). In mammals, till date 13 members of TLR family has been identified (63, 68–71). TLR1–9 is conserved in humans and mice while TLR10 is non-functional in mice because of a retroviral insertion while TLR11–13 is lost from the human genome. The first TLR identified was TLR4

and recognizes bacterial lipopolysaccharide (LPS) from Gram-negative bacteria (67, 72, 73). TLRs are classified into several groups based on the types of PAMPs they recognize. TLR1, 2, 4 and 6 recognize lipids whereas the highly related TLR7, TLR8 and TLR9 recognize nucleic acids. Murine TLR11 recognizes a protozoan derived profilin-like protein while TLR13 recognizes *Vesicular stomatitis virus* (63). TLRs are localized in the distinct cellular compartments, for example; TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are expressed on the cell surface whereas TLR3, TLR7, TLR8 TLR9, TLR11, TLR12 and TLR13 are expressed in intracellular vesicles such as the endosome and ER. The intracellular TLRs are transported to the intracellular vesicles via UNC93B1, a trans-membrane protein, which is localized in the ER of the cell (70, 71, 74–77). TLR family receptors have a common structural architecture. TLRs are type I integral membrane glycoproteins characterized by multiple extracellular leucine-rich repeats (LRRs) and a single intracellular Toll/interleukin-1 (IL-1) receptor (TIR). TLRs mostly form homo-dimers with a few exceptions, which form heterodimers to trigger a signal. For example, TLR2 forms heterodimers with TLR1 or TLR6 enabling differential recognition of lipopeptides. The TIR domain of TLRs is required for the interaction and recruitment of various adaptor molecules to activate downstream signaling pathway. After recognizing PAMPs, TLRs activate intracellular signaling pathways that lead to the induction of inflammatory cytokine genes such as TNF- α , IL-6, IL-1 β and IL-12 through the recruitment of adaptors such as MyD88, TRIF, TRAM, TIRAP and SARM1 (78). MyD88 is a universal adaptor used by all TLRs, except TLR3, to induce inflammatory pathways through activation of MAP Kinases (ERK, JNK, p38) and transcriptional factor NF- κ B (63, 79). TLR3 and TLR4 use TRIF to bring activation of alternative pathway (TRIF-dependent pathway) through transcription factors IRF3 and NF- κ B to induce type 1 IFN and inflammatory cytokines (80–82). TRAM selectively participates in the activation of the TRIF-dependent pathway downstream of TLR4, but not TLR3 (83, 84). TIRAP functions to recruit MyD88 leading to activation of MyD88-dependent pathway downstream of TLR2 and TLR4 (85, 86). Sterile- α - and armadillo-motif-containing protein 1 (SARM1), was shown to inhibit TRIF and is also critical for TLR-independent innate immunity (87). Thus, signaling pathways can be broadly classified as either MyD88-dependent pathway or TRIF-dependent pathway.

Hornung et al. have showed differential expression of TLR1–10 on human APCs and lymphocytes including T cells and their functional discrepancy in recognition of specific TLR ligands (88). CD4 $^+$ T cells express almost all TLRs at mRNA levels but may not express all as functional protein (89, 90). Moreover, they do not respond to all TLR ligands. Stimulation with TLR5, 7, or 8 agonists combined with TCR activation of CD4 $^+$ T cells resulted in increased proliferation and production of IL-2, IL-8, IL-10, IFN- γ and TNF α (91). There are other reports as well suggesting the functional modulation of subtypes of CD4 $^+$ T cells by TLR ligands. The mouse Th1 but not Th2 cells responded to TLR2 agonist and resulted in enhanced proliferation and IFN- γ production independent of TCR stimulation (92). This work validated that the TLR can regulate function of CD4 $^+$ T cells even in absence of TCR engagement. CD4 $^+$ CD25 $^+$ regulatory T cells (Tregs) express

majority of TLRs with selectively higher expression of TLR2, 4, 5, 7/8, and 10 compared to CD4 $^+$ CD25 $^-$ conventional T cells (93). Liu et al. showed that CD4 $^+$ CD25 $^+$ regulatory T cells and CD4 $^+$ CD25 $^-$ conventional T cells express TLR2 and proliferated upon stimulation with its agonist. TLR2 stimulation also led to transient loss of Treg suppressive potential through suppression of FOXP3 (94, 95). However, Tregs also express TLR5 but upon stimulation with flagellin (ligand of TLR5), do not proliferate rather showed increased suppressive capacity and enhanced expression of FOXP3 (96). These reports suggest that the suppressive function of Treg can be either enhanced or dampened by the type of TLR ligand engaged. TLR2 stimulation not only abrogates suppressive functions of CD4 $^+$ Tregs but also drives naïve as well as effector Treg population toward IL17 producing Th17 phenotype (97). Th17 cells express TLR2 along with TLR6 compared to Th1 and Th2 subsets and promote Th17 differentiation upon Pam3Cys stimulation and accelerates experimental autoimmune encephalomyelitis (98). Like TLR2, TLR4 also regulate the functions of CD4 $^+$ T cells. In a mouse model of arthritis, mice lacking TLR2 showed enhanced histopathological scores of arthritis by a shift in T cell balance from Th2 and T regulatory cells toward pathogenic Th1 cells. TLR4, in contrast, contributes to more severe disease by modulating the Th17 cell population and IL-17 production (99, 100). Recently, Li et al. showed that high-mobility group box 1 (HMGB1) proteins decrease Treg/Th17 ratio by inhibiting FOXP3 and enhancing ROR γ t in CD4 $^+$ T cells via TLR4–IL6 axis in patients with chronic hepatitis B infections (101). This shows that HMGB1 (TLR4 ligand) act as a modulator of CD4 $^+$ T cells responses in chronic viral inflammation. CD4 $^+$ T cells also express intracellular TLRs such as TLR9 and TLR3. Both these TLRs promote T cell survival via activation of NF- κ B and MAPK signaling (102). Although the effector functions of CD4 $^+$ T cells are regulated by TLRs but the molecular pathway involved in skewing of CD4 $^+$ T cell function is poorly understood.

Like CD4 $^+$ T cells, CD8 $^+$ T cells also show differential expression of TLRs with high expression of TLR3 but lower expression of TRL1,2,5,9,10 compared to CD4 $^+$ T cells at mRNA level. It is important to note that the expression of TLR2, TLR3 and TLR5 increases on CD8 T cells in infected tonsils compared to controls (89) indicating immune activating role of TLRs in infections. Stimulation of CD8 $^+$ T cells through TLR2 agonists enhances their proliferation and IFN- γ production (103, 104). It also promotes cytolytic activity of CD8 $^+$ T cells and enhances anti-tumor response mediated through MyD88-dependent TLR1/2 pathway (105). Recently, Mercier et al. showed that TLR2 cooperate with NOD-containing protein 1 (NOD1) to enhance TCR mediated activation and can serve as alternative co-stimulatory receptor in CD8 $^+$ T cells (106). CD8 $^+$ T cells also express intracellular TLRs such as TLR3, TLR9 which are more potent in inducing CD8 $^+$ T cell activation *in vivo* (107).

Natural killer (NK) cell is a vital player in innate immune system. They recognize infected and transformed cells with down-regulated major histocompatibility complex (MHC) class 1 molecules. They are the primary producers of IFN- γ and are protective against infections. Unlike CD4 and CD8 T cells NK cells as well as CD56 $^+$ CD3 $^+$ NKT cells constitutively express TLR 1–8 with high expression of TLR2 and 3 at mRNA level. They recognize

bacterial PAMPs and respond by producing α -defensins (108–111). Human NK cells can also directly recognize *Mycobacterium bovis* via TLR2 and enhance their cytolytic activity against tumor cells (112). Tumor-associated macrophages induce NK cell IFN- γ production and cytolytic activity upon TLR engagement (113). TLRs modulate NK cell function directly or indirectly to promote antibody dependent cell mediated cytotoxicity and cross presentation of viral antigens to T lymphocytes (114, 115). This highlights that the cells of adaptive immune system do express TLRs and their function can be directly or indirectly modulated by TLR ligands.

ACTIVATION OF $\gamma\delta$ T CELLS BY TLR LIGANDS

In 1997, the first human homolog of *Drosophila* Toll protein was cloned and characterized. It was also established that $\gamma\delta$ T cells also express hToll mRNA (67). Purified $\gamma\delta$ T cells were found to respond to the *E. coli* native lipid A in a TCR-independent fashion and the LPS/lipid A-reactive $\gamma\delta$ T cells strongly expressed TLR2 mRNA. TLR2 antisense oligonucleotide inhibited the proliferation of $\gamma\delta$ T cells in response to the native lipid A as well as the TLR2-deficient mice showed an impaired response of the $\gamma\delta$ T cells following injection of native lipid A. These results suggest that TLR2 is involved in the activation of canonical V γ 6/V δ 1 T cells by native lipid A (116). Again, functional presence of TLR2 on V γ 2V δ 2 T cells (also known as V γ 9V δ 2 T cells) was reported when the dual stimulation of V γ 2V δ 2 T cells with anti-TCR antibody and Pam₃Cys increased synthesis and secretion of IFN- γ and elevated the levels of CD107a expression. IFN- γ secretion and cell surface CD107a levels are markers of increased effector function in V γ 2V δ 2 T cells (117). Similarly, Bruno et al. reported that IL-23 and TLR2 co-stimulation induces IL17 expression in $\gamma\delta$ T cells. However, TLR1 and TLR2 expression was found only on CCR6 $^+$ IL-17 producing murine peritoneal $\gamma\delta$ T cells but not others. Thus, $\gamma\delta$ T cells with innate receptor expression coupled with IL-17 production establishes them as first line of defense that can orchestrate an inflammatory response to pathogen-derived and environmental signals long before Th17 can sense the bacterial invasion (118). Pam3CSK4, TLR2 agonist was able to stimulate only splenic $\gamma\delta$ T cell proliferation but not the dermal $\gamma\delta$ T cells demonstrating that TLR2 signaling shows tissue tropism. (19). Furthermore, a profound change in the circulating $\gamma\delta$ T-cell population was observed in early burn injury (24 h). These $\gamma\delta$ T-cells showed TLR2 and TLR4 expression, priming them for TLR reactivity. However TLR expression was specific to circulatory $\gamma\delta$ T cell subset and was transient, since it was not observed after post-injury (7 days). Transient nature of the post-burn increase in $\gamma\delta$ T-cell TLR expression is likely to be protective to the host, most likely via regulation of inflammation and initiation of healing processes (119). Mitochondrial danger-associated molecular patterns (MDPs) induce TLR2 and TLR4 expression on $\gamma\delta$ T cells in dose dependent manner. MDPs also induced the production of IL-1 β , IL-6, IL-10, RANTES, and vascular endothelial growth factor by $\gamma\delta$ T-cells thereby resulting in initiation of sterile inflammation leading to tissue/cellular repair (120).

Different studies have reported that $\gamma\delta$ T cells express TLR3 (121, 122). TLR3 recognizes viral dsRNA, synthetic analogs of dsRNA, polyinosinic–polycytidylic acid [poly (I:C)] and small interfering (si) RNA. The direct stimulation of freshly isolated $\gamma\delta$

T cells via TCR and surrogate TLR3 ligand poly (I:C) dramatically increased IFN- γ production. Addition of neutralizing anti-TLR3 mAb inhibited the co-stimulatory effect of poly (I:C), presumably by antagonizing the TLR3 signaling (122). Thus, the integrated signals of TLR3 and TCR induce a strong antiviral effector function in $\gamma\delta$ T cells supporting the decisive role of $\gamma\delta$ T cells in early defense against viral infection. In other study, it has been reported that $\gamma\delta$ cells of term babies and of adults express TLR3 and TLR7 while the preterm babies have reduced levels. The greater levels of IFN- γ protein was observed in adult and cord blood cells co-stimulated with anti-CD3 and poly(I:C) whereas this was not seen in $\gamma\delta$ T cell clones of preterm babies. Thus, reduced level of TLR3 expression by preterm-derived clones had an overt functional consequence on IFN- γ levels (11). Interestingly, a primary role of TLR3 in humans appears to mediate resistance to HSV-induced encephalitis (123). Hence, premature babies are particularly susceptible to HSV infection because of reduced levels of TLR3 on $\gamma\delta$ T cells.

TLR4 was reported to be absent in the $\gamma\delta$ T cells but can become functional in $\gamma\delta$ T cells depending on localization, environmental signals, or $\gamma\delta$ TCR usage (19, 118, 124). However, our own data has shown that TLR4 is expressed on human $\gamma\delta$ T cells. Stimulation of $\gamma\delta$ T cells with LPS (TLR4 ligand) increased their proliferation, IFN- γ release, and cytotoxic potential (125). DETCs lack cell surface expression of TLR4-MD2. MD-2 physically associates with TLR4 on the cell surface and is required for LPS signaling. However, TLR4-MD2 expression was upregulated when DETCs emigrated from the epidermis during cutaneous inflammation. The migration signals of DETCs may promote the TLR4-MD2 expression (126). Cairns et al. showed that late post-burn injury increased expression of TLR-4 on splenic T-cells (127). However, Martin et al. reported transient TLR-4 expression post-burn in the circulation or spleen but were specific for the $\gamma\delta$ T-cell subset (119). Several evidences suggest that murine $\gamma\delta$ T cells recognize LPS/LA through TLR2 or TLR4 (128, 129). Importantly activated $\gamma\delta$ T cells, especially V δ 2 T cells, in peripheral blood cells recognize LA, a major component of LPS, via TLR4 resulting in extensive proliferation and production of IFN- γ and TNF- α *in vitro* (130). The data suggest that $\gamma\delta$ T cells play an important role in the control of infection induced by gram negative bacteria. Reynolds et al. showed that a heterogeneous population of $\gamma\delta$ T cells responds to LPS via TLR4 dependent manner and demonstrate the crucial and innate role of TLR4 in promoting the activation of $\gamma\delta$ T cells, which contributes to the initiation of autoimmune inflammation (100). Another study showed the indirect role of TLR4 in HMGB1-TLR4-IL-23-IL17A axis between macrophages and $\gamma\delta$ T cells, which contribute to the accumulation of neutrophils and liver inflammation. Necrotic hepatocytes release HMGB1, a damage-associated molecule or TLR4 ligand, which increased IL-23 production of macrophages in a TLR4 dependent manner. IL-23 aids $\gamma\delta$ T cells in liver in the generation of IL-17A, which then recruits hepatic neutrophils (131).

Human $\gamma\delta$ T cells were found to express appreciable levels of TLR7. Costimulation with poly I:C upregulated the TLR7 expression in TCR-cross linked freshly isolated $\gamma\delta$ T cells (124). In addition, tumor-infiltrating $\gamma\delta$ T cells also express TLR7 (132). In case of mouse dermal $\gamma\delta$ T cells, both TLR7 and

TLR9 signaling promoted IL-17 production, which could be synergistically enhanced with the addition of IL-23 (19).

The identification of dominant $\gamma\delta$ T cells in the total population of tumor-infiltrating lymphocytes (TILs) in renal, breast, and prostate cancer suggested that these cells might have the potent negative immune regulatory function (132, 133). The breast tumor-derived bulk $\gamma\delta$ T cell lines and clones efficiently suppressed the proliferation and IL-2 secretion of naïve/effector T cells and inhibited DC maturation and function. Hence, their depletion or the reversal of their suppressive function could enhance anti-tumor immune responses against breast cancer. Indeed as in CD4⁺ regulatory T cells (Tregs), the immunosuppressive activity of $\gamma\delta$ T cells could be reversed by human TLR8 ligands both *in vitro* and *in vivo*. Study revealed that MyD88, TRAF6, IKK α , IKK β and p38 α molecules in $\gamma\delta$ T cells were required for these cells to respond to TLR8 ligands (132, 134, 135). **Table 2** shows expression and co-stimulatory effects mediated by TLR activation of $\gamma\delta$ T cells

TLRs MODULATE CROSSTALK BETWEEN $\gamma\delta$ T AND DENDRITIC CELLS

The functional fate of effector T cells is governed by antigen presentation and the cytokine milieu in the local environment. Dendritic cells (DCs) being professional APCs, recognize the danger signal, process it, and present it to the T lymphocytes thereby modulate adaptive immune response. $\gamma\delta$ T cells influence the antigen presenting property of DCs. DCs pre-incubated with activated $\gamma\delta$ T cells enhance the production of IFN- γ by alloreactive T cells in mixed lymphocyte reaction (136). Moreover, $\gamma\delta$ T cells not only upregulated CD86 and MHC I expression on DC but themselves get activated, leading to up-regulation of CD25, CD69, and cytokine production (137). These studies showed how $\gamma\delta$ T cell and DCs regulate each other's function. There are reports, which have shown how $\gamma\delta$ T cells interact with DC or *vice versa* via TLR ligands. Leslie et al. reported that stimulation with TLR ligands in $\gamma\delta$ /DC cocultures enhanced the maturation and production of IL12p70 by DCs (138). TLR also regulate the $\gamma\delta$ T cells and DC crosstalk in microbial context. TLR2-stimulated DCs enhanced IFN- γ production by V82 T cells; conversely, phospho-antigen activated V82 T cells enhanced TLR2-induced DC maturation via IFN- γ , which co-stimulated interleukin-12 (IL-12) p70 secretion by DCs (139). Further, $\gamma\delta$ T cells stimulated with TLR7 (CL097) or TLR3 (poly I:C) agonists produce IFN- γ , TNF α and/or IL-6 thereby inducing DC maturation, which prime effector T cells against West Nile Virus (WNV) infection (140). This study

confirmed that the antiviral effector immunity may be regulated by interplay of DCs, $\gamma\delta$ T cells and TLRs. Similarly, in human's $\gamma\delta$ T cells and DCs regulate each other's immunostimulatory functions. TLR3 and TLR4 ligands stimulation of human PBMCs induced a rapid and exclusive IFN- γ production by V γ 9V82 subset dependent on type 1 IFN secreted by monocytic DC. TLR-induced IFN- γ response of V γ 9V82 T cells led to efficient DC polarization into IL-12p70-producing cells (58). In another study, it was reported that V82 cells are indirectly activated by BCG and IL-12p70 secreted by DCs. IL-12p70 production by DC is modulated by Toll like receptor 2/4 ligands from BCG and IFN- γ secreted by memory CD4 T cells (141). This study portrayed the complex interplay between cells of the innate and adaptive immune response in contributing to immunosurveillance against pathogenic infections.

TLRs COMPLEMENT CYTOTOXIC POTENTIAL OF $\gamma\delta$ T CELLS AGAINST TUMOR CELLS

$\gamma\delta$ T cells have capability to lyse different types of tumors and tumor-derived cell lines (49, 50, 142–145). Circulating as well as tumor-infiltrating $\gamma\delta$ T cells have the ability to produce abundant proinflammatory cytokines like IFN- γ and TNF- α , cytotoxic mediators and MHC-independent recognition of antigens, render them as important players in cancer immunotherapy (143, 145). In addition to TCR, $\gamma\delta$ T cells use additional stimulatory co-receptors or ligands including TLRs to execute effector functions and TLR agonists are considered as adjuvants in clinical trial of cancer immunotherapy (146). Kalyan et al. even quoted that "TLR signaling may perfectly complement the anti-tumor synergy of aminobisphosphonates and activated $\gamma\delta$ T cells and this combined innate artillery could provide the necessary ammunition to topple malignancy's stronghold on the immune system" (147). Paradoxically, TLR agonists execute dual role of enhancing immune response (148) as well as increasing invasiveness of tumor cells (149–152). Hence, the tripartite cooperation of tumor cell, TLRs, and $\gamma\delta$ T cells should be carefully analyzed. In concordance to this, Shojaei et al. reported that Toll like receptor 3 and 7 agonists enhanced the tumor cell lysis by human $\gamma\delta$ T cells. The enhanced capability of $\gamma\delta$ T cells to lyse tumor cells was attributed to increased expression of CD54 and downregulation of MHC class 1 on tumor cells. Poly(I:C) treatment of pancreatic adenocarcinomas resulted in overexpression of CD54 and concomitant coculture of tumor cells with $\gamma\delta$ T cells led to interaction between CD54 and its ligand CD11a/CD18 triggering effector function in $\gamma\delta$ T cells. However, TLR7 surrogate ligand induced

Table 2 | Expression and functions mediated by TLRs on $\gamma\delta$ T cells.

TLR	Functions	References
TLR 2	Recognize LPS, enhance proliferation, induce IFNy and CD107a expression, enhance IL17 secretion, expression transiently increases after burn injury, mitochondrial danger-associated molecular patterns (MDTs) induce expression and production of IL-1 β , IL-6, IL-10, RANTES, and VEGF	(19, 116–120)
TLR3	Induce IFNy production in conjunction with TCR stimulation, resistance to HSV induced encephalitis	(11, 121–123)
TLR4	Increases proliferation, IFN- γ release, and cytotoxic potential, activation following burn injury	(100, 125, 127, 130)
TLR7/9	Upregulate upon poly I:C costimulation, promote IL-17 production	(19, 124, 132)
TLR8	Reversal of immunosuppressive activity	(132, 134, 135)

downregulation of MHC class 1 molecule on tumor cells resulting in a reduced affinity for inhibitory receptor NKG2A on $\gamma\delta$ T cells (59). Manipulation of TLR signaling by using TLR8 agonists reversed the suppressive potential of $\gamma\delta$ Tregs found elevated in breast cancer (132). Polysaccharide K (PSK) known for its anti-tumor and immuno-modulatory function can also activate TLR2 leading to increased secretion of IFN- γ by $\gamma\delta$ T cells on stimulation. The cell-cell contact between $\gamma\delta$ T cells and DC was required for optimal activation of $\gamma\delta$ T cells. However, PSK along with anti-TCR could co-activate $\gamma\delta$ T cells even in the absence of DC. The study confirmed that the anti-tumor effect of PSK was through activation of $\gamma\delta$ T cells (153).

Studies from our lab have shown that the TLR signaling in $\gamma\delta$ T cells derived from the oral cancer (OC) patients may be dysfunctional. We reported that $\gamma\delta$ T cells from healthy individuals (HI) and OC patients express higher levels of TLR2, TLR3, TLR4, and TLR9 than in $\alpha\beta$ T cells. Higher TLR expression was observed in HI compared to OC patients. Stimulation with IL2 and TLR agonists (Pam3CSK, Poly I:C, LPS, and CpG ODN) resulted in higher proliferative response of peripheral blood lymphocytes from HI compared to OC patients. However, the role of other immune cells that may influence the TLR ligand stimulation induced activation

status of lymphocytes cannot be ignored (125). Impairment in TLR expression/signaling can be viewed as a strategy employed by tumor cells to avoid immune recognition.

TLRs AND $\gamma\delta$ T CELLS IN DISEASES

Studies have demonstrated the protective role of $\gamma\delta$ T cells in infection and inflammation (154–157). Inoue et al. showed that during mycobacterial infection, $\gamma\delta$ T cells precedes the $\alpha\beta$ T cells, indicating role of $\gamma\delta$ T cells as first line of defense against infections (158). The conserved molecular patterns associated with pathogens are directly recognized by $\gamma\delta$ T cells leading to rapid protective response against the danger signal. Unlike $\alpha\beta$ TCR, $\gamma\delta$ TCR acts as pattern recognition receptor providing advantage in anti-infection immunity by directly initiating cytotoxicity against infected cells or through production of cytokine to involve multiple immune system components to combat infection (159, 160). Activated $\gamma\delta$ T cells through TLR3 and TLR4 ligands rescue the repressed maturation of virus-infected DCs and mount a potent antiviral response (58, 140). Malarial infection in MyD88 deficient mice resulted in impairment in CD27 $^{-}$ IL-17A-producing $\gamma\delta$ T cell without affecting the IFN- γ producing $\gamma\delta$ T cells (161). This study specifies the role of TLR in promoting proliferation

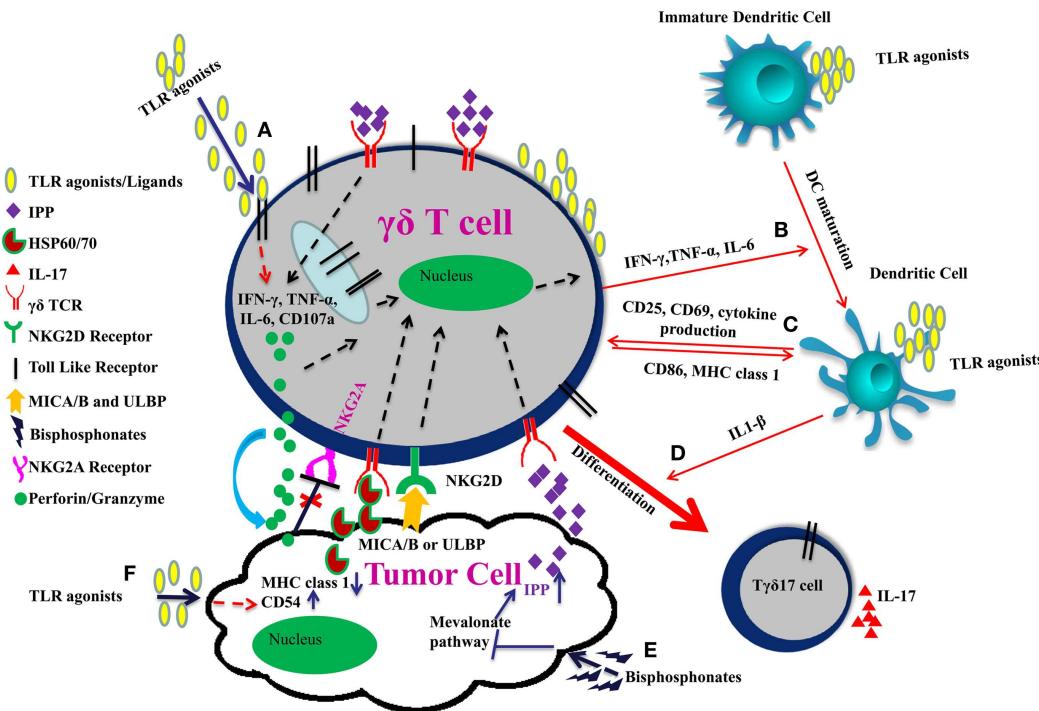


FIGURE 1 | Improving $\gamma\delta$ T cell functions by TLRs in combinatorial therapy. **(A)** TLR agonists induce effector function of $\gamma\delta$ T cells through IFN- γ , TNF- α , IL-6 secretion, and increased expression of CD107a. **(B)** IFN- γ , TNF- α , and IL-6 secreted by $\gamma\delta$ T cells and TLR agonists promote the maturation of dendritic cell. **(C)** $\gamma\delta$ T cells upregulate CD86 and MHC I expression on DCs and are themselves activated through up-regulation of CD25, CD69, and cytokine production thereby modulating each other's function. **(D)** Co-stimulation of $\gamma\delta$ T cells with TLR agonists and IL-1 β secreted by dendritic cells promote their polarization toward IL17 producing cells. **(E)** $\gamma\delta$ TCR also recognizes the specific molecular patterns

such as IPP, which are induced upon inhibition of mevalonate pathway by bisphosphonates. Moreover, NKG2D receptor on $\gamma\delta$ T cells recognizes MICA/B or ULBP expressed on tumor cells. This binding enhances release of perforins and granzymes by the $\gamma\delta$ T cells leading to tumor cell lysis. **(F)** TLR agonists act as adjuvants and can induce CD54 expression and downregulation of MHC class 1 on tumor cells. Interaction between CD54 and its ligand CD11a/CD18 trigger effector functions in $\gamma\delta$ T cells. Downregulation of MHC class 1 molecule on tumor cells result in reduced signaling through the inhibitory receptor NKG2A on $\gamma\delta$ T cells, which enhances the cytotoxic potential of $\gamma\delta$ T cell.

of proinflammatory $\gamma\delta$ T cells. Another study by Martin et al. showed that IL17 producing $\gamma\delta$ T cells express TLR1 and TLR2 and expand in response to their ligands and mount an adequate response against heat-killed *M. tuberculosis* or *C. albicans* infection (118). However, $\gamma\delta$ T cell are also known to directly recognize the pathogen-derived molecules and mediate cytotoxic effector function either through secretion of perforin and granzyme B or by secretion of proinflammatory cytokine IL17 (162–164). The involvement of TLRs in regulating anti-microbial $\gamma\delta$ T cell function should be investigated in depth to exploit it as a cell based therapy for infectious diseases.

CONCLUDING REMARKS

The characteristic copious IFN- γ or IL17 secretion, MHC-independent antigen recognition, tissue tropism, and potent cytotoxicity make $\gamma\delta$ T cells promising targets for immunotherapy. Similar to $\alpha\beta$ T cells, $\gamma\delta$ T cells exhibit functional and phenotypic plasticity, which influences the nature of the downstream adaptive immune response. The adoptive transfer of *ex vivo* expanded V γ 9V δ 2 T cells or *in vivo* activation of V γ 9V δ 2 T cells (phospho-antigens or amino-bisphosphonates) can be utilized as adjuvant to conventional therapies. Clinical trials of V γ 9V δ 2 T cells as immunotherapeutic agents have shown encouraging results that could be attributed to its low toxicity grade. Combinations of cellular immune-based therapies with chemotherapy and other anti-tumor agents may be of clinical benefit in the treatment of malignancies. Combinatorial treatment using, chemotherapeutic agents or bisphosphonate zoledronate (ZOL) sensitizes tumor-derived cell lines to rapid $\gamma\delta$ T cells killing. V γ 9V δ 2 T cell triggering may be also enhanced by combining TCR stimulation with engagement of TLRs. Various TLR agonists are currently under investigation in clinical trials for their ability to orchestrate anti-tumor immunity. In one study, simultaneous use of both Imiquimod (TLR7 agonist) and CpG-ODN (TLR9 agonist) loaded onto virus like nanoparticles was found to be effective in triggering effector and memory CD8 $^+$ T cell response (165). Similarly, combination of $\gamma\delta$ T cells and DCs along with nanoparticle loaded TLR agonists can be employed for developing effective immunotherapeutic strategies. The direct or indirect stimulation of $\gamma\delta$ T cells by TLR agonists could be a strategy to optimize Th1-mediated immune responses as adjuvant in vaccines against infectious or malignant diseases.

Administration of an “immunogenic chemotherapy” (such as oxaliplatin or anthracycline or an X-ray-based regimen) or local delivery of TLR surrogates in the tumor microenvironment (which stimulate local DCs and provides a source of IL-1 β) may be also instrumental in polarization of $\gamma\delta$ TILs into IL17 producing cells. Ty δ 17 cells play a crucial role in anti-microbial immunity but their role in tumor immunity remains controversial. Ty δ 17 have both pro and anti-tumor properties. TLR use in combinatorial therapy, therefore, could be a double edged sword. Careful use of TLR agonists in combinatorial $\gamma\delta$ T cell based therapy is needed to strike the balance between pro and anti-tumor effects (**Figure 1**).

REFERENCES

- Tipping PG. Toll-like receptors: the interface between innate and adaptive immunity. *J Am Soc Nephrol* (2006) **17**(7):1769–71. doi:10.1681/ASN.2006050489
- Holtmeier W, Kabelitz D. Gammadelta T cells link innate and adaptive immune responses. *Chem Immunol Allergy* (2005) **86**:151–83. doi:10.1159/000086659
- Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* (2001) **2**(8):675–80. doi:10.1038/90609
- Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* (2010) **10**(7):467–78. doi:10.1038/nri2781
- Hayday AC. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* (2009) **31**(2):184–96. doi:10.1016/j.immuni.2009.08.006
- Wesch D, Peters C, Oberg HH, Pietschmann K, Kabelitz D. Modulation of gammadelta T cell responses by TLR ligands. *Cell Mol Life Sci* (2011) **68**(14):2357–70. doi:10.1007/s0018-011-0699-1
- Hayday AC. [Gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol* (2000) **18**:975–1026. doi:10.1146/annurev.immunol.18.1.975
- Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annu Rev Immunol* (2003) **21**:139–76. doi:10.1146/annurev.immunol.21.120601.141107
- Velilla PA, Rugeles MT, Chougnet CA. Defective antigen-presenting cell function in human neonates. *Clin Immunol* (2006) **121**(3):251–9. doi:10.1016/j.clim.2006.08.010
- De Rosa SC, Andrus JP, Perfetto SP, Mantovani JJ, Herzenberg LA, Herzenberg LA, et al. Ontogeny of gamma delta T cells in humans. *J Immunol* (2004) **172**(3):1637–45. doi:10.4049/jimmunol.172.3.1637
- Gibbons DL, Haque SF, Silberzahn T, Hamilton K, Langford C, Ellis P, et al. Neonates harbour highly active gammadelta T cells with selective impairments in preterm infants. *Eur J Immunol* (2009) **39**(7):1794–806. doi:10.1002/eji.200939222
- Shin S, El-Diwany R, Schaffert S, Adams EJ, Garcia KC, Pereira P, et al. Antigen recognition determinants of gammadelta T cell receptors. *Science* (2005) **308**(5719):252–5. doi:10.1126/science.1106480
- Kalyan S, Kabelitz D. Defining the nature of human gammadelta T cells: a biographical sketch of the highly empathetic. *Cell Mol Immunol* (2013) **10**(1):21–9. doi:10.1038/cmi.2012.44
- Li H, Lebedeva MI, Llera AS, Fields BA, Brenner MB, Mariuzza RA. Structure of the Vdelta domain of a human gammadelta T-cell antigen receptor. *Nature* (1998) **391**(6666):502–6. doi:10.1038/35172
- Vavassori S, Kumar A, Wan GS, Ramanjaneyulu GS, Cavallari M, El Daker S, et al. Butyrophilin 3A1 binds phosphorylated antigens and stimulates human gammadelta T cells. *Nat Immunol* (2013) **14**(9):908–16. doi:10.1038/ni.2665
- Bordessoule D, Gaulard P, Mason DY. Preferential localisation of human lymphocytes bearing gamma delta T cell receptors to the red pulp of the spleen. *J Clin Pathol* (1990) **43**(6):461–4. doi:10.1136/jcp.43.6.461
- Itohara S, Farr AG, Lafaille JJ, Bonneville M, Takagaki Y, Haas W, et al. Homing of a gamma delta thymocyte subset with homogeneous T-cell receptors to mucosal epithelia. *Nature* (1990) **343**(6260):754–7. doi:10.1038/343754a0
- Goodman T, Lefrancois L. Intraepithelial lymphocytes. Anatomical site, not T cell receptor form, dictates phenotype and function. *J Exp Med* (1989) **170**(5):1569–81. doi:10.1084/jem.170.5.1569
- Cai Y, Shen X, Ding C, Qi C, Li K, Li X, et al. Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity* (2011) **35**(4):596–610. doi:10.1016/j.immuni.2011.08.001
- Gray EE, Suzuki K, Cyster JG. Cutting edge: identification of a motile IL-17-producing gammadelta T cell population in the dermis. *J Immunol* (2011) **186**(11):6091–5. doi:10.4049/jimmunol.1100427
- Havran WL, Allison JP. Origin of Thy-1+ dendritic epidermal cells of adult mice from fetal thymic precursors. *Nature* (1990) **344**(6261):68–70. doi:10.1038/344068a0
- Jameson J, Ugarte K, Chen N, Yachi P, Fuchs E, Boismenu R, et al. A role for skin gammadelta T cells in wound repair. *Science* (2002) **296**(5568):747–9. doi:10.1126/science.1069639
- Sharp LL, Jameson JM, Cauvi G, Havran WL. Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1. *Nat Immunol* (2005) **6**(1):73–9. doi:10.1038/ni1152
- Chien YH, Jones R, Crowley MP. Recognition by gamma/delta T cells. *Annu Rev Immunol* (1996) **14**:511–32. doi:10.1146/annurev.immunol.14.1.511
- Adams EJ, Chien YH, Garcia KC. Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22. *Science* (2005) **308**(5719):227–31. doi:10.1126/science.1106885

26. Bluestone JA, Cron RQ, Cotterman M, Houlden BA, Matis LA. Structure and specificity of T cell receptor gamma/delta on major histocompatibility complex antigen-specific CD3+, CD4-, CD8- T lymphocytes. *J Exp Med* (1988) **168**(5):1899–916. doi:10.1084/jem.168.5.1899
27. Crowley MP, Reich Z, Mavaddat N, Altman JD, Chien Y. The recognition of the nonclassical major histocompatibility complex (MHC) class I molecule, T10, by the gammadelta T cell, G8. *J Exp Med* (1997) **185**(7):1223–30. doi:10.1084/jem.185.7.1223
28. Bonneville M, Ito K, Krecko EG, Itohara S, Kappes D, Ishida I, et al. Recognition of a self major histocompatibility complex TL region product by gamma delta T-cell receptors. *Proc Natl Acad Sci U S A* (1989) **86**(15):5928–32. doi:10.1073/pnas.86.15.5928
29. Matis LA, Fry AM, Cron RQ, Cotterman MM, Dick RF, Bluestone JA. Structure and specificity of a class II MHC alloreactive gamma delta T cell receptor heterodimer. *Science* (1989) **245**(4919):746–9. doi:10.1126/science.2528206
30. Schild H, Mavaddat N, Litzenberger C, Ehrlich EW, Davis MM, Bluestone JA, et al. The nature of major histocompatibility complex recognition by gamma delta T cells. *Cell* (1994) **76**(1):29–37. doi:10.1016/0092-8674(94)90170-8
31. Sciammas R, Kodukula P, Tang Q, Hendricks RL, Bluestone JA. T cell receptor-gamma/delta cells protect mice from herpes simplex virus type 1-induced lethal encephalitis. *J Exp Med* (1997) **185**(11):1969–75. doi:10.1084/jem.185.11.1969
32. Johnson RM, Lancki DW, Sperling AI, Dick RF, Spear PG, Fitch FW, et al. A murine CD4-, CD8- T cell receptor-gamma delta T lymphocyte clone specific for herpes simplex virus glycoprotein I. *J Immunol* (1992) **148**(4):983–8.
33. Zeng X, Wei YL, Huang J, Newell EW, Yu H, Kidd BA, et al. Gammadelta T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific interleukin-17 response. *Immunity* (2012) **37**(3):524–34. doi:10.1016/j.immuni.2012.06.011
34. Dieudé M, Striegl H, Tyznik AJ, Wang J, Behar SM, Piccirillo CA, et al. Cardiolipin binds to CD1d and stimulates CD1d-restricted gammadelta T cells in the normal murine repertoire. *J Immunol* (2011) **186**(8):4771–81. doi:10.4049/jimmunol.1000921
35. Zhang L, Jin N, Nakayama M, O'Brien RL, Eisenbarth GS, Born WK. Gamma delta T cell receptors confer autonomous responsiveness to the insulin-peptide B9-23. *J Autoimmun* (2010) **34**(4):478–84. doi:10.1016/j.jaut.2009.12.008
36. Havran WL, Chien YH, Allison JP. Recognition of self antigens by skin-derived T cells with invariant gamma delta antigen receptors. *Science* (1991) **252**(5011):1430–2. doi:10.1126/science.1828619
37. Tanaka Y, Sano S, Nieves E, De Libero G, Rosa D, Modlin RL, et al. Nonpeptide ligands for human gamma delta T cells. *Proc Natl Acad Sci U S A* (1994) **91**(17):8175–9. doi:10.1073/pnas.91.17.8175
38. Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, Bloom BR. Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature* (1995) **375**(6527):155–8. doi:10.1038/375155a0
39. Rust CJ, Verreck F, Vietor H, Koning F. Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the V gamma 9 region. *Nature* (1990) **346**(6284):572–4. doi:10.1038/346572a0
40. Willcox CR, Pitard V, Netzer S, Couzi L, Salim M, Silberzahn T, et al. Cytomegalovirus and tumor stress surveillance by binding of a human gammadelta T cell antigen receptor to endothelial protein C receptor. *Nat Immunol* (2012) **13**(9):872–9. doi:10.1038/ni.2394
41. Scotet E, Martinez LO, Grant E, Barbaras R, Jenö P, Guiraud M, et al. Tumor recognition following Vgamma9Vdelta2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I. *Immunity* (2005) **22**(1):71–80. doi:10.1016/j.jimmuni.2004.11.012
42. Russano AM, Agea E, Corazzi L, Postle AD, De Libero G, Porcelli S, et al. Recognition of pollen-derived phosphatidyl-ethanolamine by human CD1d-restricted gamma delta T cells. *J Allergy Clin Immunol* (2006) **117**(5):1178–84. doi:10.1016/j.jaci.2006.01.001
43. Bai L, Picard D, Anderson B, Chaudhary V, Luoma A, Jabri B, et al. The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vdelta1 TCR. *Eur J Immunol* (2012) **42**(9):2505–10. doi:10.1002/eji.201242531
44. Uldrich AP, Le Nours J, Pellicci DG, Gherardin NA, McPherson KG, Lim RT, et al. CD1d-lipid antigen recognition by the gammadelta TCR. *Nat Immunol* (2013) **14**(11):1137–45. doi:10.1038/ni.2713
45. Luoma AM, Castro CD, Mayassi T, Bembinstre LA, Bai L, Picard D, et al. Crystal structure of Vdelta1 T cell receptor in complex with CD1d-sulfatide shows MHC-like recognition of a self-lipid by human gammadelta T cells. *Immunity* (2013) **39**(6):1032–42. doi:10.1016/j.immuni.2013.11.001
46. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science* (1998) **279**(5357):1737–40. doi:10.1126/science.279.5357.1737
47. Wu J, Groh V, Spies T. T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial gamma delta T cells. *J Immunol* (2002) **169**(3):1236–40. doi:10.4049/jimmunol.169.3.1236
48. Kong Y, Cao W, Xi X, Ma C, Cui L, He W. The NKG2D ligand ULBP4 binds to TCRgamma9/delta2 and induces cytotoxicity to tumor cells through both TCRgammadelta and NKG2D. *Blood* (2009) **114**(2):310–7. doi:10.1182/blood-2008-12-196287
49. Laad AD, Thomas ML, Fakih AR, Chiplunkar SV. Human gamma delta T cells recognize heat shock protein-60 on oral tumor cells. *Int J Cancer* (1999) **80**(5):709–14. doi:10.1002/(SICI)1097-0215(19990301)80:5<709::AID-IJC14>3.0.CO;2-R
50. Thomas ML, Samant UC, Deshpande RK, Chiplunkar SV. Gammadelta T cells lyse autologous and allogenic oesophageal tumours: involvement of heat-shock proteins in the tumour cell lysis. *Cancer Immunol Immunother* (2000) **48**(11):653–9. doi:10.1007/s002620050014
51. Zhang H, Hu H, Jiang X, He H, Cui L, He W. Membrane HSP70: the molecule triggering gammadelta T cells in the early stage of tumorigenesis. *Immunol Invest* (2005) **34**(4):453–68. doi:10.1080/08820130500265349
52. Hirsh MI, Hashiguchi N, Chen Y, Yip L, Junger WG. Surface expression of HSP72 by LPS-stimulated neutrophils facilitates gammadelta T cell-mediated killing. *Eur J Immunol* (2006) **36**(3):712–21. doi:10.1002/eji.200535422
53. Kotsopoulos M, Tanner JE, Alfieri C. Heat shock protein 90 expression in Epstein-Barr virus-infected B cells promotes gammadelta T-cell proliferation in vitro. *J Virol* (2005) **79**(11):7255–61. doi:10.1128/JVI.79.11.7255-7261.2005
54. Morita CT, Beckman EM, Bukowski JF, Tanaka Y, Band H, Bloom BR, et al. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells. *Immunity* (1995) **3**(4):495–507. doi:10.1016/1074-7613(95)90178-7
55. Gogoi D, Dar AA, Chiplunkar SV. Involvement of Notch in activation and effector functions of gammadelta T cells. *J Immunol* (2014) **192**(5):2054–62. doi:10.4049/jimmunol.1300369
56. Rincon-Orozco B, Kunzmann V, Wrobel P, Kabelitz D, Steinle A, Herrmann T. Activation of V gamma 9V delta 2 T cells by NKG2D. *J Immunol* (2005) **175**(4):2144–51. doi:10.4049/jimmunol.175.4.2144
57. Nedellec S, Sabourin C, Bonneville M, Scotet E. NKG2D costimulates human V gamma 9V delta 2 T cell antitumor cytotoxicity through protein kinase C theta-dependent modulation of early TCR-induced calcium and transduction signals. *J Immunol* (2010) **185**(1):55–63. doi:10.4049/jimmunol.1000373
58. Devilder MC, Allain S, Dousset C, Bonneville M, Scotet E. Early triggering of exclusive IFN-gamma responses of human Vgamma9Vdelta2 T cells by TLR-activated myeloid and plasmacytoid dendritic cells. *J Immunol* (2009) **183**(6):3625–33. doi:10.4049/jimmunol.0901571
59. Shojaei H, Oberg HH, Juricke M, Marischen L, Kunz M, Mundhenke C, et al. Toll-like receptors 3 and 7 agonists enhance tumor cell lysis by human gammadelta T cells. *Cancer Res* (2009) **69**(22):8710–7. doi:10.1158/0008-5472.CAN-09-1602
60. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* (2002) **20**:197–216. doi:10.1146/annurev.immunol.20.083001.084359
61. Mogeness TH. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev* (2009) **22**(2):240–73. doi:10.1128/CMR.00046-08
62. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* (1989) **54**(Pt 1):1–13. doi:10.1101/SQB.1989.054.01.003
63. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* (2006) **124**(4):783–801. doi:10.1016/j.cell.2006.02.015
64. Beutler BA. TLRs and innate immunity. *Blood* (2009) **113**(7):1399–407. doi:10.1182/blood-2008-07-019307

65. Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature* (2007) **449**(7164):819–26. doi:10.1038/nature06246
66. Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. *Microbes Infect* (2004) **6**(15):1382–7. doi:10.1016/j.micinf.2004.08.018
67. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* toll protein signals activation of adaptive immunity. *Nature* (1997) **388**(6640):394–7. doi:10.1038/41131
68. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol* (2005) **17**(1):1–14. doi:10.1093/intimm/dxh186
69. Shi Z, Cai Z, Sanchez A, Zhang T, Wen S, Wang J, et al. A novel toll-like receptor that recognizes vesicular stomatitis virus. *J Biol Chem* (2011) **286**(6):4517–24. doi:10.1074/jbc.M110.159590
70. Oldenburg M, Krüger A, Ferstl R, Kaufmann A, Nees G, Sigmund A, et al. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. *Science* (2012) **337**(6098):1111–5. doi:10.1126/science.1220363
71. Koblancky AA, Jankovic D, Oh H, Hiieny S, Sungnak W, Mathur R, et al. Recognition of profilin by toll-like receptor 12 is critical for host resistance to *Toxoplasma gondii*. *Immunity* (2013) **38**(1):119–30. doi:10.1016/j.immuni.2012.09.016
72. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to *Drosophila* toll. *Proc Natl Acad Sci U S A* (1998) **95**(2):588–93. doi:10.1073/pnas.95.2.588
73. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* (1998) **282**(5396):2085–8. doi:10.1126/science.282.5396.2085
74. Tabata K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K, et al. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc Natl Acad Sci U S A* (2004) **101**(10):3516–21. doi:10.1073/pnas.0400525101
75. Brinkmann MM, Spooner E, Hoebe K, Beutler B, Ploegh HL, Kim YM. The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling. *J Cell Biol* (2007) **177**(2):265–75. doi:10.1083/jcb.200612056
76. Kim YM, Brinkmann MM, Paquet ME, Ploegh HL. UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes. *Nature* (2008) **452**(7184):234–8. doi:10.1038/nature06726
77. Ewald SE, Lee BL, Lau L, Wickliffe KE, Shi GP, Chapman HA, et al. The ectodomain of toll-like receptor 9 is cleaved to generate a functional receptor. *Nature* (2008) **456**(7222):658–62. doi:10.1038/nature07405
78. O'Neill LA, Golenbock D, Bowie AG. The history of toll-like receptors—redefining innate immunity. *Nat Rev Immunol* (2013) **13**(6):453–60. doi:10.1038/nri3446
79. Wesche H, Henzel WJ, Shillinglaw W, Li S, Cao Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* (1997) **7**(6):837–47. doi:10.1016/S1074-7613(00)80402-1
80. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the toll-like receptor signaling. *J Immunol* (2002) **169**(12):6668–72. doi:10.4049/jimmunol.169.12.6668
81. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* (2003) **4**(2):161–7. doi:10.1038/ni886
82. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* (2003) **301**(5633):640–3. doi:10.1126/science.1087262
83. Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, et al. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *J Exp Med* (2003) **198**(7):1043–55. doi:10.1084/jem.20031023
84. Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, et al. TRAM is specifically involved in the toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol* (2003) **4**(11):1144–50. doi:10.1038/ni986
85. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, et al. Mal (MyD88-adapter-like) is required for toll-like receptor-4 signal transduction. *Nature* (2001) **413**(6851):78–83. doi:10.1038/35092578
86. Horng T, Barton GM, Medzhitov R. TIRAP: an adapter molecule in the toll signaling pathway. *Nat Immunol* (2001) **2**(9):835–41. doi:10.1038/ni0901-835
87. Carty M, Goodbody R, Schröder M, Stack J, Moynagh PN, Bowie AG. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent toll-like receptor signaling. *Nat Immunol* (2006) **7**(10):1074–81. doi:10.1038/ni1382
88. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdörfer B, Giese T, et al. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* (2002) **168**(9):4531–7. doi:10.4049/jimmunol.168.9.4531
89. Mansson A, Adner M, Cardell LO. Toll-like receptors in cellular subsets of human tonsil T cells: altered expression during recurrent tonsillitis. *Respir Res* (2006) **7**:36. doi:10.1186/1465-9921-7-36
90. Kabelitz D. Expression and function of toll-like receptors in T lymphocytes. *Curr Opin Immunol* (2007) **19**(1):39–45. doi:10.1016/j.coim.2006.11.007
91. Caron G, Duluc D, Frémaux I, Jeannin P, David C, Gascan H, et al. Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-gamma production by memory CD4+ T cells. *J Immunol* (2005) **175**(3):1551–7. doi:10.4049/jimmunol.175.3.1551
92. Imanishi T, Hara H, Suzuki S, Suzuki N, Akira S, Saito T. Cutting edge: TLR2 directly triggers Th1 effector functions. *J Immunol* (2007) **178**(11):6715–9. doi:10.4049/jimmunol.178.11.6715
93. Dai J, Liu B, Li Z. Regulatory T cells and toll-like receptors: what is the missing link? *Int Immunopharmacol* (2009) **9**(5):528–33. doi:10.1016/j.intimp.2009.01.027
94. Liu H, Komai-Koma M, Xu D, Liew FY. Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. *Proc Natl Acad Sci U S A* (2006) **103**(18):7048–53. doi:10.1073/pnas.0601554103
95. Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J Clin Invest* (2006) **116**(2):485–94. doi:10.1172/JCI25439
96. Crellin NK, Garcia RV, Hadisfar O, Allan SE, Steiner TS, Levington MK. Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. *J Immunol* (2005) **175**(12):8051–9. doi:10.4049/jimmunol.175.12.8051
97. Nyirenda MH, Sanvito L, Darlington PJ, O'Brien K, Zhang GX, Constantinescu CS, et al. TLR2 stimulation drives human naïve and effector regulatory T cells into a Th17-like phenotype with reduced suppressive function. *J Immunol* (2011) **187**(5):2278–90. doi:10.4049/jimmunol.1003715
98. Reynolds JM, Pappu BP, Peng J, Martinez GJ, Zhang Y, Chung Y, et al. Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. *Immunity* (2010) **32**(5):692–702. doi:10.1016/j.jimmuni.2010.04.010
99. Abdollahi-Roodsaz S, Joosten LA, Koenders MI, Devesa I, Roelofs MF, Radstake TR, et al. Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis. *J Clin Invest* (2008) **118**(1):205–16. doi:10.1172/JCI32639
100. Reynolds JM, Martinez GJ, Chung Y, Dong C. Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. *Proc Natl Acad Sci U S A* (2012) **109**(32):13064–9. doi:10.1073/pnas.1120585109
101. Li J, Wang FP, She WM, Yang CQ, Li L, Tu CT, et al. Enhanced high-mobility group box 1 (HMGB1) modulates regulatory T cells (Treg)/T helper 17 (Th17) balance via toll-like receptor (TLR)-4-interleukin (IL)-6 pathway in patients with chronic hepatitis B. *J Viral Hepat* (2014) **21**(2):129–40. doi:10.1111/jvh.12152
102. Reynolds JM, Dong C. Toll-like receptor regulation of effector T lymphocyte function. *Trends Immunol* (2013) **34**(10):511–9. doi:10.1016/j.it.2013.06.003
103. Mercier BC, Cottalorda A, Coupet CA, Marvel J, Bonnefoy-Bérard N. TLR2 engagement on CD8 T cells enables generation of functional memory cells in response to a suboptimal TCR signal. *J Immunol* (2009) **182**(4):1860–7. doi:10.4049/jimmunol.0801167
104. Cottalorda A, Mercier BC, Mbitikon-Kobo FM, Arpin C, Teoh DY, McMichael A, et al. TLR2 engagement on memory CD8(+) T cells improves their cytokine-mediated proliferation and IFN-gamma secretion in the absence of Ag. *Eur J Immunol* (2009) **39**(10):2673–81. doi:10.1002/eji.200939627
105. Asprodites N, Zheng L, Geng D, Velasco-Gonzalez C, Sanchez-Perez L, Davila E. Engagement of toll-like receptor-2 on cytotoxic T-lymphocytes occurs in vivo and augments antitumor activity. *FASEB J* (2008) **22**(10):3628–37. doi:10.1096/fj.08-108274

106. Mercier BC, Ventre E, Fogeron ML, Debaud AL, Tomkowiak M, Marvel J, et al. NOD1 cooperates with TLR2 to enhance T cell receptor-mediated activation in CD8 T cells. *PLoS One* (2012) **7**(7):e42170. doi:10.1371/journal.pone.0042170
107. Mandraru R, Murray S, Forman J, Pasare C. Differential ability of surface and endosomal TLRs to induce CD8 T cell responses in vivo. *J Immunol* (2014) **192**(9):4303–15. doi:10.4049/jimmunol.1302244
108. Chalifour A, Jeannin P, Gauchat JF, Blaecke A, Malissard M, N'Guyen T, et al. Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers alpha-defensin production. *Blood* (2004) **104**(6):1778–83. doi:10.1182/blood-2003-08-2820
109. Sivori S, Falco M, Della Chiesa M, Carluomagno S, Vitale M, Moretta L, et al. CpG and double-stranded RNA trigger human NK cells by toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc Natl Acad Sci U S A* (2004) **101**(27):10116–21. doi:10.1073/pnas.0403744101
110. Hart OM, Athie-Morales V, O'Connor GM, Gardiner CM. TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production. *J Immunol* (2005) **175**(3):1636–42. doi:10.4049/jimmunol.175.3.1636
111. Souza-Fonseca-Guimaraes F, Adib-Conquy M, Cavaillon JM. Natural killer (NK) cells in antibacterial innate immunity: angels or devils? *Mol Med* (2012) **18**:270–85. doi:10.2119/molmed.2011.00201
112. Marcenaro E, Ferranti B, Falco M, Moretta L, Moretta A. Human NK cells directly recognize *Mycobacterium bovis* via TLR2 and acquire the ability to kill monocyte-derived DC. *Int Immunopharmacol* (2008) **20**(9):1155–67. doi:10.1093/intimm/dxn073
113. Bellora F, Castriconi R, Dondero A, Pessino A, Nencioni A, Liggieri G, et al. TLR activation of tumor-associated macrophages from ovarian cancer patients triggers cytolytic activity of NK cells. *Eur J Immunol* (2014) **44**(6):1814–22. doi:10.1002/eji.201344130
114. Kim M, Osborne NR, Zeng W, Donaghy H, McKinnon K, Jackson DC, et al. Herpes simplex virus antigens directly activate NK cells via TLR2, thus facilitating their presentation to CD4 T lymphocytes. *J Immunol* (2012) **188**(9):4158–70. doi:10.4049/jimmunol.1103450
115. Lu H, Dietsch GN, Matthews MA, Yang Y, Ghanekar S, Inokuma M, et al. VTX-2337 is a novel TLR8 agonist that activates NK cells and augments ADCC. *Clin Cancer Res* (2012) **18**(2):499–509. doi:10.1158/1078-0432.CCR-11-1625
116. Mokuno Y, Matsuguchi T, Takano M, Nishimura H, Washizu J, Ogawa T, et al. Expression of toll-like receptor 2 on gamma delta T cells bearing invariant V gamma 6/V delta 1 induced by Escherichia coli infection in mice. *J Immunol* (2000) **165**(2):931–40. doi:10.4049/jimmunol.165.2.931
117. Deetz CO, Hebbeler AM, Propp NA, Cairo C, Tikhonov I, Pauza CD. Gamma interferon secretion by human Vgamma2Vdelta2 T cells after stimulation with antibody against the T-cell receptor plus the toll-like receptor 2 agonist Pam3Cys. *Infect Immun* (2006) **74**(8):4505–11. doi:10.1128/IAI.00088-06
118. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* (2009) **31**(2):321–30. doi:10.1016/j.jimmuni.2009.06.020
119. Schwacha MG, Daniel T. Up-regulation of cell surface toll-like receptors on circulating gammadelta T-cells following burn injury. *Cytokine* (2008) **44**(3):328–34. doi:10.1016/j.cyto.2008.09.001
120. Schwacha MG, Rani M, Zhang Q, Nunez-Cantu O, Cap AP. Mitochondrial damage-associated molecular patterns activate gammadelta T-cells. *Innate Immun* (2014) **20**(3):261–8. doi:10.1177/1753425913488969
121. Hedges JF, Lubick KJ, Jutila MA. Gamma delta T cells respond directly to pathogen-associated molecular patterns. *J Immunol* (2005) **174**(10):6045–53. doi:10.4049/jimmunol.174.10.6045
122. Wesch D, Beetz S, Oberg HH, Marget M, Krengel K, Kabelitz D. Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes. *J Immunol* (2006) **176**(3):1348–54. doi:10.4049/jimmunol.176.3.1348
123. Zhang SY, Jouanguy E, Ugolini S, Smahi A, Elain G, Romero P, et al. TLR3 deficiency in patients with herpes simplex encephalitis. *Science* (2007) **317**(5844):1522–7. doi:10.1126/science.1139522
124. Pietschmann K, Beetz S, Welte S, Martens I, Gruen J, Oberg HH, et al. Toll-like receptor expression and function in subsets of human gammadelta T lymphocytes. *Scand J Immunol* (2009) **70**(3):245–55. doi:10.1111/j.1365-3083.2009.02290.x
125. Paleja B, Anand A, Chaukar D, D'Cruz A, Chiplunkar S. Decreased functional response to toll like receptor ligands in patients with oral cancer. *Hum Immunol* (2013) **74**(8):927–36. doi:10.1016/j.humimm.2013.04.018
126. Shimura H, Nitahara A, Ito A, Tomiyama K, Ito M, Kawai K. Up-regulation of cell surface toll-like receptor 4-MD2 expression on dendritic epidermal T cells after the emigration from epidermis during cutaneous inflammation. *J Dermatol Sci* (2005) **37**(2):101–10. doi:10.1016/j.jdermsci.2004.11.006
127. Cairns B, Maile R, Barnes CM, Frelinger JA, Meyer AA. Increased toll-like receptor 4 expression on T cells may be a mechanism for enhanced T cell response late after burn injury. *J Trauma* (2006) **61**(2):293–8; discussion 298–9. doi:10.1097/01.ta.0000228969.46633.bb
128. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* (1999) **274**(16):10689–92. doi:10.1074/jbc.274.16.10689
129. Yang RB, Mark MR, Gray A, Huang A, Xie MH, Zhang M, et al. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* (1998) **395**(6699):284–8. doi:10.1038/26239
130. Cui Y, Kang L, Cui L, He W. Human gammadelta T cell recognition of lipid A is predominately presented by CD1b or CD1c on dendritic cells. *Biol Direct* (2009) **4**:47. doi:10.1186/1745-6150-4-47
131. Wang X, Sun R, Wei H, Tian Z. High-mobility group box 1 (HMGB1)-toll-like receptor (TLR4)-interleukin (IL)-23-IL-17A axis in drug-induced damage-associated lethal hepatitis: interaction of gammadelta T cells with macrophages. *Hepatology* (2013) **57**(1):373–84. doi:10.1002/hep.25982
132. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang RF. Tumor-infiltrating gammadelta T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway. *Immunity* (2007) **27**(2):334–48. doi:10.1016/j.jimmuni.2007.05.020
133. Choudhary A, Davodeau F, Moreau A, Peyrat MA, Bonneville M, Jotereau F. Selective lysis of autologous tumor cells by recurrent gamma delta tumor-infiltrating lymphocytes from renal carcinoma. *J Immunol* (1995) **154**(8):3932–40.
134. Peng G, Guo Z, Kiniwa Y, Voo KS, Peng W, Fu T, et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science* (2005) **309**(5739):1380–4. doi:10.1126/science.1113401
135. Ye J, Ma C, Hsueh EC, Eickhoff CS, Zhang Y, Varvares MA, et al. Tumor-derived gammadelta regulatory T cells suppress innate and adaptive immunity through the induction of immunosenescence. *J Immunol* (2013) **190**(5):2403–14. doi:10.4049/jimmunol.1202369
136. Ismaili J, Olislagers V, Poupot R, Fournié JJ, Goldman M. Human gamma delta T cells induce dendritic cell maturation. *Clin Immunol* (2002) **103**(3 Pt 1):296–302. doi:10.1006/clim.2002.5218
137. Conti L, Casetti R, Cardone M, Varano B, Martino A, Belardelli F, et al. Reciprocal activating interaction between dendritic cells and pamidronate-stimulated gammadelta T cells: role of CD86 and inflammatory cytokines. *J Immunol* (2005) **174**(1):252–60. doi:10.4049/jimmunol.174.1.252
138. Leslie DS, Vincent MS, Spada FM, Das H, Sugita M, Morita CT, et al. CD1-mediated gamma/delta T cell maturation of dendritic cells. *J Exp Med* (2002) **196**(12):1575–84. doi:10.1084/jem.20021515
139. Shrestha N, Ida JA, Lubinski AS, Pallin M, Kaplan G, Haslett PA. Regulation of acquired immunity by gamma delta T-cell/dendritic-cell interactions. *Ann NY Acad Sci* (2005) **1062**:79–94. doi:10.1196/annals.1358.011
140. Fang H, Welte T, Zheng X, Chang GJ, Holbrook MR, Soong L, et al. Gammadelta T cells promote the maturation of dendritic cells during West Nile virus infection. *FEMS Immunol Med Microbiol* (2010) **59**(1):71–80. doi:10.1111/j.1574-695X.2010.00663.x
141. Fowler DW, Copier J, Dalgleish AG, Bodman-Smith MD. Tripartite immune cell co-operation in the bacillus Calmette Guerin-induced activation of gammadelta T cells. *Immunol Cell Biol* (2013) **91**(7):461–8. doi:10.1038/icb.2013.30
142. Dhar S, Chiplunkar SV. Lysis of aminobisphosphonate-sensitized MCF-7 breast tumor cells by Vgamma9Vdelta2 T cells. *Cancer Immun* (2010) **10**:10.
143. D'Asaro M, La Mendola C, Di Liberto D, Orlando V, Todaro M, Spina M, et al. V gamma 9V delta 2 T lymphocytes efficiently recognize and kill zoledronate-sensitized, imatinib-sensitive, and imatinib-resistant chronic myelogenous leukemia cells. *J Immunol* (2010) **184**(6):3260–8. doi:10.4049/jimmunol.0903454
144. Chargui J, Combaret V, Scaglione V, Iacono I, Péri V, Valteau-Couanet D, et al. Bromohydron pyrophosphate-stimulated Vgamma9delta2 T cells expanded

- ex vivo from patients with poor-prognosis neuroblastoma lyse autologous primary tumor cells. *J Immunother* (2010) **33**(6):591–8. doi:10.1097/CJI.0b013e3181dda207
145. Gogoi D, Chiplunkar SV. Targeting gamma delta T cells for cancer immunotherapy: bench to bedside. *Indian J Med Res* (2013) **138**(5):755–61.
146. Weeratna RD, Makinen SR, McCluskie MJ, Davis HL. TLR agonists as vaccine adjuvants: comparison of CpG ODN and resiquimod (R-848). *Vaccine* (2005) **23**(45):5263–70. doi:10.1016/j.vaccine.2005.06.024
147. Kalyan S, Wesch D, Kabelitz D. Aminobisphosphonates and toll-like receptor ligands: recruiting Vgamma9Vdelta2 T cells for the treatment of hematologic malignancy. *Curr Med Chem* (2011) **18**(34):5206–16. doi:10.2174/092986711798184280
148. Paulos CM, Kaiser A, Wrzesinski C, Hinrichs CS, Cassard L, Boni A, et al. Toll-like receptors in tumor immunotherapy. *Clin Cancer Res* (2007) **13**(18 Pt 1):5280–9. doi:10.1158/1078-0432.CCR-07-1378
149. Smits EL, Ponsaerts P, Berneman ZN, Van Tendeloo VF. The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. *Oncologist* (2008) **13**(8):859–75. doi:10.1634/theoncologist.2008-0097
150. Yu L, Chen S. Toll-like receptors expressed in tumor cells: targets for therapy. *Cancer Immunol Immunother* (2008) **57**(9):1271–8. doi:10.1007/s00262-008-0459-8
151. Conroy H, Marshall NA, Mills KH. TLR ligand suppression or enhancement of Treg cells? A double-edged sword in immunity to tumours. *Oncogene* (2008) **27**(2):168–80. doi:10.1038/sj.onc.1210910
152. Ridnour LA, Cheng RY, Switzer CH, Heinecke JL, Ambros S, Glynn S, et al. Molecular pathways: toll-like receptors in the tumor microenvironment – poor prognosis or new therapeutic opportunity. *Clin Cancer Res* (2013) **19**(6):1340–6. doi:10.1158/1078-0432.CCR-12-0408
153. Inatsuka C, Yang Y, Gad E, Rastetter L, Disis ML, Lu H. Gamma delta T cells are activated by polysaccharide K (PSK) and contribute to the anti-tumor effect of PSK. *Cancer Immunol Immunother* (2013) **62**(8):1335–45. doi:10.1007/s00262-013-1436-4
154. Hiromatsu K, Yoshikai Y, Matsuzaki G, Ohga S, Muramori K, Matsumoto K, et al. A protective role of gamma/delta T cells in primary infection with *Listeria monocytogenes* in mice. *J Exp Med* (1992) **175**(1):49–56. doi:10.1084/jem.175.1.49
155. Hara T, Mizuno Y, Takaki K, Takada H, Akeda H, Aoki T, et al. Predominant activation and expansion of V gamma 9-bearing gamma delta T cells in vivo as well as in vitro in *Salmonella* infection. *J Clin Invest* (1992) **90**(1):204–10. doi:10.1172/JCI115837
156. Kühl AA, Pawlowski NN, Grollich K, Loddenkemper C, Zeitz M, Hoffmann JC. Aggravation of intestinal inflammation by depletion/deficiency of gammadelta T cells in different types of IBD animal models. *J Leukoc Biol* (2007) **81**(1):168–75. doi:10.1189/jlb.1105696
157. Poccia F, Agrati C, Martini F, Capobianchi MR, Wallace M, Malkovsky M. Antiviral reactivities of gammadelta T cells. *Microbes Infect* (2005) **7**(3):518–28. doi:10.1016/j.micinf.2004.12.009
158. Inoue T, Yoshikai Y, Matsuzaki G, Nomoto K. Early appearing gamma/delta-bearing T cells during infection with Calmette Guerin bacillus. *J Immunol* (1991) **146**(8):2754–62.
159. Beetz S, Wesch D, Marischen L, Welte S, Oberg HH, Kabelitz D. Innate immune functions of human gammadelta T cells. *Immunobiology* (2008) **213**(3–4):173–82. doi:10.1016/j.imbio.2007.10.006
160. Zheng J, Liu Y, Lau YL, Tu W. Gammadelta-T cells: an unpolished sword in human anti-infection immunity. *Cell Mol Immunol* (2013) **10**(1):50–7. doi:10.1038/cmi.2012.43
161. Ribot JC, Chaves-Ferreira M, d’Orey F, Wencker M, Gonçalves-Sousa N, Decalf J, et al. Cutting edge: adaptive versus innate receptor signals selectively control the pool sizes of murine IFN-gamma- or IL-17-producing gammadelta T cells upon infection. *J Immunol* (2010) **185**(11):6421–5. doi:10.4049/jimmunol.1002283
162. Born WK, Zhang L, Nakayama M, Jin N, Chain JL, Huang Y, et al. Peptide antigens for gamma/delta T cells. *Cell Mol Life Sci* (2011) **68**(14):2335–43. doi:10.1007/s00018-011-0697-3
163. Nedellec S, Bonneville M, Scotet E. Human Vgamma9Vdelta2 T cells: from signals to functions. *Semin Immunol* (2010) **22**(4):199–206. doi:10.1016/j.smim.2010.04.004
164. Qin G, Mao H, Zheng J, Sia SF, Liu Y, Chan PL, et al. Phosphoantigen-expanded human gammadelta T cells display potent cytotoxicity against monocyte-derived macrophages infected with human and avian influenza viruses. *J Infect Dis* (2009) **200**(6):858–65. doi:10.1086/605413
165. Goldinger SM, Dummer R, Baumgaertner P, Mihic-Probst D, Schwarz K, Hammann-Haenni A, et al. Nano-particle vaccination combined with TLR-7 and -9 ligands triggers memory and effector CD8(+) T-cell responses in melanoma patients. *Eur J Immunol* (2012) **42**(11):3049–61. doi:10.1002/eji.201142361

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Toll-like receptors and cancer: MYD88 mutation and inflammation

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Pattern recognition receptors (PRRs) expressed on immune cells are crucial for the early detection of invading pathogens, in initiating early innate immune response and in orchestrating the adaptive immune response. PRRs are activated by specific pathogen-associated molecular patterns that are present in pathogenic microbes or nucleic acids of viruses or bacteria. However, inappropriate activation of these PRRs, such as the Toll-like receptors (TLRs), due to genetic lesions or chronic inflammation has been demonstrated to be a major cause of many hematological malignancies. Gain-of-function mutations in the TLR adaptor protein MYD88 found in 39% of the activated B cell type of diffuse large B cell lymphomas and almost 100% of Waldenström's macroglobulinemia further highlight the involvement of TLRs in these malignancies. MYD88 mutations result in the chronic activation of TLR signaling pathways, thus the constitutive activation of the transcription factor NF_κB to promote cell survival and proliferation. These recent insights into TLR pathway driven malignancies warrant the need for a better understanding of TLRs in cancers and the development of novel anti-cancer therapies targeting TLRs. This review focuses on TLR function and signaling in normal or inflammatory conditions, and how mutations can hijack the TLR signaling pathways to give rise to cancer. Finally, we discuss how potential therapeutic agents could be used to restore normal responses to TLRs and have long lasting anti-tumor effects.

Keywords: cancer, drug targets, inflammation, lymphoma, MYD88 L265P, pattern recognition receptors, self-nucleic acid, Toll-like receptors

INTRODUCTION

Pattern recognition receptors (PRRs) are germline-encoded receptors with the ability to relay "danger signals" to the host in order to mediate an early innate immune response. The term "pattern recognition receptors" comes from their ability to recognize specific pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) (1, 2). PRRs can be broadly divided into five distinct subfamilies: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), RIG-1-like receptors (RLRs), and AIM2-like receptors (ALRs). These PRR subfamilies differ in their structures, localization patterns, the distinct types of ligands they recognize, and the activation of specific intracellular signaling cascades to mediate a range of responses such as the regulation of gene transcription, cell activation, and proliferation, and the production of pro-inflammatory cytokines, chemokines, and anti-viral molecules (3).

One of the most well characterized PRR is the TLR (4). TLRs are type I transmembrane proteins with an extracellular domain consisting of leucine-rich repeats and a cytoplasmic domain homologous to that of the interleukin (IL)-1 receptor (5, 6). These evolutionarily conserved receptors are absolutely critical for the host innate immune response against many pathogens (7). Activation of TLRs depends on the number of different ligands they may encounter, which is by large, governed by their subcellular localization. Much insight has been gained in recent years on the localization and trafficking of TLRs and the important roles their

localization play in the way they recognize their ligands. TLRs can be divided into two groups based on their subcellular localization, either on the cell surface or within intracellular compartments (8). Given the ability of TLRs to recognize a large number of pathogen-associated ligands such as glycoproteins, lipopolysaccharides, flagellin, and viral double-strand or single-strand RNAs or DNAs, TLRs have emerged as an important family of PRRs in shaping both the innate and adaptive immunity (7). However, inappropriate activation of these pathways can often lead to chronic inflammatory diseases or cancer.

This review will focus on TLRs and malignancies associated with the dysregulation of TLR signaling pathways. TLR activation by somatic MYD88 mutations and chronic inflammations has been implicated in a number of hematological malignancies. Targeting the TLR signaling network has gained increasing attention from researchers and clinicians seeking strategies to achieve long lasting anti-tumor outcomes. Here, we discuss the signal transduction and immune regulation by TLRs and the immunological malignancies that manifest from dysregulation of TLR pathways, including how targeting these pathways could be an attractive therapeutic regime.

TOLL-LIKE RECEPTORS

Toll-like receptors are probably the best studied PRRs that participate in the first line of host defense against pathogens. TLRs belong to an evolutionarily conserved family of adaptors sharing

homology with the *Drosophila* protein Toll, which is best known for its essential role in establishing dorsoventral polarity during embryogenesis in insects (9). Amino acid sequencing and hydropathy profiling identified Toll as a type I transmembrane protein with a membrane-spanning segment and multiple tandem leucine-rich repeats directed at the extracellular surface (9). Further biochemical and functional studies conducted on the receptor Toll and its leucine repeats established it as a critical pathogen sensing receptor for recognizing bacteria and fungus in *Drosophila* (10). This study later became critical for the discovery of Toll-like homologs (TLRs) in mammals as mediators of the innate immunity (4, 10, 11).

A total of 10 TLRs have been identified in humans and 12 in mice (7). Due to the small repertoire of TLRs available to recognize a virtually unlimited combination of pathogen-associated patterns, each individual TLR must be able to detect and respond to a large number of pathogens ranging from bacteria, fungi, protozoa, and viruses (12, 13). For instance, TLRs 1, 2, and 6 recognize lipo-, glycol-, and acyl-peptides expressed on the surfaces of many Gram-positive and Gram-negative bacteria and mycobacteria (7). Additional cooperation between TLRs 1, 2, and 6 enables them to further discriminate different microbial components (14). TLR4 recognizes lipopolysaccharide components of the cell wall of Gram-negative bacteria through its co-receptor MD-2 (15, 16). In addition, TLR4 can also recognize endogenous ligands such as heat-shock proteins, extracellular matrix components including fibronectin, hyaluronic acid, and heparin sulfate in response to tissue injury (7). The nucleic acid sensing subfamily of TLRs consists of TLRs 3, 7, and 9 and exhibit unique endosomal localization in contrast to the surface expression of the other TLRs (17). These TLRs have the ability to detect nuclear material such as ssRNAs, dsRNAs, and dsDNAs and are vital for anti-viral responses (18–21). Importantly, these nucleic acid sensing TLRs must discriminate between foreign and self-nuclear material to prevent autoimmunity. Due to the relative lack of specificity of TLRs compared to the B cell receptors (BCRs), restriction of self-TLR activation must be achieved through other means. TLRs are protected from engaging self-nuclear material by Unc93b mediated restriction to the endosome (22). In such way, self-nucleic acids are prevented from entering the endosome, but foreign material can enter via endocytosis and be processed in the acidified endosomes in order to activate the endosomal TLRs (23, 24).

Together, the 10 human TLRs can recognize a virtually unlimited combination of pathogens, however, the downstream signaling pathways they share are striking. All TLRs except for TLR3 signal through the adaptor protein MYD88 (25). Upon ligand binding, TLRs induce the dimerization of their ectodomains, bringing the cytoplasmic TIR domains together, and initiating a signaling cascade via signal adaptor molecules. The four main TLR adaptor molecules are the myeloid differentiation response protein 88 (MYD88), Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP; also known as MAL), TIRAP inducing IFN- β (TRIF), and TRIF-related adaptor molecule (TRAM) (Figure 1). These adaptors are used in various combinations by the different TLRs, but these signaling pathways can be broadly classified into either MYD88 dependent or MYD88 independent.

MYD88 DEPENDENT TLR SIGNALING

With the exception of TLR3, all TLRs initiate a MYD88-dependent signaling pathway (26). The signal adaptor protein MYD88 contains two main conserved protein domains; a C-terminal TIR and a N-terminal death domain (DD) (27, 28). Upon TLR activation, MYD88 is recruited to the TIR domain of the activated TLR via TIR–TIR interaction (29). The serine–threonine kinase, IL1-receptor associated kinase 4 (IRAK4), is then recruited to MYD88 through the interaction of their DD domains. IRAK4 then recruits and phosphorylates IRAK1 and IRAK2 to form a structure known as the “Myddosome” (30). Phosphorylation of IRAKs 1 and 2 allows them to interact with the E3 ubiquitin ligase, TRAF6, via their TRAF binding domain (31). TRAF6 then ubiquitylates and activates TAK1 (32), which has the dual ability to activate both the NF κ B pathway and the mitogen-activated protein kinase (MAPK) pathway (26). In resting cells, NF κ B dimers are sequestered in an inactive form in the cytoplasm by the I κ B protein (33). During NF κ B activation, TAK1 phosphorylates and activates I κ B kinase β (IKK β), which in turn phosphorylates I κ B, targeting it for proteosomal degradation (34). The degradation of I κ B releases NF κ B, enabling it to enter the nucleus and bind to sequences known as κ B sites to activate transcription of genes (35). TAK1 also activates the MAPK pathway, leading to the activation of c-Jun N-terminal kinase (JNK), which activates the Jun family of transcription factors (36) (Figure 1).

The MYD88-dependent pathway can be initiated by TLR5 and TLR7-9 using the adaptor MYD88 alone, while the adaptor protein TIRAP is required with MYD88 to initiate signaling downstream of TLR2 and TLR4 (37, 38). In this subset of TLRs, TIRAP acts as a sorting molecule that is necessary for efficient recruitment of MYD88 to the activated TLRs to initiate signal transduction to activate NF κ B and produce pro-inflammatory cytokines (39). During TLR 7 and 9 activation, MYD88 also recruits TRAF3 to activate TBK1 and IKK ϵ , which phosphorylates the transcription factor interferon-regulatory factor 7 (IRF7) and leads to IFN- α production (40, 41). IFN- α production, as with production of other IFNs, is particularly important for anti-viral responses (42) (Figure 1).

MYD88 INDEPENDENT TLR SIGNALING

MYD88-independent TLR3 signaling requires the adaptor molecule TRIF to activate downstream signaling pathways, including the activation of IRF3 and the production of IFN- β (43). TRIF has also been known to participate in signaling downstream of TLR4 for type 1 interferon responses (44). Upon ligand binding, TRIF recruits TRAF3, which acts as a scaffold for the activation of the IKKs, TBK1, and IKK ϵ , leading to the phosphorylation and activation of the transcription factor IRF3 and IFN- β transcription (45, 46). While TLR3 can activate this pathway using TRIF alone, the adaptor TRAM is required for TLR4, where TRAM facilitates the recruitment of TRIF to TLR4 (47). Upon the activation of TRIF, TRAF6 is recruited, which then activates TAK1 through ubiquitination and leading to the subsequent activation of NF κ B (48) (Figure 1). Interestingly, TRAF3 has been shown to play important roles in regulating both the MYD88 dependent and independent response through its differential ubiquitination (49). MYD88-independent signaling triggers the non-degradative

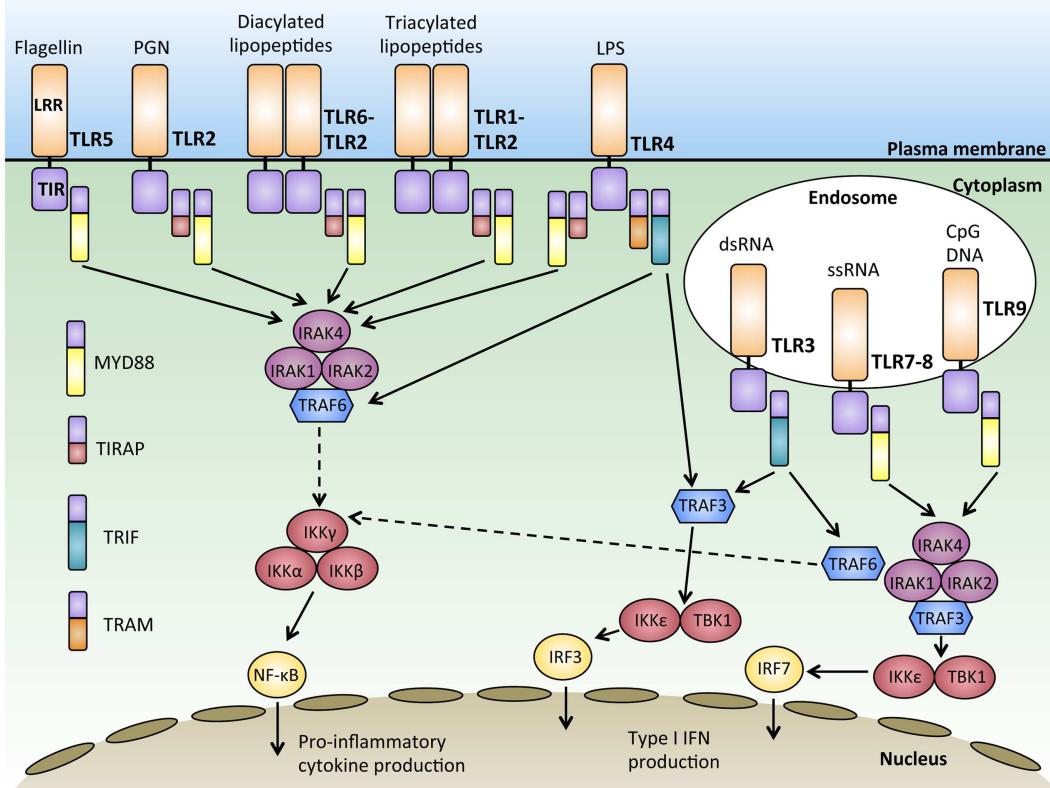


FIGURE 1 | Signal transduction downstream of MYD88-dependent and independent pathways. Activation of Toll-like receptors (TLRs) through binding of their ligand leads to receptor dimerization and the recruitment of adaptor proteins such as MYD88, TIRAP, TRIF, and TRAM. Most of the TLRs form homodimers upon activation while TLR2 can also form heterodimers with either TLR6 or TLR1 to recognize

diacylated and triacylated lipopeptides, respectively. Downstream signals are propagated through the activation of IRAKs-TRAF6 and the IKK complex, culminating in the activation of transcription factors such as nuclear factor- κ B (NF- κ B) and interferon-regulatory factors (IRFs), which regulate the production of pro-inflammatory cytokines and type 1 interferon (IFNs).

self-ubiquitination of TRAF3, promoting IRF3 activation. On the other hand, the MYD88-dependent pathway results in the degradative ubiquitination of TRAF3 and the activation of TAK1 (49). In this manner, TRAF3 acts to balance pro-inflammatory and IFN response by the MYD88 dependent and independent pathways.

HEMATOLOGICAL MALIGNANCY AND MYD88 MUTATION

Inappropriate activation of TLRs due to the somatic acquisition of gain-of-function mutations in the TLR adaptor protein MYD88 has been implicated in many hematological malignancies. Activated B cell type diffuse large B cell lymphoma (ABC-DLBCL), a particularly aggressive subtype of DLBCL whose pathogenesis relies on constitutively active NF- κ B, frequently accumulates MYD88 mutations. 39% of tumor samples contain mutations in MYD88, and strikingly, 29% of those mutations result in a single nucleotide change from leucine into proline at position 265 (L265P) (50). shRNA knockdown of MYD88 in lymphoma cell lines demonstrated that MYD88 mutations are critical for their survival and high NF- κ B transcription factor activity (50). A hyperphosphorylated isoform of IRAK1 was strongly associated with the

L265P mutant form of MYD88, suggesting that this mutation is a gain-of-function mutation that leads to the constitutive activation of downstream IRAKs (50). The effects of the L265P mutation include increased NF- κ B activity as well as increased JAK-STAT3 signaling and the production of pro-inflammatory cytokines such as IL6, IL10, and IFN- β (50). The production of these cytokines further activates JAK-STAT3 signaling as part of an autocrine loop that enhances the survival of the lymphoma cells (51, 52) (Figure 2).

MYD88 mutations have since emerged in a number of other human malignancies, with the L265P mutation found in including almost 100% of Waldenström's macroglobulinemia (WM), 2–10% of chronic lymphocytic leukemia (CLL), 69% of cutaneous diffuse large B cell lymphoma (CBCL), and 38% of primary central nervous system lymphoma (PCNSL) (previously reviewed in Ref. (53)). However, the effect of single MYD88 L265P mutation on tumor growth is confounded by the accumulation of other potential damaging mutations in the same malignant clones. Recently, a retroviral gene transfer strategy to study the effects of single MYD88 mutation in otherwise normal mature B cells found that the MYD88 L265P mutation alone was able to drive limited

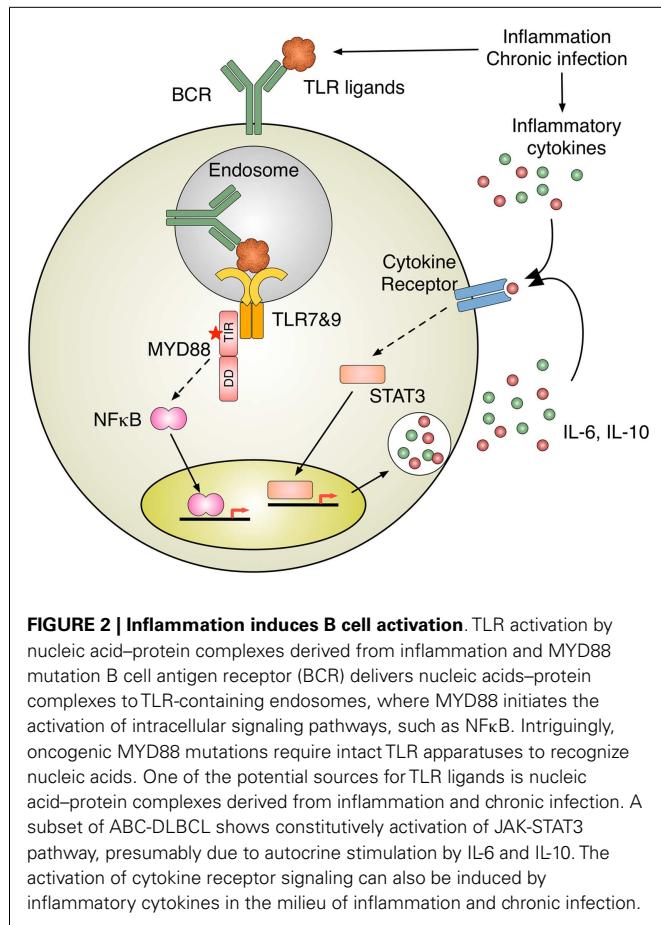


FIGURE 2 | Inflammation induces B cell activation. TLR activation by nucleic acid–protein complexes derived from inflammation and MYD88 mutation B cell antigen receptor (BCR) delivers nucleic acids–protein complexes to TLR-containing endosomes, where MYD88 initiates the activation of intracellular signaling pathways, such as NF κ B. Intriguingly, oncogenic MYD88 mutations require intact TLR apparatuses to recognize nucleic acids. One of the potential sources for TLR ligands is nucleic acid–protein complexes derived from inflammation and chronic infection. A subset of ABC-DLBCL shows constitutively activation of JAK-STAT3 pathway, presumably due to autocrine stimulation by IL-6 and IL-10. The activation of cytokine receptor signaling can also be induced by inflammatory cytokines in the milieu of inflammation and chronic infection.

rounds of mitogen independent B cell proliferation both *in vitro* and *in vivo* (54). Nevertheless, the drive for B cell proliferation was dependent on intact nucleic acid sensing TLR activity since *Unc93b1*^{3d} mutation or *Tlr9* deficiency inhibited the proliferation of MYD88 L265P B cells *in vitro* (54). Other studies have also shown that oncogenic MYD88 depends on TLRs by using the depletion of UNC91B1, PRAT4A, and CD14 in ABC-DLBCL lines as well as by using pharmacological inhibitors to TLR7 and TLR9 (55). Given that intact TLR activity is critical for lymphoma cells carrying MYD88 mutations, targeting this pathway appears to be attractive for treating these malignancies. Indeed, blocking endosome acidification using chloroquine selectively inhibits MYD88 L265P mutation driven B cell proliferation *in vitro* (54). The use of chloroquine to treat hematological malignancies should be further explored, as evidence suggests that there is a strong involvement of the activation of nucleic acid sensing TLRs that depends on normal endosome acidification in promoting proliferative abnormality in these tumors.

HEMATOLOGICAL MALIGNANCY AND INFLAMMATION

Remarkably, inflammation enables most of the key cellular and molecular capabilities that are required for carcinogenesis, such as genomic instability, proliferative abnormality, and reprogramming of the stromal environment (56). Although, the mechanisms by which inflammation promotes neoplastic transformation are

not fully understood, it is apparent that, in many cases, tumor development is linked to chronic inflammation (57, 58).

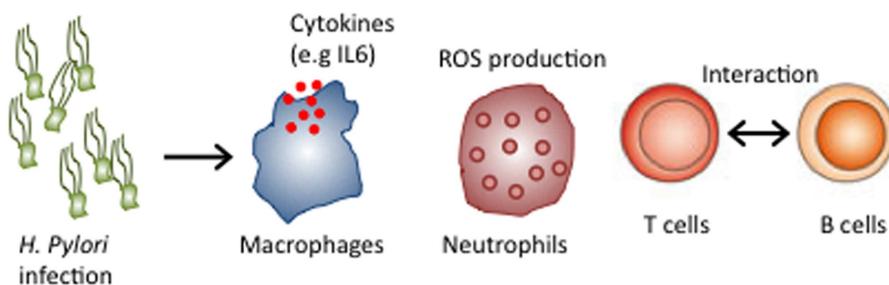
The link between inflammation and tumor formation was first speculated by Virchow in the 1800s as he observed that tissue injury and inflammation induced by irritants could promote cell proliferation (59). Infection has been accepted as a major driver of inflammation-induced tumorigenesis, with up to one-fifth of all cases of cancer associated with infection (60, 61). For instance, persistent *Helicobacter pylori* infection is associated with gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma, infections with hepatitis B and C viruses are associated with hepatocellular carcinoma, and infections with *Bacteroides* species are linked to colon cancer (62, 63). The inflammatory response triggered by infection is a part of normal host defense to eliminate the pathogen. However, some tumorigenic pathogens subvert host immunity and establish persisting infections, leading to chronic inflammation (64, 65).

Persistent inflammation establishes a microenvironment, which contains macrophages, dendritic cells, natural killer cells, and T and B lymphocytes in addition to the surrounding stroma (66) (Figure 3A). These diverse cells communicate with each other by means of direct contact or cytokine and chemokines, which influence tumor formation and growth (67, 68). This network of inflammatory cells promotes the formation of cancerous cells, which further complicates the initial chronic inflammation induced by infection. The neoplastic cells trigger anti-tumor immunity, which further adds to the established inflammation. Early during tumor formation, whether tumor-promoting inflammation or anti-tumor immunity follows seems to be stochastic and is influenced by a combination of cell-intrinsic and cell-extrinsic processes (69, 70). In established cancers, pro-tumor inflammation seems to be favored, as without therapeutic intervention advanced tumors rarely regress.

Continuous stimulation of TLRs by microbial products constitutively engages the activation of the NF κ B and STAT3 transcription factors, which exert pro-cancerous activity through multiple effectors (62, 71, 72). Additionally, the production of cytokines by the host inflammatory cells activates these transcription factors (62) (Figure 2). These cytokines facilitate the establishment of feed-forward signal amplification loops, which ultimately promote cell proliferation and resistance to cell death. For instance, the expression of the anti-apoptotic proteins Bcl-2 and Bcl-X_L is promoted by both NF κ B and STAT3, as is the expression of c-IAP1, c-IAP2, Mcl-1, c-FLIP, and survivin (62, 72). Moreover, both transcription factors interfere with p53 expression and function, representing another potential tumor-promoting mechanism (73).

An additional mechanism linking inflammation to tumor formation is the expression of activation-induced cytidine deaminase (AID), an enzyme that promotes immunoglobulin gene class switching by catalyzing deamination of cytosines in DNA (74). In addition to B lymphocytes, where it was originally discovered, AID is overexpressed in many cancers of diverse origin, and its expression is induced by inflammatory cytokines in a NF κ B-dependent manner (74). AID induces genomic instability and increases mutation probability during the error-prone joining of double-stranded DNA breaks. This mutagenic process causes

A Recruitment of immune cells and establishment of chronic inflammatory millieu



B Hypothetical model of MALT lymphoma formation

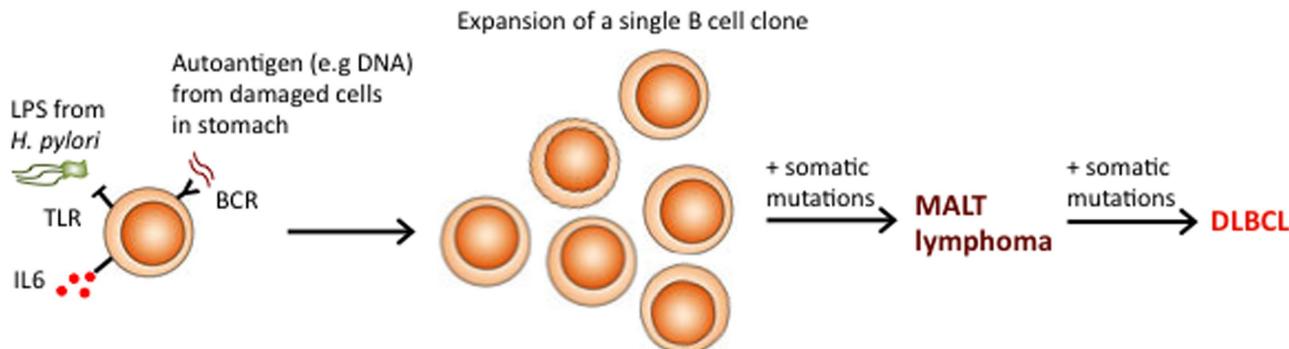


FIGURE 3 | Role of *Helicobacter pylori* in the pathogenesis of gastric MALT lymphoma. (A) *H. pylori* infection results in buffering of the gastric pH, which allows immune cell infiltration and the establishment of MALT. The presentation of *H. pylori* antigens by dendritic cells recruits and activates T cell responses, which enhance B cell activation through CD40–CD40L interactions. **(B)** MALT lymphoma may result from the transformation of a single B cell clone, which

initially formed part of the polyclonal B lymphocyte response against *H. pylori*. Both direct activation of TLR signaling by *H. pylori* and chronic BCR signaling from engagement of autoantigens from damaged stomach cells and the B cell receptor, in addition to T cell–B cell co-stimulation could be involved in the expansion of the single neoplastic B cell clone. Acquisition of additional genomic lesions could transform MALT lymphomas into more aggressive DLBCL.

mutations in critical cancer-associated genes such as *Tp53* and *c-Myc* (75, 76).

HELICOBACTER PYLORI, INFLAMMATION AND MALT LYMPHOMA

MALT lymphomas, which occur in the context of chronic inflammation caused by infectious agents, such as *H. pylori* (gastric lymphoma), *Chlamydia psittaci* (ocular adnexal lymphoma), and *Borrelia burgdorferi* (cutaneous lymphoma) are a prime example of lymphoid malignancies associated with chronic inflammation (77, 78). Interestingly, in some patients, gastric MALT lymphoma and diffuse large B cell lymphoma (DLBCL) co-occur, indicating that MALT lymphomas can develop into more aggressive DLBCL (79). The pathogenesis of MALT lymphoma involves several steps, which result in the transformation of a single B cell clone, initially part of the polyclonal B lymphocyte response against *H. pylori* into a monoclonal tumor (78) (Figure 3B). Under physiological conditions, the stomach lacks MALT because the low pH prevents the survival of lymphocytes in the gastric wall. However, *H. pylori* infection results in buffering of the gastric pH owing to the secretion of bacterial urease. The decreased acidity of stomach

environment, along with the presence of the infection, triggers lymphoid infiltration and the establishment of MALT (78).

Subsequently, the continuous presence of *H. pylori* induces an upregulation of TLR4 and MD-2 expression in gastric epithelial cells, which contributes to establishing a persistent inflammatory environment (80–82). Although, the role of TLRs in the pathogenesis of MALT lymphoma has been poorly investigated, the immune response to chronic stimulation by *H. pylori* infection is thought to induce NF κ B activation in B cells, which plays a crucial part in the development of MALT lymphoma (83, 84). In addition, the presentation of *H. pylori* by dendritic cells recruits and activates T cell responses, which enhance B cell activation through CD40–CD40L interactions (85) (Figure 3A). Thus, both direct activation of TLR signaling by *H. pylori* and T cell-mediated B cell activation could be involved in the pathogenesis of MALT lymphoma (86).

Interestingly, several lines of evidence indicate that chronic antigen stimulation precedes MALT lymphoma pathogenesis. The rearranged *IGVH* genes from MALT lymphomas have a high frequency of variants, which have been implicated in autoantibody production (87). In addition, approximately half of the MALT lymphoma cases display evidence of intraclonal variation in the

IGVH locus, indicating that continued antigenic stimulation is a key driver of clonal B cell expansion (87). As both somatic hypermutation and intraclonal variations are antigen-driven processes, their occurrence in gastric MALT lymphoma strongly indicates a role for antigens during both initiation and progression of this neoplasm.

Remarkably, tumor-derived immunoglobulins from MALT lymphomas bind to various autoantigens as well as *H. pylori* with varying affinities (87). The autoantigens include DNA and stomach-associated antigens, which could be abundant in the MALT-microenvironment under a situation of continuous inflammation. Given that *H. pylori* eradication with antibiotics is the preferred therapy for patients with *H. pylori*-positive gastric MALT lymphoma (88, 89), and the evidence that MALT lymphoma cells proliferate when stimulated with *H. pylori* in tissue culture, one possible hypothesis is that neoplastic B cells receive proliferative signals from both the B cell receptor and TLRs, which are continuously and simultaneously engaged by self-antigens and LPS from *H. pylori* respectively. Thus, the eradication of *H. pylori* by antibiotics disrupts a critical ‘weak’ link in the inflammatory process, which gradually resolves and shuts off the supply of autoantigens available to lymphoma cells.

ROLE OF INFLAMMATION AND CYTOKINES IN CLL AND MULTIPLE MYELOMA

It is apparent that antigenic stimulation, autoimmunity, and inflammation contribute to the development of CLL (90). One mechanism through which these stimuli promote CLL development is induction of B cell activating factor (BAFF), a member of the TNF family, recently shown to accelerate development of CLL-like disease in mice (91). In addition, cytokines such as IL6 and interactions with bone marrow stromal cells support CLL expansion and suppress apoptosis through the expression of Bcl-2, Survivin, and Mcl-1 (92, 93). Increased IL6 production activates the JAK-STAT, MAPK, and PI3K pathways to promote cell survival, proliferation, and resistance to apoptosis (94–96), with the constitutive activation of STAT3 being a hallmark for CLLs (97, 98). Similarly, through the secretion of IL6, TNF- α , and BAFF, bone marrow stromal cells promote the survival of neoplastic plasma cells and also confer drug resistance in multiple myeloma (99). Interestingly, IL6-deficient mice are resistant to induction of multiple myeloma (100, 101). Thus, despite cell-intrinsic constitutive NF κ B activation, multiple myeloma cells depend on an extrinsic source of IL6 for their development and survival. High levels of plasma IL6 have been associated with increased disease progression and decreased survival, thus providing the rationale for the evaluation of combination therapies including drugs targeting IL6 for the treatment of this malignancy (102–104).

TARGETING INFLAMMATION AND TLRs IN CANCER

Constitutively, active NF κ B signaling due to the aberrant activation of TLRs during chronic inflammation or by MYD88 mutation determines the poor clinical outcome of many hematological malignancies. Desirable outcomes in treating these diseases can be achieved by using a combination of inhibition of signal transducers and transcription factors, sequestration of chemokines

and cytokines that sustain inflammatory cells, and the depletion of immune or inflammatory cells that promote tumor development.

Gain-of-function MYD88 mutations have emerged as a potent driver of constitutively active NF κ B signaling in many tumors. Targeting this pathway is likely going to be useful as part of a multi-component therapy for many hematological malignancies that are addicted to NF κ B activity for their survival. MYD88 signaling is critically dependent on its homo-dimerization through conserved residues within the BB-loop structure of the TIR domain (29, 105). Interfering with this interaction by heptapeptides mimicking the BB-loop has achieved significant reduction in NF κ B activity (106). Another novel synthetic compound, ST2825, developed by the same group of researchers is currently under pre-clinical evaluation for the treatment of chronic inflammatory diseases (107). Other peptide-based synthetic small molecule inhibitors such as hydrocinnamoyl-L-valyl pyrrolidine (compound 4a) and Pephinh-MYD88 have also been developed to target MYD88 dimerization in the treatment of lymphoma patients with MYD88 mutations (108). However, these potential MYD88 specific therapeutic options are yet to be trialled in large clinical cohorts.

Constitutive NF κ B activity in certain lymphoid tumors suggests that the activation of this pathway is crucial for their survival and thus making them attractive drug targets for anti-cancer therapy (62, 72, 109, 110). However in most cases, such therapy is likely to be effective only in combination with more conventional approaches. Furthermore, as genotoxic therapies often lead to NF κ B activation in remaining malignant cells, it makes sense to combine genotoxic drugs with NF κ B inhibitors to overcome drug resistance. However, prolonged NF κ B inhibition can result in severe immune deficiency and may lead to neutropenia and greatly enhanced acute inflammation due to enhanced IL1 β secretion. Such complications as well as increase propensity for liver damage have hindered the clinical development of NF κ B and IKK β inhibitors (57, 111, 112). An attractive alternative target is the STAT3 transcription factor and the signaling pathway that leads to its activation (113, 114). Several STAT3 and JAK2 inhibitors have been described and shown to inhibit the growth of various cancers that exhibit STAT3 activation (115, 116). So far, none of the complications associated with NF κ B inhibitor have been reported for STAT3 or JAK2 inhibitors.

It is unlikely that inhibition of NF κ B or STAT signaling alone will be sufficient for tumor regression, yet the combination of an NF κ B inhibitor and an apoptosis inducing drug or cytokine could be highly effective. Selective inhibition of NF κ B in cancer cells blocks the stimulatory effect of TNF and markedly increases susceptibility to TRAIL-induced cell death, resulting in tumor regression (117, 118). NF κ B inhibition and anti-TNF therapy, together with the administration of IFN or TRAIL might offer an attractive combined strategy for immunomodulatory cancer therapy. A recent study has found such synergy between lenalidomide and the BTK inhibitor Ibrutinib in killing ABC-DLBCL by the induction of IRF7 and IFN- β production to cause cell cycle arrest and apoptosis (119, 120). Combinatorial strategies provide a distinct advantage where by certain IFN induced side-effects might be diminished after NF κ B inhibitor treatment, shifting the

balance of cytokines in the tumor microenvironment to promote tumor regression.

Although it is widely accepted that dampening inflammation and diminishing TLR activity are beneficial for tumor regression, several new lines of evidence have emerged to suggest that TLR agonists could be used as potent anti-tumor agents. When treated with a TLR9 agonist, type B CpG oligodeoxynucleotides (CpG-B ODNs), and CLL B cells that selectively express high levels of TLR9 undergo profound apoptosis by the activation of STAT1, reduction of Bcl-xL pro-survival protein, and elevation of Fas and Fas ligand (121). TLR9 triggered apoptosis seems to be dependent on the altered NFκB status of lymphoma cells compared to normal cells. Moreover, the use of TLR agonists has been known to activate the cognate immune system against cancer cells (122–124). TLRs in lymphoid malignancies appear to be a “double-edged sword” in actively driving disease progression in some but exhibit tumor regressive roles in others. Activation of TLRs by MYD88 mutations has often been associated with poor clinical outcome in lymphoma patients. However, a recent study has reported improved patient survival in a subset of young CLL patients with the identical mutation (125). Interestingly, patients with MYD88 mutations were much younger and had lower expression of CD38 and ZAP-70 than patients with unmutated MYD88. CD38 expression on CLL cells is important for their proliferation and chemotaxis through a signaling pathway involving ZAP-70 (90). Elevated CD38 expression often marks CLL patients with poor clinical outcome and responsiveness to therapy (90). Complex interactions between MYD88 mutation, IGHV mutation status, and CD38 and ZAP-70 levels confound the explanation behind why patients with MYD88 mutations had reduced CD38 expression and show better survival (125).

CONCLUSION

Pattern recognition receptors protect us from danger and damage associated signals, however, inappropriate activation of these pathways can cause cancer. TLRs can also use ubiquitously available self-ligands such as our own DNA to drive aberrant cell growth when the adaptor protein MYD88 is mutated. This recent finding is one of the many pieces of supportive evidence for Virchow’s hypothesis that chronic inflammation is linked with cancer development. Studies into mutations in the TLR signaling pathways have significantly advanced our understanding on the involvement of TLRs in cancer. However, the potential for targeting TLRs as anti-cancer therapy remains an area that is not yet fully understood. Often TLRs act as a “double-edged sword” in cancer, over active TLR signal provides a microenvironment that is necessary for malignant cell proliferation; on the other hand, TLR agonists can also be used to inhibit cancer cell growth. A better understanding of the involvement of TLRs in cancer would help in tipping the balance between tumor stimulatory and inhibitory effects and the development of novel anti-cancer agents.

REFERENCES

- Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* (1989) **54**(Pt 1):1–13. doi:10.1101/SQB.1989.054.01.003
- Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* (2002) **20**:197–216. doi:10.1146/annurev.immunol.20.083001.084359
- Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* (2010) **140**:805–20. doi:10.1016/j.cell.2010.01.022
- Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* (1997) **388**:394–7. doi:10.1038/41131
- Gay NJ, Keith FJ. *Drosophila* toll and IL-1 receptor. *Nature* (1991) **351**:355–6. doi:10.1038/351355b0
- Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B. The product of the tobacco mosaic virus resistance gene N: similarity to toll and the interleukin-1 receptor. *Cell* (1994) **78**:1101–15. doi:10.1016/0092-8674(94)90283-6
- Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* (2004) **4**:499–511. doi:10.1038/nri1391
- McGettrick AF, O’Neill LA. Localisation and trafficking of Toll-like receptors: an important mode of regulation. *Curr Opin Immunol* (2010) **22**:20–7. doi:10.1016/j.coim.2009.12.002
- Hashimoto C, Hudson KL, Anderson KV. The Toll gene of *Drosophila*, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* (1988) **52**:269–79. doi:10.1016/0092-8674(88)90516-8
- Lemaître B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell* (1996) **86**:973–83. doi:10.1016/S0092-8674(00)80172-5
- Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* (1998) **282**:2085–8. doi:10.1126/science.282.5396.2085
- Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* (2003) **21**:335–76. doi:10.1146/annurev.immunol.21.120601.141126
- Akira S. Mammalian Toll-like receptors. *Curr Opin Immunol* (2003) **15**:5–11. doi:10.1016/S0952-7915(03)00005-0
- Farhat K, Riekenberg S, Heine H, Debarry J, Lang R, Mages J, et al. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. *J Leukoc Biol* (2008) **83**:692–701. doi:10.1189/jlb.0807586
- Re F, Strominger JL. Monomeric recombinant MD-2 binds toll-like receptor 4 tightly and confers lipopolysaccharide responsiveness. *J Biol Chem* (2002) **277**:23427–32. doi:10.1074/jbc.M202554200
- Nagai Y, Akashi S, Nagafuku M, Ogata M, Iwakura Y, Akira S, et al. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol* (2002) **3**:667–72. doi:10.1038/ni809
- Blasius AL, Beutler B. Intracellular toll-like receptors. *Immunity* (2010) **32**:305–15. doi:10.1016/j.immuni.2010.03.012
- Krug A, French AR, Barchet W, Fischer JA, Dzionek A, Pingel JT, et al. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* (2004) **21**:107–19. doi:10.1016/j.immuni.2004.06.007
- Krug A, Luker GD, Barchet W, Leib DA, Akira S, Colonna M. Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. *Blood* (2004) **103**:1433–7. doi:10.1182/blood-2003-08-2674
- Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* (2004) **303**:1529–31. doi:10.1126/science.1093616
- Tabeta K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K, et al. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proc Natl Acad Sci U S A* (2004) **101**:3516–21. doi:10.1073/pnas.0400525101
- Tabeta K, Hoebe K, Janssen EM, Du X, Georgel P, Crozat K, et al. The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9. *Nat Immunol* (2006) **7**:156–64. doi:10.1038/ni1297
- Groves E, Dart AE, Covarelli V, Caron E. Molecular mechanisms of phagocytic uptake in mammalian cells. *Cell Mol Life Sci* (2008) **65**:1957–76. doi:10.1007/s00018-008-7578-4
- Conner SD, Schmid SL. Regulated portals of entry into the cell. *Nature* (2003) **422**:37–44. doi:10.1038/nature01451
- Takeda K, Akira S. TLR signaling pathways. *Semin Immunol* (2004) **16**:3–9. doi:10.1016/j.smim.2003.10.003
- Compagno M, Lim WK, Grunn A, Nandula SV, Brahmacary M, Shen Q, et al. Mutations of multiple genes cause deregulation of NF-κappaB in diffuse large B-cell lymphoma. *Nature* (2009) **459**:717–21. doi:10.1038/nature07968

27. Nishiya T, Kajita E, Horinouchi T, Nishimoto A, Miwa S. Distinct roles of TIR and non-TIR regions in the subcellular localization and signaling properties of MyD88. *FEBS Lett* (2007) **581**:3223–9. doi:10.1016/j.febslet.2007.06.008
28. Wesche H, Henzel WJ, Shillinglaw W, Li S, Cao Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* (1997) **7**:837–47. doi:10.1016/S1074-7613(00)80402-1
29. Loiarro M, Volpe E, Ruggiero V, Gallo G, Furlan R, Maiorino C, et al. Mutational analysis identifies residues crucial for homodimerization of myeloid differentiation factor 88 (MyD88) and for its function in immune cells. *J Biol Chem* (2013) **288**:30210–22. doi:10.1074/jbc.M113.490946
30. Lin SC, Lo YC, Wu H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature* (2010) **465**:885–90. doi:10.1038/nature09121
31. Ye H, Arron JR, Lamotte B, Cirilli M, Kobayashi T, Shevde NK, et al. Distinct molecular mechanism for initiating TRAF6 signalling. *Nature* (2002) **418**:443–7. doi:10.1038/nature00888
32. Xia ZP, Sun L, Chen X, Pineda G, Jiang X, Adhikari A, et al. Direct activation of protein kinases by unanchored polyubiquitin chains. *Nature* (2009) **461**:114–9. doi:10.1038/nature08247
33. Jacobs MD, Harrison SC. Structure of an IkappaBalpha/NF-kappaB complex. *Cell* (1998) **95**:749–58. doi:10.1016/S0092-8674(00)81698-0
34. Li Q, Lu Q, Bottero V, Estepa G, Morrison L, Mercurio F, et al. Enhanced NF-kappaB activation and cellular function in macrophages lacking IkappaB kinase 1 (IKK1). *Proc Natl Acad Sci U S A* (2005) **102**:12425–30. doi:10.1073/pnas.0505997102
35. Solt LA, May MJ. The IkappaB kinase complex: master regulator of NF-kappaB signaling. *Immunol Res* (2008) **42**:3–18. doi:10.1007/s12026-008-8025-1
36. Liu W, Ouyang X, Yang J, Liu J, Li Q, Gu Y, et al. AP-1 activated by toll-like receptors regulates expression of IL-23 p19. *J Biol Chem* (2009) **284**:24006–16. doi:10.1074/jbc.M109.025528
37. Verstak B, Nagpal K, Bottomley SP, Golenbock DT, Hertzog PJ, Mansell A. MyD88 adapter-like (Mal)/TIRAP interaction with TRAF6 is critical for TLR2- and TLR4-mediated NF-kappaB proinflammatory responses. *J Biol Chem* (2009) **284**:24192–203. doi:10.1074/jbc.M109.023044
38. Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, et al. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* (2002) **420**:324–9. doi:10.1038/nature01182
39. Ohnishi H, Tochio H, Kato Z, Orii KE, Li A, Kimura T, et al. Structural basis for the multiple interactions of the MyD88 TIR domain in TLR4 signaling. *Proc Natl Acad Sci U S A* (2009) **106**:10260–5. doi:10.1073/pnas.0812956106
40. Ning S, Pagano JS, Barber GN. IRF7: activation, regulation, modification and function. *Genes Immun* (2011) **12**:399–414. doi:10.1038/gene.2011.21
41. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* (2010) **11**:373–84. doi:10.1038/ni.1863
42. Kawai T, Sato S, Ishii KJ, Coban C, Hemmi H, Yamamoto M, et al. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol* (2004) **5**:1061–8. doi:10.1038/ni1118
43. Doyle S, Vaidya S, O’Connell R, Dadgostar H, Dempsey P, Wu T, et al. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* (2002) **17**:251–63. doi:10.1016/S1074-7613(02)00390-4
44. Kagan JC, Su T, Horng T, Chow A, Akira S, Medzhitov R. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nat Immunol* (2008) **9**:361–8. doi:10.1038/ni1569
45. Häcker H, Reddecke V, Blagojev B, Kratchmarova I, Hsu LC, Wang GG, et al. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* (2006) **439**:204–7. doi:10.1038/nature04369
46. Oganesyan G, Saha SK, Guo B, He JQ, Shahangian A, Zarnegar B, et al. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature* (2006) **439**:208–11. doi:10.1038/nature04374
47. Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, et al. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol* (2003) **4**:1144–50. doi:10.1038/ni986
48. Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, et al. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity* (2000) **13**:539–48. doi:10.1016/S1074-7613(00)00053-4
49. Tseng PH, Matsuzawa A, Zhang W, Mino T, Vignali DA, Karin M. Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. *Nat Immunol* (2010) **11**:70–5. doi:10.1038/ni.1819
50. Ngo VN, Young RM, Schmitz R, Jhavar S, Xiao W, Lim KH, et al. Oncogenically active MYD88 mutations in human lymphoma. *Nature* (2011) **470**:115–9. doi:10.1038/nature09671
51. Lam LT, Wright G, Davis RE, Lenz G, Farinha P, Dang L, et al. Cooperative signaling through the signal transducer and activator of transcription 3 and nuclear factor- κ B pathways in subtypes of diffuse large B-cell lymphoma. *Blood* (2008) **111**:3701–13. doi:10.1182/blood-2007-09-111948
52. Ding BB, Yu JJ, Yu RY, Mendez LM, Shaknovich R, Zhang Y, et al. Constitutively activated STAT3 promotes cell proliferation and survival in the activated B-cell subtype of diffuse large B-cell lymphomas. *Blood* (2008) **111**:1515–23. doi:10.1182/blood-2007-04-087734
53. Wang JQ, Jeelall YS, Horikawa K. Emerging targets in human lymphoma: targeting the MYD88 mutation. *Blood Lymphat Cancer* (2013) **2013**:53–61. doi:10.2147/BLCTT.S35292
54. Wang JQ, Jeelall YS, Beutler B, Horikawa K, Goodnow CC. Consequences of the recurrent MYD88(L265P) somatic mutation for B cell tolerance. *J Exp Med* (2014) **211**:413–26. doi:10.1084/jem.20131424
55. Lim K-H, Barton GM, Staudt LM. Oncogenic MYD88 mutants require Toll-like receptors [abstract]. In: *Proceedings of the 104th Annual Meeting of the American Association for Cancer Research*; 2013 Apr 6–10; Washington, DC. Philadelphia: AACR; *Cancer Res* (2013) **73**(8 Suppl):Abst 2332. doi:10.1158/1538-7445.AM2013-2332
56. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) **144**:646–74. doi:10.1016/j.cell.2011.02.013
57. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* (2010) **140**:883–99. doi:10.1016/j.cell.2010.01.025
58. Medzhitov R. Origin and physiological roles of inflammation. *Nature* (2008) **454**:428–35. doi:10.1038/nature07201
59. Virchow R. An address on the value of pathological experiments. *Br Med J* (1881) **2**:198–203. doi:10.1136/bmjj.2.1075.198
60. Kuper H, Adamo HO, Trichopoulos D. Infections as a major preventable cause of human cancer. *J Intern Med* (2000) **248**:171–83. doi:10.1046/j.1365-2796.2000.00742.x
61. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* (2014) **64**:9–29. doi:10.3322/caac.21208
62. Karin M. Nuclear factor- κ B in cancer development and progression. *Nature* (2006) **441**:431–6. doi:10.1038/nature04870
63. Wu S, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, et al. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med* (2009) **15**:1016–22. doi:10.1038/nm.2015
64. Coussens LM, Werb Z. Inflammation and cancer. *Nature* (2002) **420**:860–7. doi:10.1038/nature01322
65. Virgin HW, Wherry EJ, Ahmed R. Redefining chronic viral infection. *Cell* (2009) **138**:30–50. doi:10.1016/j.cell.2009.06.036
66. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* (2006) **6**:24–37. doi:10.1038/nrc1782
67. Bui JD, Schreiber RD. Cancer immunosurveillance, immunoediting and inflammation: independent or interdependent processes? *Curr Opin Immunol* (2007) **19**:203–8. doi:10.1016/j.coi.2007.02.001
68. Swann JB, Vesely MD, Silva A, Sharkey J, Akira S, Schreiber RD, et al. Demonstration of inflammation-induced cancer and cancer immunoediting during primary tumorigenesis. *Proc Natl Acad Sci U S A* (2008) **105**:652–6. doi:10.1073/pnas.0708594105
69. Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. *J Clin Invest* (2007) **117**:1175–83. doi:10.1172/JCI31537
70. Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol* (2006) **90**:1–50. doi:10.1016/S0065-2776(06)90001-7
71. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* (2011) **34**:637–50. doi:10.1016/j.immuni.2011.05.006

72. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat Rev Immunol* (2007) 7:41–51. doi:10.1038/nri1995
73. Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis* (2009) 30:1073–81. doi:10.1093/carcin/bgp127
74. Okazaki IM, Kotani A, Honjo T. Role of AID in tumorigenesis. *Adv Immunol* (2007) 94:245–73. doi:10.1016/S0065-2776(06)94008-5
75. Liu M, Duke JL, Richter DJ, Vinuesa CG, Goodnow CC, Kleinsteine SH, et al. Two levels of protection for the B cell genome during somatic hypermutation. *Nature* (2008) 451:841–5. doi:10.1038/nature06547
76. Ramiro AR, Jankovic M, Callen E, Difilippantonio S, Chen HT, McBride KM, et al. Role of genomic instability and p53 in AID-induced c-myc-Igh translocations. *Nature* (2006) 440:105–9. doi:10.1038/nature04495
77. Isaacson P, Wright DH. Malignant lymphoma of mucosa-associated lymphoid tissue. A distinctive type of B-cell lymphoma. *Cancer* (1983) 52:1410–6. doi:10.1002/1097-0142(19831015)52:8<1410::AID-CNCR2820520813>3.0.CO;2-3
78. Ferreri AJ, Ernberg I, Copie-Bergman C. Infectious agents and lymphoma development: molecular and clinical aspects. *J Intern Med* (2009) 265:421–38. doi:10.1111/j.1365-2796.2009.02083.x
79. Barth TF, Barth CA, Kestler HA, Michl P, Weniger MA, Buchholz M, et al. Transcriptional profiling suggests that secondary and primary large B-cell lymphomas of the gastrointestinal (GI) tract are blastic variants of GI marginal zone lymphoma. *J Pathol* (2007) 211:305–13. doi:10.1002/path.2096
80. Kawahara T, Teshima S, Oka A, Sugiyama T, Kishi K, Rokutan K. Type I *Helicobacter pylori* lipopolysaccharide stimulates toll-like receptor 4 and activates mitogen oxidase 1 in gastric pit cells. *Infect Immun* (2001) 69:4382–9. doi:10.1128/IAI.69.7.4382-4389.2001
81. Eisenhofer G, Kopin IJ, Goldstein DS. Leaky catecholamine stores: undue waste or a stress response coping mechanism? *Ann NY Acad Sci* (2004) 1018:224–30. doi:10.1196/annals.1296.027
82. Schmausser B, Andrusil M, Endrich S, Muller-Hermelink HK, Eck M. Toll-like receptors TLR4, TLR5 and TLR9 on gastric carcinoma cells: an implication for interaction with *Helicobacter pylori*. *Int J Med Microbiol* (2005) 295:179–85. doi:10.1016/j.ijmm.2005.02.009
83. Fukata M, Abreu MT. Role of Toll-like receptors in gastrointestinal malignancies. *Oncogene* (2008) 27:234–43. doi:10.1038/sj.onc.1210908
84. Farinha P, Gascoyne RD. Molecular pathogenesis of mucosa-associated lymphoid tissue lymphoma. *J Clin Oncol* (2005) 23:6370–8. doi:10.1200/JCO.2005.05.011
85. Guindi M. Role of activated host T cells in the promotion of MALT lymphoma growth. *Semin Cancer Biol* (2000) 10:341–4. doi:10.1006/scbi.2000.0351
86. Sagaert X, Van Cutsem E, De Hertog G, Geboes K, Tousseyn T. Gastric MALT lymphoma: a model of chronic inflammation-induced tumor development. *Nat Rev Gastroenterol Hepatol* (2010) 7:336–46. doi:10.1038/nrgastro.2010.58
87. Craig VJ, Arnold I, Gerke C, Huynh MQ, Wündisch T, Neubauer A, et al. Gastric MALT lymphoma B cells express polyreactive, somatically mutated immunoglobulins. *Blood* (2010) 115:581–91. doi:10.1182/blood-2009-06-228015
88. Ferrucci PF, Zucca E. Primary gastric lymphoma pathogenesis and treatment: what has changed over the past 10 years? *Br J Haematol* (2007) 136:521–38. doi:10.1111/j.1365-2141.2006.06444.x
89. Zullo A, Hassan C, Andriani A, Cristofari F, De FrancescoV, Ierardi E, et al. Eradication therapy for *Helicobacter pylori* in patients with gastric MALT lymphoma: a pooled data analysis. *Am J Gastroenterol* (2009) 104:1932–1937;quiz1938. doi:10.1038/ajg.2009.314
90. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. *N Engl J Med* (2005) 352:804–15. doi:10.1056/NEJMra041720
91. Enzler T, Kater AP, Zhang W, Widhopf GF2nd, Chuang HY, Lee J, et al. Chronic lymphocytic leukemia of Emu-TCL1 transgenic mice undergoes rapid cell turnover that can be offset by extrinsic CD257 to accelerate disease progression. *Blood* (2009) 114:4469–76. doi:10.1182/blood-2009-06-230169
92. Granziero L, Ghia P, Circosta P, Gottardi D, Stroila G, Geuna M, et al. Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia. *Blood* (2001) 97:2777–83. doi:10.1182/blood.V97.9.2777
93. Pedersen IM, Kitada S, Leoni LM, Zapata JM, Karras JG, Tsukada N, et al. Protection of CLL B cells by a follicular dendritic cell line is dependent on induction of McI-1. *Blood* (2002) 100:1795–801.
94. Heinrich PC, Behrmann I, Muller-Newen G, Schaper F, Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J* (1998) 334(Pt 2):297–314.
95. Murakami M, Hibi M, Nakagawa N, Nakagawa T, Yasukawa K, Yamanishi K, et al. IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. *Science* (1993) 260:1808–10. doi:10.1126/science.8511589
96. Wegiel B, Bjartell A, Culig Z, Persson JL. Interleukin-6 activates PI3K/Akt pathway and regulates cyclin A1 to promote prostate cancer cell survival. *Int J Cancer* (2008) 122:1521–9. doi:10.1002/ijc.23261
97. Hazan-Halevy I, Harris D, Liu Z, Liu J, Li P, Chen X, et al. STAT3 is constitutively phosphorylated on serine 727 residues, binds DNA, and activates transcription in CLL cells. *Blood* (2010) 115:2852–63. doi:10.1182/blood-2009-10-230060
98. Frank DA, Mahajan S, Ritz J. B lymphocytes from patients with chronic lymphocytic leukemia contain signal transducer and activator of transcription (STAT) 1 and STAT3 constitutively phosphorylated on serine residues. *J Clin Invest* (1997) 100:3140–8. doi:10.1172/JCI119869
99. Kastritis E, Palumbo A, Dimopoulos MA. Treatment of relapsed/refractory multiple myeloma. *Semin Hematol* (2009) 46:143–57. doi:10.1053/j.seminhematol.2009.01.004
100. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. *Eur J Cancer* (2005) 41:2502–12. doi:10.1016/j.ejca.2005.08.016
101. Gadó K, Silva S, Pálóczi K, Domján G, Falus A. Mouse plasmacytoma: an experimental model of human multiple myeloma. *Haematologica* (2001) 86:227–36.
102. Kurzrock R, Voorhees PM, Casper C, Furman RR, Fayad L, Lonial S, et al. A phase I, open-label study of siltuximab, an anti-IL-6 monoclonal antibody, in patients with B-cell non-Hodgkin lymphoma, multiple myeloma, or Castleman disease. *Clin Cancer Res* (2013) 19:3659–70. doi:10.1158/1078-0432.CCR-12-3349
103. Voorhees PM, Manges RF, Sonneveld P, Jagannath S, Somlo G, Krishnan A, et al. A phase 2 multicentre study of siltuximab, an anti-interleukin-6 monoclonal antibody, in patients with relapsed or refractory multiple myeloma. *Br J Haematol* (2013) 161:357–66. doi:10.1111/bjh.12266
104. Lai R, O'Brien S, Maushouri T, Rogers A, Kantarjian H, Keating M, et al. Prognostic value of plasma interleukin-6 levels in patients with chronic lymphocytic leukemia. *Cancer* (2002) 95:1071–5. doi:10.1002/cncr.10772
105. Burns K, Martinon F, Esslinger C, Pahl H, Schneider P, Bodmer JL, et al. MyD88, an adapter protein involved in interleukin-1 signaling. *J Biol Chem* (1998) 273:12203–9. doi:10.1074/jbc.273.20.12203
106. Loiarro M, Sette C, Gallo G, Ciacci A, Fantò N, Mastroianni D, et al. Peptide-mediated interference of TIR domain dimerization in MyD88 inhibits interleukin-1-dependent activation of NF- κ B. *J Biol Chem* (2005) 280:15809–14. doi:10.1074/jbc.C400613200
107. Loiarro M, Capolunghi F, Fantò N, Gallo G, Campo S, Arseni B, et al. Pivotal advance: inhibition of MyD88 dimerization and recruitment of IRAK1 and IRAK4 by a novel peptidomimetic compound. *J Leukoc Biol* (2007) 82:801–10. doi:10.1189/jlb.1206746
108. Bartfai T, Behrens MM, Gaidarov S, Pemberton J, Shivanyuk A, Rebek Jr. A low molecular weight mimic of the Toll/IL-1 receptor/resistance domain inhibits IL-1 receptor-mediated responses. *Proc Natl Acad Sci U S A* (2003) 100:7971–6. doi:10.1073/pnas.0932746100
109. Mackenzie GG, Queisser N, Wolfson ML, Fraga CG, Adamo AM, Oteiza PI. Curcumin induces cell-arrest and apoptosis in association with the inhibition of constitutively active NF- κ B and STAT3 pathways in Hodgkin's lymphoma cells. *Int J Cancer* (2008) 123:56–65. doi:10.1002/ijc.23477
110. Coppo P, Gouilleux-Gruart V, Huang Y, Bouhal H, Bouamar H, Bouchet S, et al. STAT3 transcription factor is constitutively activated and is oncogenic in nasal-type NK/T-cell lymphoma. *Leukemia* (2009) 23:1667–78. doi:10.1038/leu.2009.91
111. Greten FR, Arkan MC, Bollrath J, Hsu LC, Goode J, Miethling C, et al. NF- κ B is a negative regulator of IL-1 β secretion as revealed by genetic and pharmacological inhibition of IKK β . *Cell* (2007) 130:918–31. doi:10.1016/j.cell.2007.07.009
112. He G, Karin M. NF- κ B and STAT3 – key players in liver inflammation and cancer. *Cell Res* (2011) 21:159–68. doi:10.1038/cr.2010.183
113. Kortylewski M, Kujawski M, Wang T, Wei S, Zhang S, Pilon-Thomas S, et al. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat Med* (2005) 11:1314–21. doi:10.1038/nm1325
114. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer* (2009) 9:798–809. doi:10.1038/nrc2734

115. Hedvat M, Huszar D, Herrmann A, Gozgit JM, Schroeder A, Sheehy A, et al. The JAK2 inhibitor AZD1480 potently blocks Stat3 signaling and oncogenesis in solid tumors. *Cancer Cell* (2009) **16**:487–97. doi:10.1016/j.ccr.2009.10.015
116. Lin L, Amin R, Gallicano GI, Glasgow E, Jogunoori W, Jessup JM, et al. The STAT3 inhibitor NSC74859 is effective in hepatocellular cancers with disrupted TGF-beta signaling. *Oncogene* (2009) **28**:961–72. doi:10.1038/onc.2008.448
117. Kim YS, Schwabe RF, Qian T, Lemasters JJ, Brenner DA. TRAIL-mediated apoptosis requires NF-kappaB inhibition and the mitochondrial permeability transition in human hepatoma cells. *Hepatology* (2002) **36**:1498–508. doi:10.1053/jhep.2002.36942
118. Braeuer SJ, Buneker C, Mohr A, Zwacka RM. Constitutively activated nuclear factor-kappaB, but not induced NF-kappaB, leads to TRAIL resistance by up-regulation of X-linked inhibitor of apoptosis protein in human cancer cells. *Mol Cancer Res* (2006) **4**:715–28. doi:10.1158/1541-7786.MCR-05-0231
119. Yang Y, Shaffer AL3rd, Emre NC, Ceribelli M, Zhang M, Wright G, et al. Exploiting synthetic lethality for the therapy of ABC diffuse large B cell lymphoma. *Cancer Cell* (2012) **21**:723–37. doi:10.1016/j.ccr.2012.05.024
120. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* (2005) **434**:772–7. doi:10.1038/nature03464
121. Liang X, Moseman EA, Farrar MA, Bachanova V, Weisdorf DJ, Blazar BR, et al. Toll-like receptor 9 signaling by CpG-B oligodeoxynucleotides induces an apoptotic pathway in human chronic lymphocytic leukemia B cells. *Blood* (2010) **115**:5041–52. doi:10.1182/blood-2009-03-213363
122. Vacchelli E, Galluzzi L, Eggermont A, Fridman WH, Galon J, Sautès-Fridman C, et al. Trial watch: FDA-approved Toll-like receptor agonists for cancer therapy. *Oncioimmunology* (2012) **1**:894–907. doi:10.4161/onci.20931
123. Galluzzi L, Vacchelli E, Eggermont A, Fridman WH, Galon J, Sautès-Fridman C, et al. Trial watch: experimental Toll-like receptor agonists for cancer therapy. *Oncioimmunology* (2012) **1**:699–716. doi:10.4161/onci.20696
124. Adams S. Toll-like receptor agonists in cancer therapy. *Immunotherapy* (2009) **1**:949–64. doi:10.2217/int.09.70
125. Martinez-Trillo A, Pinyol M, Navarro A, Aymerich M, Jares P, Juan M, et al. Mutations in the Toll-like receptor/MYD88 pathway in chronic lymphocytic leukemia identify a subset of young patients with favorable outcome. *Blood* (2014) **123**:3790–6. doi:10.1182/blood-2013-12-543306

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IL-1 receptor-associated kinase signaling and its role in inflammation, cancer progression, and therapy resistance

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Chronic inflammation has long been associated with the development of cancer. Among the various signaling pathways within cancer cells that can incite the expression of inflammatory molecules are those that activate IL-1 receptor-associated kinases (IRAK). The IRAK family is comprised of four family members, IRAK-1, IRAK-2, IRAK-3 (also known as IRAK-M), and IRAK-4, which play important roles in both positively and negatively regulating the expression of inflammatory molecules. The wide array of inflammatory molecules that are expressed in response to IRAK signaling within the tumor microenvironment regulate the production of factors which promote tumor growth, metastasis, immune suppression, and chemotherapy resistance. Based on published reports we propose that dysregulated activation of the IRAK signaling pathway in cancer cells contributes to disease progression by creating a highly inflammatory tumor environment. In this article, we present both theoretical arguments and reference experimental data in support of this hypothesis.

Keywords: IRAK-4, cancer, toll-like receptors, therapeutics, inflammation

INTRODUCTION

Interleukin-1 receptor-associated kinases (IRAK) play a central role in inflammatory responses by regulating the expression of various inflammatory genes in immune cells. These signals are critical for elimination of viruses, bacteria, and cancer cells, as well as for wound healing. Inflammation plays contradictory roles in tumor development, exhibiting both the potential to promote anti-tumor immune responses and also paradoxically to support tumor growth and metastases. What role the expression of IRAK family members in cancer cells plays in tumorigenesis and cancer progression remains relatively unknown and is the focus of this review. We also describe how these proteins may be novel therapeutic targets that can be inhibited in order to sensitize cancer cells to cytotoxic therapies.

The IRAK family is composed of IRAK-1, -2, and -4, which are expressed in a variety of human immune cell types and IRAK-M whose expression is largely limited to monocytes and macrophages (1), **Figure 1**. Greater details regarding the structures of the IRAK family proteins were extensively described in a recent review by Flannery and Bowie (1). All four IRAK family proteins contain an N-terminal death domain (DD), a ProST domain, and a centrally located kinase domain (1). With the exception of IRAK-4, all IRAK family members also contain a C-terminal domain. The DD serves as a platform that allows protein–protein interaction with other DD-containing proteins, the most important of which is the adaptor protein myeloid differentiation factor 88 (MyD88) (1, 2).

The proST domain, which contains serine, proline, and threonine residues, is important for regulating some of the IRAK family

proteins. For example, in IRAK-1, auto-phosphorylation occurs several times in the ProST domain, which is located between the N-terminal DD and the kinase domain. Phosphorylation at multiple sites allows IRAK to dissociate from MyD88 while maintaining interactions with downstream proteins such as TNF receptor-associated factor 6 (TRAF-6) to initiate signaling (1, 3). Furthermore, all IRAK proteins contain an invariant lysine in sub-domain II of the kinase domain. This invariant lysine is essential for ATP binding and catalytic function, and disruption of this lysine abrogates kinase activity (1, 4). IRAKs also contain a tyrosine “gatekeeper” residue (Tyr²⁶²) that alters the conformation of the IRAK protein, allowing it to maintain an active orientation. The term “gatekeeper” arises from its role in blocking a hydrophilic pocket located behind the ATP-binding site where small-molecule ATP competitive inhibitors bind and impair function (5). In a database search of over 400 kinases, this Tyr²⁶² residue was seen exclusively on IRAK family members (5). Finally, IRAK proteins can initiate downstream activation of NF-κB and JNK through engagement and activation of TRAF-6 (1, 6). Interaction with TRAF-6 occurs through Pro-X-Glu-X-X-(Ar/Ac) motifs located in the C-terminal region of IRAK1-3 (1, 6).

IRAK ACTIVATION

IL-1 receptor-associated kinase signaling can be initiated from Toll-like receptors (TLRs) or from the interleukin-1 family receptors (IL-1R), **Figure 2** (7, 8). Thirteen TLRs have been identified in human beings. TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) expressed on a variety of microbes including bacteria, fungus, yeast, and viruses. Some

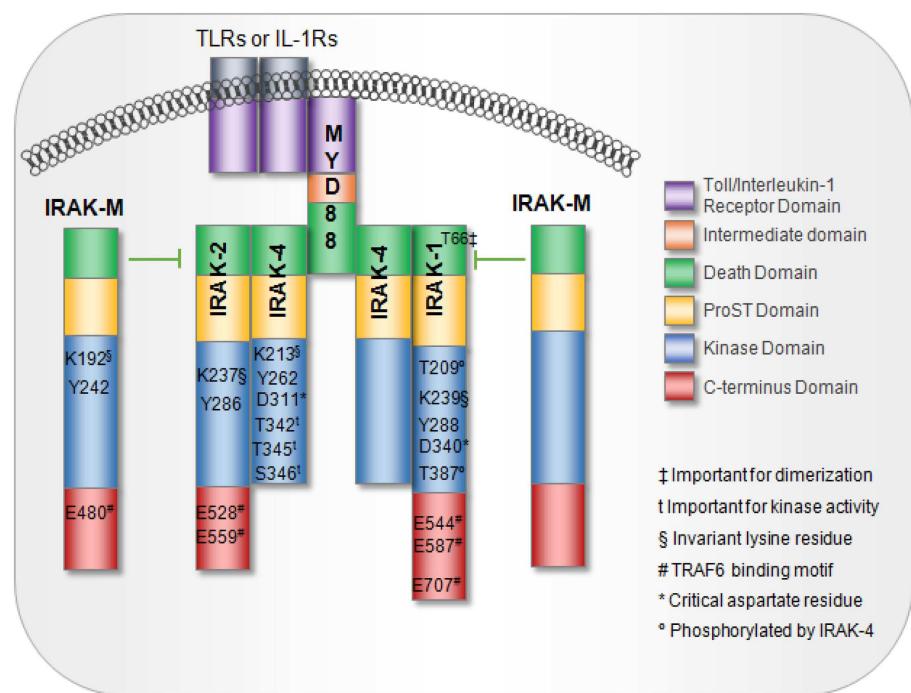


FIGURE 1 | IL-1 receptor-associated kinase family members and domains. MyD88 interaction with TLRs or IL-1R receptors is mediated via interactions between the toll-interleukin receptor (TIR) domains. MyD88 recruitment to TLRs or IL-1R induces IRAK proteins to

associate with MyD88 through death domains. IRAK-M blocks IRAK dissociation from the receptor complex, thus, acting as a negative regulator of downstream signaling. Key residues important for activation are noted.

TLRs can also be stimulated by endogenous danger signals released from stressed or dying cells such as HMBG-1 and A100 (9, 10). A wide variety of cancers have been shown to express functional TLRs. A detailed review regarding the expression of TLRs and the consequence of ligating these receptors on tumor cells was recently published by Kaczanowska et al. (11). The IL-1Rs bind pro-inflammatory cytokines in the IL-1 family, the most well-known of which are IL-1 α , IL-1 β , and IL-18. The signaling cascade is initiated by the adaptor MyD88 binding to the toll/interleukin-1 receptor (TIR) domain, which is shared by these receptors. MyD88 oligomerizes and recruits IRAK-4 via the DD. IRAK multimerization is dependent on DD interactions, which in turn result in kinase activation and propagation of the downstream signal.

Of the four IRAK proteins, IRAK-1 and IRAK-4 are active serine/threonine kinases (12). IRAK-4, the most recent IRAK family protein to be discovered, is the most proximal IRAK family protein in the TIR-mediated signaling pathway and directly downstream of MyD88 (8, 13, 14). IRAK-4 and IRAK-1 are able to associate with each other upon engaging MyD88 through their DD. IRAK-4 is thought to phosphorylate IRAK-1, which allows IRAK-1 to initiate an auto-phosphorylation cascade occurring in three sequential steps (15). IRAK-1 is first phosphorylated at Thr²⁰⁹, which causes a conformational change in the protein (14, 15). The second step is phosphorylation at Thr³⁸⁷. IRAK-1 does not become fully active until this residue is phosphorylated. There are data suggesting that either Thr²⁰⁹ or Thr³⁸⁷ may be sites for initial IRAK-1 phosphorylation by IRAK-4. However, this question remains unresolved as

both of these residues are also sites of auto-phosphorylation. The third step is auto-phosphorylation at several residues in the proST region; this allows IRAK-1 to be released from the active receptor complex. IRAK-1 and TRAF-6 dissociate from the complex, bind TAB-1 (TAK-1 binding protein-1) followed by binding of TAK-1 (transforming growth factor- β -activated kinase) and TAB-2. IRAK-1 ubiquitination and degradation are rapidly induced. The remaining complex translocates into the cytoplasm, associates with ubiquitin ligase such as ubiquitin conjugating enzyme-13 (UBC-13) and ubiquitin conjugating enzyme E2 variant-1 (UEV-1a), leading to ubiquitination and degradation of TRAF-6. This activates TAK-1 and phosphorylation of the inhibitor of κ B kinase (IKK) complex (IKK α , IKK β , and IKK γ), as well as mitogen activated protein kinases (MAPKs). The resulting NF- κ B activation regulates the transcription of pro-inflammatory genes. IRAK-1 activity and induction of NF- κ B is also regulated by ubiquitination at Lys¹³⁴ and Lys¹⁸⁰. It is worth noting that mutant forms of IRAK containing arginine at these sites have an impaired capacity to induce NF- κ B (16).

While the IRAK-1 kinase activity is also not essential for IL-1R-mediated NF- κ B activation, its role as an adaptor protein that brings together MyD88, IRAK-4, and Tollip is essential for IL-1R-mediated NF- κ B activation (17–19). IRAK-1 expression and activation is, of course, subjected to regulation. In addition to inducing activation, auto-phosphorylation renders IRAK-1 susceptible to proteasome-mediated degradation (17, 19). Regulation may also occur at a transcriptional level (19). For example, a

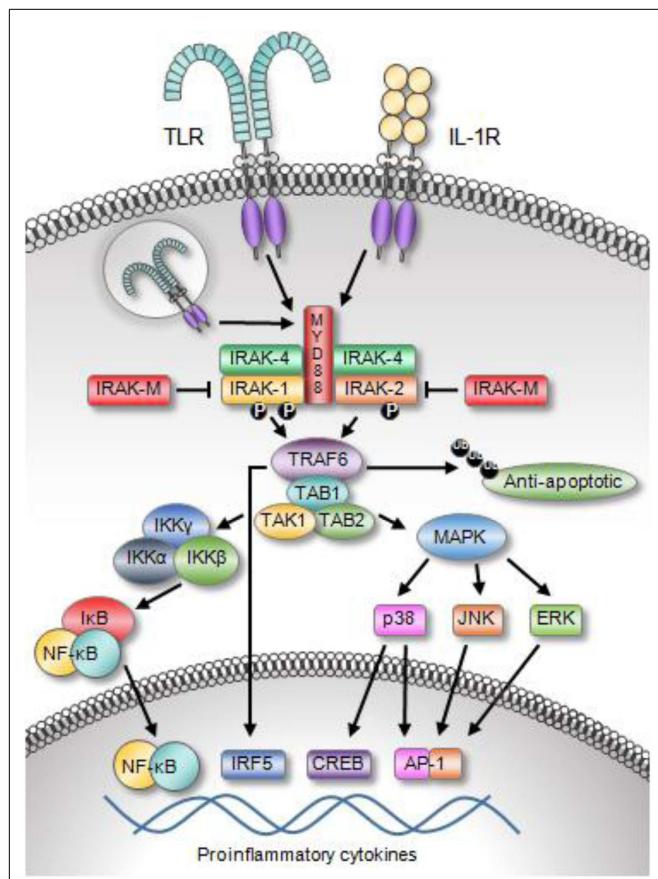


FIGURE 2 | Toll-like receptor and IL-1R family members activate IRAK signaling. The engagement of TLRs or the IL-1R recruits MyD88 and IRAK family proteins to the receptor complex. Upon activation, IRAK members associate with TRAF6, which leads to the activation of a variety of transcription factors, including NF- κ B, IRF5, AP-1, and CREB. The activation of these transcription factors results in the expression of a broad array of inflammatory molecules and apoptosis-related proteins. Moreover, TRAF6 can alter protein stability through its ability to polyubiquitinate various proteins including anti-apoptotic proteins.

human IRAK-1b splice variant that lacks kinase activity is resistant to proteasome-mediated degradation, and an IRAK-1c splice variant with a truncated sequence at the C-terminal end of the kinase domain functions as a *negative* regulator of TLR and IL-1R signaling (17, 20, 21).

IRAK-2 was initially thought to be a “pseudokinase” because a critical aspartate residue in the catalytic domain is replaced with asparagine and unlike IRAK-1 and IRAK-4, IRAK-2 cannot autophosphorylate (22–25). However, IRAK-2 possesses catalytic activity and has been implicated in maintenance of proinflammatory cytokine release induced by TLR4 and TLR9 engagement (24). Wesche et al. demonstrated that wild-type IRAK-2 can be phosphorylated when co-cultured with IRAK-1. Although it is not as good a substrate as wild-type IRAK-3, it can replace IRAK-1 when IRAK-1 is knocked down (25). However, a mutant IRAK-2 containing a substitution (K237A) in its ATP-binding pocket is not able to be phosphorylated (23, 25). Kawagoe et al. confirmed

that IRAK-4, and not IRAK-1, phosphorylates IRAK-2, resulting in activation which is essential for IRAK-2 kinase and effector function.

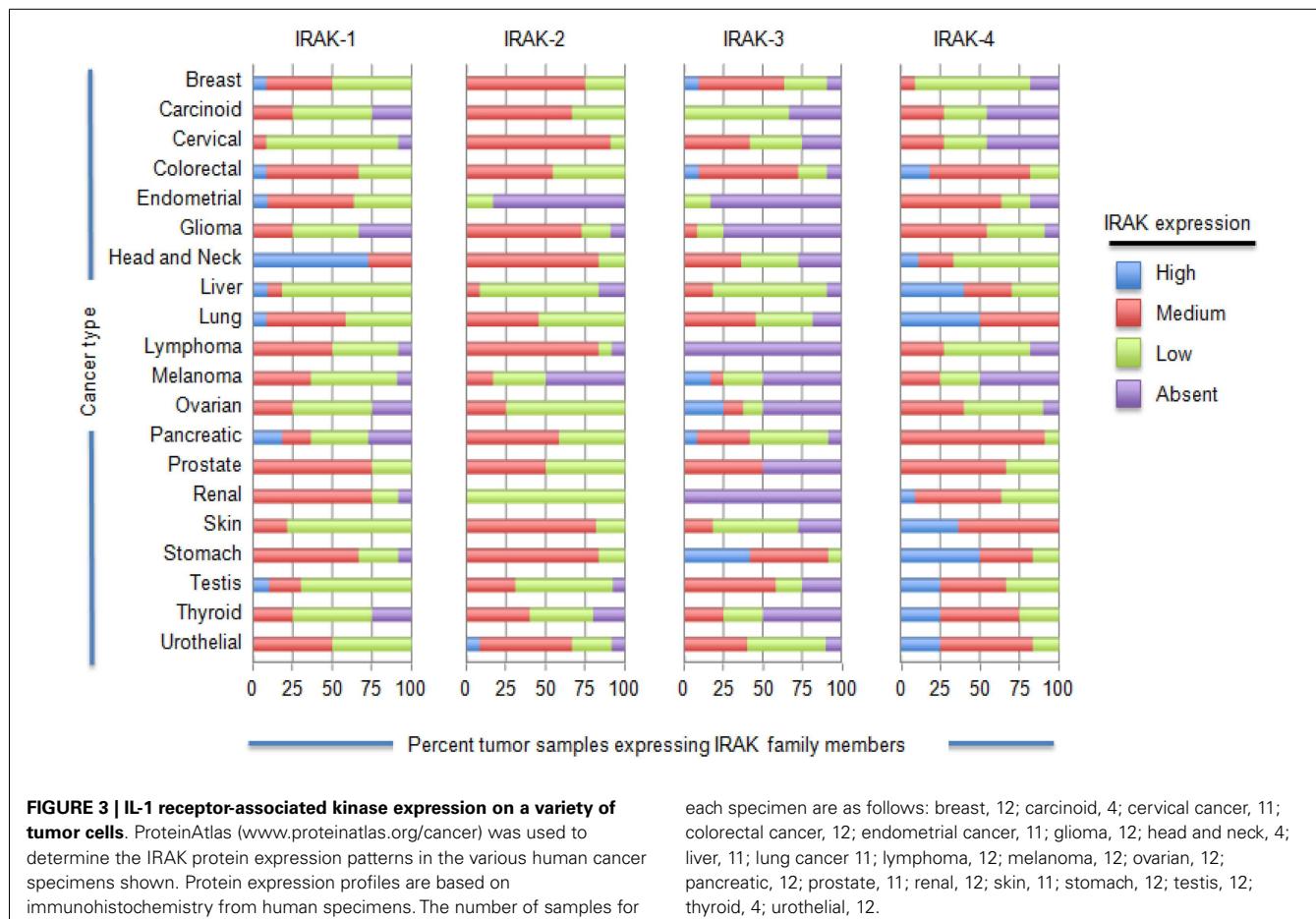
Similar to the other IRAK proteins, IRAK-3 (a.k.a. IRAK-M) can form complexes with MyD88 and TRAF-6. Like IRAK-2, it is considered to be a pseudokinase with very limited capacity for auto-phosphorylation, but with the potential to become activated by other IRAK proteins and serve as a functional kinase. In contrast to other IRAK proteins, IRAK-M is thought to function as a negative regulator that prevents the dissociation of IRAK-1 and IRAK-2 from the receptor complex, inhibiting their interaction with TRAF-6 and interrupting the downstream inflammatory cascade (26, 27).

More recent data show that IRAK-M may promote anti-inflammatory effects through a paradoxical “second wave” of NF- κ B activation. In this model, IRAK-M interacts with the MyD88/IRAK-4 complex to form an IRAK-M Myddosome. Upon ligation of the IL-1R, the IRAK-M Myddosome can induce a second wave of NF- κ B activation and is dependent on MEKK3 signaling (26). However, this secondary NF- κ B activation is believed to decrease overall inflammation by inducing the expression of several inhibitory molecules such as SOCS1, SHIP1, A20, and I κ B α (20). IRAK-M can also interact with IRAK-2 in order to inhibit mRNA transcription of inflammatory cytokines and chemokines.

ROLES OF THE DIFFERENT IRAK FAMILY PROTEINS IN CANCER

IRAK-1

There is an increasing body of data to suggest that IRAK-1 signaling may be important to the development and progression of cancer. *Helicobacter pylori*, bacteria strongly associated with gastric inflammation and the development of gastric cancer has been shown to cause upregulation of TLR2 and TLR5 expression in various cell types and subsequent engagement of these receptors increases IRAK-1 phosphorylation and NF- κ B activation (1). Importantly, gastric carcinogenesis was recently reported to be associated with increased TLR expression and reduced expression of the TLR inhibitors Tollip and PPAR (2). As another example, an evaluation of over 300 tumor samples from non-squamous cell lung cancer (NSCLC) patients showed that tumor tissue had significantly increased cytosolic IRAK-1 expression and decreased nuclear expression relative to adjacent normal tissue (3). Our group has also found IRAK-1 and/or IRAK-4 to localize to the nucleus of melanoma cells, but not melanocytes (Geng, unpublished data). IRAK’s role in the nucleus and how this contributes to tumor progression has not been defined. In order to gain a better sense of the expression levels of each IRAK family member in various cancer types, we analyzed immunohistochemistry data using the online data base ProteinAtlas (<http://www.proteinatlas.org/>), **Figure 3**. These data highlight the heterogeneity of different IRAK family members in different cancer types. Of all the IRAK family members, IRAK-4 was the most frequently expressed (at the medium to high range) and found on the highest percentage of tumor samples. IRAK-1 was the next most frequently expressed with appreciable levels (medium to high) in all tumor samples analyzed. IRAK-2 and IRAK-3 were the least detected IRAK family members, respectively. Despite the high-expression levels of IRAK-1 and IRAK-4,



it is important to note that the level of activation (phosphorylation) was not examined but plays an important role in IRAK signaling.

Additional evidence indicating the importance of IRAK-1 in cancer came from studies of microRNAs (miRNAs) (4). miRNAs are small non-coding RNA sequences that play critical roles in regulating cellular mRNA stability, protein expression, proliferation, apoptosis, and cancer metastasis (5, 6). It has been shown that expression of a specific miRNA (miR-146a) is frequently diminished in metastatic prostate cancers. Intriguingly, upregulation of miR-146a and miR-146b in metastatic breast cancer cell lines has been shown to downregulate TRAF-6 and IRAK-1 expression and subsequently reduce NF- κ B expression (5, 28, 29). Moreover, inhibiting miR-146a expression also reduced cancer cell invasiveness of pancreatic and colon cancer cell lines. Panc-1 and Colo-1 pancreas and colon cancer cell lines, respectively, also have lower miR-146 expression in comparison to non-malignant pancreas cells, and induction of miRNA in these cancer lines decreases their invasiveness. This phenotypic change is also accompanied by down-regulation of EGFR and metastasis-associated protein 2 (MTA-2) (5).

IRAK-1 may be particularly relevant to the pathogenesis of melanoma. The use of rapid subtraction hybridization analysis was used to identify IRAK-1 as one of eight genes that are

differentially expressed in metastatic cells compared to parental human melanoma cell lines, with IRAK-1 expression being upregulated in the metastatic variants (5, 30). Srivastava et al. reported that a large percentage of established human melanoma cell lines exhibit constitutive expression of phosphorylated forms of IRAK-1 and IRAK-4 (31). Patient-derived melanoma tumor samples also exhibited increased expression of phosphorylated IRAK-4 although there did not appear to be a correlation between p-IRAK levels and melanoma stage. Inhibition of IRAK-1 and IRAK-4, using pharmacological inhibitor or siRNA, sensitized melanoma tumors expressing phosphorylated forms of these IRAKs to cytotoxic chemotherapies *in vivo*, raising the possibility that IRAK family proteins may be potential therapeutic targets in cancer. In agreement with these studies, recent data indicate that inhibiting IRAK-1-4 signaling in a variety of leukemias including Waldenstrom macroglobulinemia, diffuse large B-cell lymphoma, myelodysplasia, and acute myeloid leukemia substantially impaired proliferation *in vitro* and *in vivo*, and treatment with IRAK inhibitors prolonged mouse survival (32, 33). We recently found that IRAK-4 signaling in T cell acute lymphoblastic leukemia (T-ALL) is critical for their ability to proliferate but did not induce cell death (Li, unpublished data). In order to determine whether IRAK inhibitors could enhance the cytotoxic effects of chemotherapeutic agents, we screened nearly 500 FDA-approved drugs for their ability to

kill T-ALL cells when combined with IRAK inhibitors. We identified three classes of drugs that worked synergistically with IRAK inhibitors and, in some cases, restored sensitivity of chemoresistant samples. Whether a similar effect will be observed in other cancer types merits further investigation. This is especially true given that many cancers exhibit increased protein levels of IRAK-1 and IRAK-4 and are resistant to chemotherapy (**Figure 3**).

Finally, IRAK-1 activation may also be important for cross talk between cancer cells and other cell populations present in the tumor microenvironment. IL-1 β release by lingual squamous cell carcinomas causes upregulation of the IL-1R and increased levels of p-IRAK-1 in cancer associated fibroblasts. This results in nuclear translocation of NF- κ B and induction of genes important for tumor progression including IL-6, Cox-2, BDNF, and IRF-1 (34).

IRAK-2

In terms of signaling and function, there is some redundancy between IRAK-2 and IRAK-1. Using single and double IRAK knockout mice, Kawagoe and colleagues confirmed that both IRAK1 and IRAK2 have common functionality in the early phase of TLR signaling (23). IRAK2 kinase activity, however, was longer sustained than that of IRAK-1, and IRAK-2 was critical in late-phase TLR responses. This raises the possibility that IRAK-2 may be relevant to chronic inflammatory responses often associated with cancer. Whether downstream signaling differs between IRAK-1 and IRAK-2 remain to be determined. Recent studies by Cui and colleagues suggest that a stress-induced NF- κ B-activated, miRNA-146a-mediated down-regulation of IRAK-1 coupled to an NF- κ B-driven upregulation of IRAK-2 supports a self-perpetuating inflammatory signaling loop (35).

The role of IRAK-2 as a regulator of TLR signaling may be more complex than originally thought. IRAK-2 is known to induce NF- κ B activation through TLR3, TLR4, and TLR8 (14). Of note, IRAK-2 is the only member of the family thought to mediate signaling through TLR3. Interestingly, IRAK-2 has recently been shown to have a dual function (immunosuppressive and immunostimulatory) in TLR9 related signaling and inflammatory responses. Wan and colleagues demonstrated that IRAK-2 suppresses TLR9 signaling in the early post-stimulation phase, raising the activation threshold for TLR9-induced inflammatory response and potentially preventing autoimmunity (36). However, if the higher activation threshold is successfully triggered through a strong stimulus, IRAK-2 mediates a positive feedback loop allowing for sustained release of pro-inflammatory cytokines. It is conceivable that loss of negative regulatory function could allow sustained IRAK-2 activation and inflammation, thus, promoting carcinogenesis. Importantly, whereas TLR9 was previously thought to be expressed only on immune cells, it has been shown that it also expressed on a number of different cancers (oral, prostate, breast, lung, Burkitt lymphoma), and signaling through TLR9 promotes proliferation and/or cell survival (37–44).

IRAK-3 (a.k.a. IRAK-M)

Unlike other IRAK family members that are widely expressed on a variety of cell types, IRAK-M is thought to chiefly reside in monocyte and macrophage populations. As mentioned previously, IRAK-M activation generally acts as a negative regulator

of NF- κ B activation in TLR and IL-1R signaling (45). Also, even though IRAK-M induces a paradoxical “second wave” of MEKK3 dependent NF- κ B activation, the overall effect of IRAK-M favors immunosuppression (26).

IRAK-M is a negative regulator of IRAK-4/IRAK-1 and IRAK-4/IRAK-2 and thus serves to inhibit the expression of a variety of inflammatory molecules induced by IRAK-4. Our working hypothesis is that in cancers with reduced levels of IRAK-M but elevated levels of IRAK-1, -2, and/or -4 will show increased IRAK-4 signaling and consequently elevated levels of inflammatory molecules. In addition to augmenting the amounts of inflammatory factors, the lack of IRAK-M might further sustain IRAK-4 signaling and perpetuate a chronically inflamed tumor environment; chronic inflammation is a hallmark of tumorigenesis and tumor progression (46). That IRAK-3 expression levels are reduced in some cancer types is further highlighted in **Figure 3** and supports our hypothesis.

Even though it is an anti-inflammatory mediator, IRAK-M may still play an important role in tumorigenesis through modulation of the activity of tumor-associated macrophages (TAMs). It is generally thought that there are two types of macrophages associated with cancer (47). These include classically activated (M1) macrophages that secrete pro-inflammatory cytokines and present antigens to cytotoxic immune effector cells, and alternatively activated (M2) macrophages with impaired Th1-like cytokine release (and one favoring Th2 cytokines) and decreased capacity to activate T cells. The M1 type is thought to play a more prominent role in the early stages of carcinogenesis through NF- κ B activation and chronic inflammation to initiate carcinogenesis. As cancers become more established, M1 macrophages may become “re-educated” to take on a M2 phenotype. M2 macrophages can secrete tumor growth factors, promote angiogenesis and invasiveness through remodeling of the tumor matrix, and induce immune tolerance. The term “tumor-associated macrophage” or TAM is typically associated with the M2 phenotype. Indeed, macrophage re-education may be a critical aspect of cancer pathogenesis, and IRAK-M may play a significant role in this process.

IRAK-M may promote cancer progression through modulation of macrophage activity. IRAK-M is known to be an important negative regulator in macrophages in models of inflammation. For example, in mouse models of myocardial infarction, upregulation of IRAK-M in cardiac macrophages reduces myocardial inflammation and prevents adverse cardiac remodeling (45). Naïve monocytes and macrophages exposed to tumor cell lines exhibited decreased expression of TNF α , IL-12p40, and IRAK-1 (48, 49). Moreover, these characteristics, as well as the ability to present antigens, were diminished with prolonged exposure to tumor cells as the macrophages take on an M2 phenotype. A hallmark feature of this transition is the rapid upregulation of IRAK-M in macrophages upon exposure to tumor cells (48, 49). *In vivo* mouse studies using Lewis lung cancer (LLC) cell lines have shown that tumor infiltrating macrophages have higher IRAK-M expression and impaired ability to secrete IL-12, TNF α , and IFN- γ compared to peritoneal macrophages isolated from the same mouse (50). Interestingly, the ability of TAMs to secrete TNF α could be restored by knocking down IRAK-M expression using siRNA (48). These data indicate that IRAK-M upregulation can be induced

by surface-associated or soluble factors from tumor cells to promote tumor growth and immune evasion. Proposed mechanisms include the engagement of hyaluronan (a tumor cell surface glycosaminoglycan) to monocyte-expressed CD44 or secretion of TGF- β . Furthermore, monocytes isolated from patients with chronic myelogenous show upregulation of IRAK-M mRNA, monocytes from chronic lymphocytic leukemia patients (in whom IRAK-M expression was not evaluated) showed impaired ability to secrete cytokines and present antigen. Analysis of a cohort of 439 lung cancer patients showed that the level of IRAK-M expression on tumor cells was a significant and independent predictor of mortality. In contrast, these data suggest that IRAK-M is a critical mediator of cross talk that occurs between tumor cells and macrophages to allow a more favorable tumor microenvironment and facilitate cancer progression (48, 49).

IRAK-4

IRAK-4, the most recently identified member of the family, is considered the “master IRAK” because it is required for all MyD88-dependent NF- κ B activation and for inducing IFN α expression through TLR 7, 8, and 9 (51). Loss of IRAK-4 renders mice completely resistant to LPS-induced shock, and deficiencies in human beings have been associated with increased susceptibility to encapsulated bacterial infections (especially pneumococcal) (52, 53). Data regarding the specific role of IRAK-4 in cancer have not been fully investigated, and its potential role in cancer progression is just now beginning to emerge. As previously discussed (in the Section IRAK-1) some melanomas constitutively express active, phosphorylated forms of IRAK-1 and IRAK-4. Inhibiting IRAK-4 rather than IRAK-1 using shRNA was more effective at sensitizing melanoma tumors and T-ALL cells to chemotherapies. It is still unclear, however, whether this is a direct phenomenon or whether upstream signaling events drive phosphorylation. As IRAK-4 is a lynchpin for MyD88-mediated pro-inflammatory signaling, it can promote carcinogenesis regardless of whether it is directly mutated or not. For example, a subset (29%) of activated B-cell type diffuse large B-cell lymphomas (ABC DLBCL) with a very aggressive phenotype were recently found to carry an oncogenic MyD88 mutation (L265P) that promotes survival. This mutation allowed spontaneous formation of a stable complex between MyD88, IRAK-4, and a phosphorylated form of IRAK-1. However, knockdown of IRAK-1 kinase activity was not required for survival of ABC DLBCLs, while IRAK-4 kinase activity was essential (54). To date, no group has reported any mutations in any of the IRAK family members specifically in cancer but this subject merits further investigation considering recent data uncovering an important role for dysregulated IRAK signaling via MyD88 mutations.

IRAK FAMILY PROTEIN INHIBITORS AS NOVEL CANCER THERAPEUTICS

SMALL-MOLECULE INHIBITORS

Given the strong data indicating that IRAK family proteins are critical mediators of inflammation, there has been considerable interest in developing targeted agents to treat autoimmune and inflammatory diseases. As we previously addressed, IRAK inhibitors (especially IRAK-1 and -4) may also have therapeutic

applications in cancer. Several classes of IRAK-4 inhibitors have been developed, including amino-benzimidazole, thiazole, or pyridine amides, imidazo[1,2-*a*] pyridines, imidazo[1,2-*b*]pyridazines, and benzimidazole-indazoles (47–50, 52, 54). IRAK inhibitors may have particular utility in the treatment of Waldenstrom’s macroglobulinemia, a B-cell lymphoproliferative disorder that is critically dependent upon NF- κ B activation. *However, compounds that target molecules downstream of IRAK-1 are also potential candidates.* One such compound is 5Z-7-oxozeanol, which selectively inhibits TAK-1 and has been shown to reduce inflammation and enhance the sensitivity of breast and pancreatic cancer cells to various chemotherapeutic agents, further highlighting the central role that IRAK signaling plays in chemotherapy resistance (54–56).

BOTANICAL DERIVATIVES

It is possible that plant-derived compounds may also induce anti-inflammatory and anti-cancer therapeutic effects through inhibition of IRAK family members. For example, ginseng (*Panax ginseng*), which is anecdotally described to have many health benefits including anti-inflammatory and anti-cancer properties, contains protopanaxatriol ginsenoside. This agent has been shown to inhibit IRAK-1 and IKK- β phosphorylation in LPS stimulated macrophages, as well as alleviate inflammation induced by 2,4,6-trinitrobenzene sulfonic acid-induced colitis in mice (54, 56–59). The xanthone derivative 1,3,5-trihydroxy-4-prenylxanthone (TH-4-PX) isolated from *Cudrania cochinchinensis*, a plant used as a traditional remedy for diseases in Asia, inhibits LPS/TLR-mediated release of nitrous oxide through inhibition of IRAK-1 (60). A second agent from this plant (isoalvaxanthone) has anti-neoplastic properties, as it can inhibit matrix metalloproteinase-2 expression (a factor associated with tumor invasiveness) *in vitro* in SW620 colon cancer cells. Admittedly, it is unclear if the isoalvaxanthone effects are the result of IRAK family member inhibition, as this agent did not inhibit expression of NF- κ B.

NITROGEN BISPHOSPHONATES

There has been increasing evidence that nitrogen bisphosphonates (NPBs), a class of drugs used to treat osteoporosis, may also have potential for treating cancer. Paradoxically, NPBs are associated with inhibition of IRAK-M expression. The NBP zoledronate reduces IRAK-M levels when cultured with PBMCs from a subset of human blood donors (50%). In these individuals, the reduction in IRAK-M is associated with enhanced cytokine release after TLR stimulation or administration of IL-1 (61). Depletion of IRAK-M in dendritic cells (DCs) using siRNA has been shown to enhance DC migration to lymph nodes, augment cytokine release, and enhance antigen presentation, proliferation, and activation of antigen-specific T cells. Thus, pharmacologic inhibition of IRAK-M using NPBs may likewise improve the induction of cell-based anti-tumor immune responses. A summary of the various IRAK inhibitors is shown in Table 1.

SUMMARY

Dysregulated IRAK signaling in tumors is beginning to emerge as an important factor in cancer initiation, tumor progression, and therapy resistance. Studies from several groups highlight the

Table 1 | A summary of small molecules that can inhibit IRAK family members.

Target	
SMALL-MOLECULE INHIBITORS	
Amino-benzimidazole	IRAK-4
Thiazole/pyridine amides	IRAK-4
Imidazo[1,2-a] pyridines	IRAK-4 and IRAK-1
Imidazo[1,2-b]pyridazines	IRAK-4 and IRAK-1
Benzimidazole-indazoles	IRAK-4 and IRAK-1
5Z-7-Oxozeanol	TAK1
BOTANICAL DERIVATIVES	
Protopanaxatriol ginsenoside	IRAK-1, IKK- β
1,3,5-Trihydroxy-4-prenylxanthone (TH-4-PH)	IRAK-1
NITROGEN BISPHOSPHONATES	
Xoledronate	IRAK-M

potential of IRAK family members as therapeutic targets for cancer treatment alone or when combined with other therapies. A better understanding of how IRAK signaling drives inflammation through interaction with TLR and IL-1 family members will be critical for developing targeted therapies that work synergistically with systemic chemotherapies. Furthermore, such an understanding may allow manipulation of these proteins to favor anti-tumor cytotoxicity rather than carcinogenic downstream effects.

REFERENCES

- Kumar PS, Brandt S, Madassery J, Backert S. Induction of TLR-2 and TLR-5 expression by *Helicobacter pylori* switches cagPAI-dependent signalling leading to the secretion of IL-8 and TNF-alpha. *PLoS One* (2011) 6(5):e19614. doi:10.1371/journal.pone.0019614
- Pimentel-Nunes P, Goncalves N, Boal-Carvalho I, Afonso L, Lopes P, Roncon-Albuquerque R Jr, et al. *Helicobacter pylori* induces increased expression of toll-like receptors and decreased toll-interacting protein in gastric mucosa that persists throughout gastric carcinogenesis. *Helicobacter* (2013) 18(1):22–32. doi:10.1111/hel.12008
- Behrens C, Feng L, Kadara H, Kim HJ, Lee JJ, Mehran R, et al. Expression of interleukin-1 receptor-associated kinase-1 in non-small cell lung carcinoma and preneoplastic lesions. *Clin Cancer Res* (2010) 16(1):34–44. doi:10.1158/1078-0432.CCR-09-0650
- Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med* (2011) 208(6):1189–201. doi:10.1084/jem.20101823
- Li Y, Vandenboom TG, Wang Z, Kong D, Ali S, Philip PA, et al. miR-146a suppresses invasion of pancreatic cancer cells. *Cancer Res* (2010) 70(4):1486–95. doi:10.1158/0008-5472.CAN-09-2792
- Ambros V. microRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell* (2003) 113(6):673–6. doi:10.1016/S0092-8674(03)00428-8
- O’Neill LA. The interleukin-1 receptor/toll-like receptor superfamily: 10 years of progress. *Immunol Rev* (2008) 226:10–8. doi:10.1111/j.1600-065X.2008.00701.x
- Wang Z, Wesche H, Stevens T, Walker N, Yeh WC. IRAK-4 inhibitors for inflammation. *Curr Top Med Chem* (2009) 9(8):724–37. doi:10.2174/156802609789044407
- Park JS, Gamboni-Robertson F, He Q, Svetkauskaitė D, Kim JY, Strassheim D, et al. High mobility group box 1 protein interacts with multiple toll-like receptors. *Am J Physiol Cell Physiol* (2006) 290(3):C917–24. doi:10.1152/ajpcell.00401.2005
- Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, et al. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock* (2006) 26(2):174–9. doi:10.1097/01.shk.0000225404.51320.82
- Kaczanowska S, Joseph AM, Davila E. TLR agonists: our best frenemy in cancer immunotherapy. *J Leukoc Biol* (2013) 93(6):847–63. doi:10.1189/jlb.1012501
- Bowie AG. Insights from vaccinia virus into toll-like receptor signalling proteins and their regulation by ubiquitin: role of IRAK-2. *Biochem Soc Trans* (2008) 36(Pt 3):449–52. doi:10.1042/BST0360449
- Li L, Cousart S, Hu J, McCall CE. Characterization of interleukin-1 receptor-associated kinase in normal and endotoxin-tolerant cells. *J Biol Chem* (2000) 275(30):23340–5. doi:10.1074/jbc.M001950200
- Flannery S, Bowie AG. The interleukin-1 receptor-associated kinases: critical regulators of innate immune signalling. *Biochem Pharmacol* (2010) 80(12):1981–91. doi:10.1016/j.bcp.2010.06.020
- Kollewe C, Mackensen AC, Neumann D, Knop J, Cao P, Li S, et al. Sequential autoprophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. *J Biol Chem* (2004) 279(7):5227–36. doi:10.1074/jbc.M309251200
- Conze DB, Wu CJ, Thomas JA, Landstrom A, Ashwell JD. Lys63-linked polyubiquitination of IRAK-1 is required for interleukin-1 receptor- and toll-like receptor-mediated NF-kappaB activation. *Mol Cell Biol* (2008) 28(10):3538–47. doi:10.1128/MCB.02098-07
- Gottipati S, Rao NL, Fung-Leung WP. IRAK1: a critical signaling mediator of innate immunity. *Cell Signal* (2008) 20(2):269–76. doi:10.1016/j.cellsig.2007.08.009
- Swantek JL, Tsen MF, Cobb MH, Thomas JA. IL-1 receptor-associated kinase modulates host responsiveness to endotoxin. *J Immunol* (2000) 164(8):4301–6. doi:10.4049/jimmunol.164.8.4301
- Yamin TT, Miller DK. The interleukin-1 receptor-associated kinase is degraded by proteasomes following its phosphorylation. *J Biol Chem* (1997) 272(34):21540–7. doi:10.1074/jbc.272.34.21540
- Jensen LE, Whitehead AS. IRAK1b, a novel alternative splice variant of interleukin-1 receptor-associated kinase (IRAK), mediates interleukin-1 signaling and has prolonged stability. *J Biol Chem* (2001) 276(31):29037–44. doi:10.1074/jbc.M103815200
- Rao N, Nguyen S, Ngo K, Fung-Leung WP. A novel splice variant of interleukin-1 receptor (IL-1R)-associated kinase 1 plays a negative regulatory role in toll/IL-1R-induced inflammatory signaling. *Mol Cell Biol* (2005) 25(15):6521–32. doi:10.1128/MCB.25.15.6521-6532.2005
- Brown J, Wang H, Hajishengallis GN, Martin M. TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. *J Dent Res* (2011) 90(4):417–27. doi:10.1177/0022034510381264
- Kawagoe T, Sato S, Matsushita K, Kato H, Matsui K, Kumagai Y, et al. Sequential control of toll-like receptor-dependent responses by IRAK1 and IRAK2. *Nat Immunol* (2008) 9(6):684–91. doi:10.1038/ni.1606
- Cohen P. Targeting protein kinases for the development of anti-inflammatory drugs. *Curr Opin Cell Biol* (2009) 21(2):317–24. doi:10.1016/j.ceb.2009.01.015
- Wesche H, Gao X, Li X, Kirschning CJ, Stark GR, Cao Z. IRAK-M is a novel member of the Pelle/interleukin-1 receptor-associated kinase (IRAK) family. *J Biol Chem* (1999) 274(27):19403–10. doi:10.1074/jbc.274.27.19403
- Zhou H, Yu M, Fukuda K, Im J, Yao P, Cui W, et al. IRAK-M mediates toll-like receptor/IL-1R-induced NFkappaB activation and cytokine production. *EMBO J* (2013) 32(4):583–96. doi:10.1038/embj.2013.2
- Kobayashi K, Hernandez LD, Galan JE, Janeway CA Jr, Medzhitov R, Flavell RA. IRAK-M is a negative regulator of toll-like receptor signaling. *Cell* (2002) 110(2):191–202. doi:10.1016/S0092-8674(02)00827-9
- Bhaumik D, Scott GK, Schokrpur S, Patil CK, Campisi J, Benz CC. Expression of microRNA-146 suppresses NF-kappaB activity with reduction of metastatic potential in breast cancer cells. *Oncogene* (2008) 27(42):5643–7. doi:10.1038/onc.2008.171
- Hurst DR, Edmonds MD, Scott GK, Benz CC, Vaidya KS, Welch DR. Breast cancer metastasis suppressor 1 up-regulates miR-146, which suppresses breast cancer metastasis. *Cancer Res* (2009) 69(4):1279–83. doi:10.1158/0008-5472.CAN-08-3559
- Boukerche H, Su ZZ, Kang DC, Fisher PB. Identification and cloning of genes displaying elevated expression as a consequence of metastatic progression

- in human melanoma cells by rapid subtraction hybridization. *Gene* (2004) **343**(1):191–201. doi:10.1016/j.gene.2004.09.002
31. Srivastava R, Geng D, Liu Y, Zheng L, Li Z, Joseph MA, et al. Augmentation of therapeutic responses in melanoma by inhibition of IRAK-1/-4. *Cancer Res* (2012) **72**(23):6209–16. doi:10.1158/0008-5472.CAN-12-0337
32. Rhyasen GW, Bolanos L, Fang J, Jerez A, Wunderlich M, Rigolino C, et al. Targeting IRAK1 as a therapeutic approach for myelodysplastic syndrome. *Cancer Cell* (2013) **24**(1):90–104. doi:10.1016/j.ccr.2013.05.006
33. Poulin S, Roumier C, Decambron A, Renneville A, Herbaux C, Bertrand E, et al. MYD88 L265P mutation in Waldenstrom macroglobulinemia. *Blood* (2013) **121**(22):4504–11. doi:10.1182/blood-2012-06-436329
34. Dudas J, Fullar A, Bitsche M, Schartinger V, Kovalszky I, Sprinzl GM, et al. Tumor-produced, active interleukin-1beta regulates gene expression in carcinoma-associated fibroblasts. *Exp Cell Res* (2011) **317**(15):2222–9. doi:10.1016/j.yexcr.2011.05.023
35. Cui JG, Li YY, Zhao Y, Bhattacharjee S, Lukiw WJ. Differential regulation of interleukin-1 receptor-associated kinase-1 (IRAK-1) and IRAK-2 by microRNA-146a and NF-kappaB in stressed human astroglial cells and in Alzheimer disease. *J Biol Chem* (2010) **285**(50):38951–60. doi:10.1074/jbc.M110.178848
36. Wan Y, Kim TW, Yu M, Zhou H, Yamashita M, Kang Z, et al. The dual functions of IL-1 receptor-associated kinase 2 in TLR9-mediated IFN and proinflammatory cytokine production. *J Immunol* (2011) **186**(5):3006–14. doi:10.4049/jimmunol.1003217
37. Droemann D, Albrecht D, Gerdes J, Ulmer AJ, Branscheid D, Vollmer E, et al. Human lung cancer cells express functionally active toll-like receptor 9. *Respir Res* (2005) **6**:1. doi:10.1186/1465-9921-6-1
38. Henault M, Lee LN, Evans GF, Zuckerman SH. The human Burkitt lymphoma cell line Namalwa represents a homogenous cell system characterized by high levels of toll-like receptor 9 and activation by CpG oligonucleotides. *J Immunol Methods* (2005) **300**(1–2):93–9. doi:10.1016/j.jimm.2005.02.012
39. Jukkola-Vuorinen A, Rahko E, Vuopala KS, Desmond R, Lehenkari PP, Harris KW, et al. Toll-like receptor-9 expression is inversely correlated with estrogen receptor status in breast cancer. *J Innate Immun* (2009) **1**(1):59–68. doi:10.1159/000151602
40. Lee JW, Choi JJ, Seo ES, Kim MJ, Kim WY, Choi CH, et al. Increased toll-like receptor 9 expression in cervical neoplasia. *Mol Carcinog* (2007) **46**(11):941–7. doi:10.1002/mc.20325
41. Ren T, Xu L, Jiao S, Wang Y, Cai Y, Liang Y, et al. TLR9 signaling promotes tumor progression of human lung cancer cell in vivo. *Pathol Oncol Res* (2009) **15**(4):623–30. doi:10.1007/s12253-009-9162-0
42. Vaisanen MR, Vaisanen T, Jukkola-Vuorinen A, Vuopala KS, Desmond R, Selander KS, et al. Expression of toll-like receptor-9 is increased in poorly differentiated prostate tumors. *Prostate* (2010) **70**(8):817–24. doi:10.1002/pros.21115
43. Xu L, Wang C, Wen Z, Yao X, Liu Z, Li Q, et al. Selective up-regulation of CDK2 is critical for TLR9 signaling stimulated proliferation of human lung cancer cell. *Immunol Lett* (2010) **127**(2):93–9. doi:10.1016/j.imlet.2009.10.002
44. Zhu J, Mohan C. Toll-like receptor signaling pathways – therapeutic opportunities. *Mediators Inflamm* (2010) **2010**:781235. doi:10.1155/2010/781235
45. Chen W, Saxena A, Li N, Sun J, Gupta A, Lee DW, et al. Endogenous IRAK-M attenuates postinfarction remodeling through effects on macrophages and fibroblasts. *Arterioscler Thromb Vasc Biol* (2012) **32**(11):2598–608. doi:10.1161/ATVBAHA.112.300310
46. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) **144**(5):646–74. doi:10.1016/j.cell.2011.02.013
47. Schmieder A, Michel J, Schonhaar K, Goerdt S, Schledzewski K. Differentiation and gene expression profile of tumor-associated macrophages. *Semin Cancer Biol* (2012) **22**(4):289–97. doi:10.1016/j.semcan.2012.02.002
48. del FC, Otero K, Gomez-Garcia L, Gonzalez-Leon MC, Soler-Ranger L, Fuentes-Prior P, et al. Tumor cells deactivate human monocytes by up-regulating IL-1 receptor associated kinase-M expression via CD44 and TLR4. *J Immunol* (2005) **174**(5):3032–40. doi:10.4049/jimmunol.174.5.3032
49. Soares-Schanoski A, Jurado T, Cordoba R, Siliceo M, Fresno CD, Gomez-Pina V, et al. Impaired antigen presentation and potent phagocytic activity identifying tumor-tolerant human monocytes. *Biochem Biophys Res Commun* (2012) **423**(2):331–7. doi:10.1016/j.bbrc.2012.05.124
50. Standiford TJ, Kuick R, Bhan U, Chen J, Newstead M, Keshamouni VG. TGF-beta-induced IRAK-M expression in tumor-associated macrophages regulates lung tumor growth. *Oncogene* (2011) **30**(21):2475–84. doi:10.1038/onc.2010.619
51. Li S, Strelow A, Fontana EJ, Wesche H. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc Natl Acad Sci U S A* (2002) **99**(8):5567–72. doi:10.1073/pnas.082100399
52. Picard C, Puel A, Bonnet M, Ku CL, Bustamante J, Yang K, et al. Pyogenic bacterial infections in humans with IRAK-4 deficiency. *Science* (2003) **299**(5615):2076–9. doi:10.1126/science.1081902
53. Suzuki N, Suzuki S, Duncan GS, Millar DG, Wada T, Mirtsos C, et al. Severe impairment of interleukin-1 and toll-like receptor signalling in mice lacking IRAK-4. *Nature* (2002) **416**(6882):750–6. doi:10.1038/nature736
54. Ngo VN, Young RM, Schmitz R, Jhavar S, Xiao W, Lim KH, et al. Oncogenically active MYD88 mutations in human lymphoma. *Nature* (2011) **470**(7332):115–9. doi:10.1038/nature09671
55. Acuna UM, Wittwer J, Ayers S, Pearce CJ, Oberlies NH, DE Blanco Ej. Effects of (5Z)-7-oxozeanolin on MDA-MB-231 breast cancer cells. *Anticancer Res* (2012) **32**(7):2415–21.
56. Melisi D, Xia Q, Paradiso G, Ling J, Moccia T, Carbone C, et al. Modulation of pancreatic cancer chemoresistance by inhibition of TAK1. *J Natl Cancer Inst* (2011) **103**(15):1190–204. doi:10.1093/jnci/djr243
57. Lee IA, Hyam SR, Jang SE, Han MJ, Kim DH. Ginsenoside Re ameliorates inflammation by inhibiting the binding of lipopolysaccharide to TLR4 on macrophages. *J Agric Food Chem* (2012) **60**(38):9595–602. doi:10.1021/jf301372g
58. Hu Q, He G, Zhao J, Soshilov A, Denison MS, Zhang A, et al. Ginsenosides are novel naturally-occurring aryl hydrocarbon receptor ligands. *PLoS One* (2013) **8**(6):e66258. doi:10.1371/journal.pone.0066258
59. Joh EH, Lee IA, Jung IH, Kim DH. Ginsenoside Rb1 and its metabolite compound K inhibit IRAK-1 activation – the key step of inflammation. *Biochem Pharmacol* (2011) **82**(3):278–86. doi:10.1016/j.bcp.2011.05.003
60. Chiou WF, Chen CC, Lin IH, Chiu JH, Chen YJ. 1,3,5-Trihydroxy-4-prenylxanthone represses lipopolysaccharide-induced iNOS expression via impeding posttranslational modification of IRAK-1. *Biochem Pharmacol* (2011) **81**(6):752–60. doi:10.1016/j.bcp.2010.12.022
61. Norton JT, Hayashi T, Crain B, Corr M, Carson DA. Role of IL-1 receptor-associated kinase-M (IRAK-M) in priming of immune and inflammatory responses by nitrogen bisphosphonates. *Proc Natl Acad Sci U S A* (2011) **108**(27):11163–8. doi:10.1073/pnas.1107899108

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NOD-like receptors: master regulators of inflammation and cancer

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Cytosolic NOD-like receptors (NLRs) have been associated with human diseases including infections, cancer, and autoimmune and inflammatory disorders. These innate immune pattern recognition molecules are essential for controlling inflammatory mechanisms through induction of cytokines, chemokines, and anti-microbial genes. Upon activation, some NLRs form multi-protein complexes called inflammasomes, while others orchestrate caspase-independent nuclear factor kappa B (NF-κB) and mitogen activated protein kinase (MAPK) signaling. Moreover, NLRs and their downstream signaling components engage in an intricate crosstalk with cell death and autophagy pathways, both critical processes for cancer development. Recently, increasing evidence has extended the concept that chronic inflammation caused by aberrant NLR signaling is a powerful driver of carcinogenesis, where it abets genetic mutations, tumor growth, and progression. In this review, we explore the rapidly expanding area of research regarding the expression and functions of NLRs in different types of cancers. Furthermore, we particularly focus on how maintaining tissue homeostasis and regulating tissue repair may provide a logical platform for understanding the liaisons between the NLR-driven inflammatory responses and cancer. Finally, we outline novel therapeutic approaches that target NLR signaling and speculate how these could be developed as potential pharmaceutical alternatives for cancer treatment.

Keywords: apoptosis, autophagy, colorectal cancer, innate immunity, intestinal inflammation, inflammasome, nod-like receptors, nodosome

INTRODUCTION

Over the past two decades, immunologists have begun to appreciate the complexity of the innate immune system, its importance as the first wave of defensive action against perceived harmful microbes or foreign particles and its functions in triggering antigen-specific responses by engaging the adaptive immune system. Innate immune responses are orchestrated by germline-encoded pattern recognition receptors (PRRs) (1). PRRs recognize conserved pathogen-derived and damaged self-derived molecular components, commonly referred to as pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs), respectively (2, 3). PRR superfamilies are broadly classified based upon structural homology and the requirement of different adaptor proteins that ensure their function and downstream signal transduction (4). The PRRs include members of the Toll-like receptors (TLRs) (3), nucleotide-binding, and oligomerization domain containing receptors [NOD-like receptors (NLRs)] (5, 6), retinoic acid-inducible gene (RIG) I-like RNA helicases (7), C-type lectins (8), and AIM2 like receptors (ALRs) (9). Evidence in the field points to a paramount importance of NLRs in human diseases with increasing interest in translating this knowledge toward clinical benefits. Due to the active role of NLRs in regulating pro-inflammatory signals and recruiting the adaptive arm of the immune system, dysregulation of microbial sensing has been reported to influence disease outcomes and tumorigenesis

(10). In this review, we will describe the crucial roles of NLRs in cancer development and progression, and discuss the possibility of NLRs as targets for tumor therapy.

FACTORS THAT INFLUENCE TUMORIGENESIS

Observations by Rudolf Virchow in the nineteenth century indicated a link between inflammation and cancer, and suggested that immune and inflammatory cells are frequently present within tumors. Indeed, chronic inflammation plays critical roles in various stages of cancer development and progression (11–13). Many cancer risk factors are associated with a source of inflammation or act through inflammatory mechanisms such as those evoked by bacterial and viral infections (14), tobacco smoke (15), obesity (16, 17), and aging or cell senescence (18, 19). While some cancers arise from chronic inflammation or after immune deregulation and autoimmunity, solid malignancies elicit intrinsic immune mechanisms that guide the construction of a tumorigenic microenvironment (12, 13, 20). Although the exact mechanism of how inflammation leads to neoplastic transformation is not fully known, it is suggested that inflammatory immune cells like macrophages and T cells are the main orchestrators of inflammation-mediated tumor progression. These cells secrete cytokines and chemokines that cause DNA damage, generate mutagenic reactive oxygen species (ROS), and supply cancer cells with growth factors (13). In addition, inflammatory mechanisms

were shown to promote genetic instability by impairing DNA repair mechanisms, altering cell cycle checkpoints, and often facilitating epigenetic silencing of anti-tumor genes, thus contributing to the high degree of genetic heterogeneity in tumors (21). Oncogenic mutations prompted by an inflammatory microenvironment frequently cause neoplastic transformation by promoting excessive proliferation and resistance to cell death (22). Indeed, impaired expression and activity of proteins that control cell survival, such as the inhibitor of apoptosis proteins (IAPs) and the BCL2 family of proteins, is a common occurrence in many cancers (23, 24). Typically known to exert strong anti-apoptotic functions, IAPs neutralize pro-apoptotic second mitochondrial activator of caspases (SMAC) and inhibit activation of apoptotic caspases, thereby promoting cell survival during both physiological stresses and pathogenic stimulations (25–29). Owing to their strong pro-survival potency, enhanced expression of IAPs has been correlated with several human cancers (22). Unlike IAPs, the BCL2 family of proteins consists of both pro- and anti-apoptotic proteins that control critical checkpoints of intrinsic apoptosis by regulating mitochondrial integrity and release of cytochrome *c* into the cytosol (30). Deregulation of the functions of BCL2 proteins, i.e., down-regulation of pro-apoptotic members and overexpression of pro-survival members, has been strongly correlated with tumorigenesis and resistance to chemotherapy (31). Interestingly, the pro-apoptotic BID, PUMA, and NOXA are transcriptional targets of the tumor suppressor gene p53 and loss of their expression enhances tumorigenesis and morbidity of MYC overexpressing transgenic mice (32, 33). It was described that the transcription factor p53 senses physiological stresses and is critical for restraining tumor growth. Indeed, loss of p53 expression or function in both humans and mice has been proven to promote sporadic tumorigenesis (34, 35). Induction of target genes that inhibit cancer progression is generally considered to be the canonical mechanism of p53-mediated tumor-suppression. These target genes directly modulate cellular programs involving induction of apoptosis, cell cycle arrest, and promotion of cellular senescence and DNA repair (36). Recently, non-canonical functions of p53 have come to light, like the regulation of cellular metabolism, cell-to-cell communication, autophagy, tumor invasion, and metastasis, making p53 an attractive pharmaceutical target for treating cancers [reviewed in Ref. (37)]. Early detection of rogue tumor cells by the innate immune cells and their rapid removal is a key host defense strategy for evading tumorigenesis. In particular, natural killer (NK) cells are primary sentinels that guarantee such immune surveillance by differentiating normal cells from stressed or tumor cells via the expression of specific NK receptors (38). Indeed, increased presence of NK cells at tumor sites has been reported to improve remission, whereas decreased NK cell anti-tumor activity has been correlated with a greater likelihood for developing cancer (39).

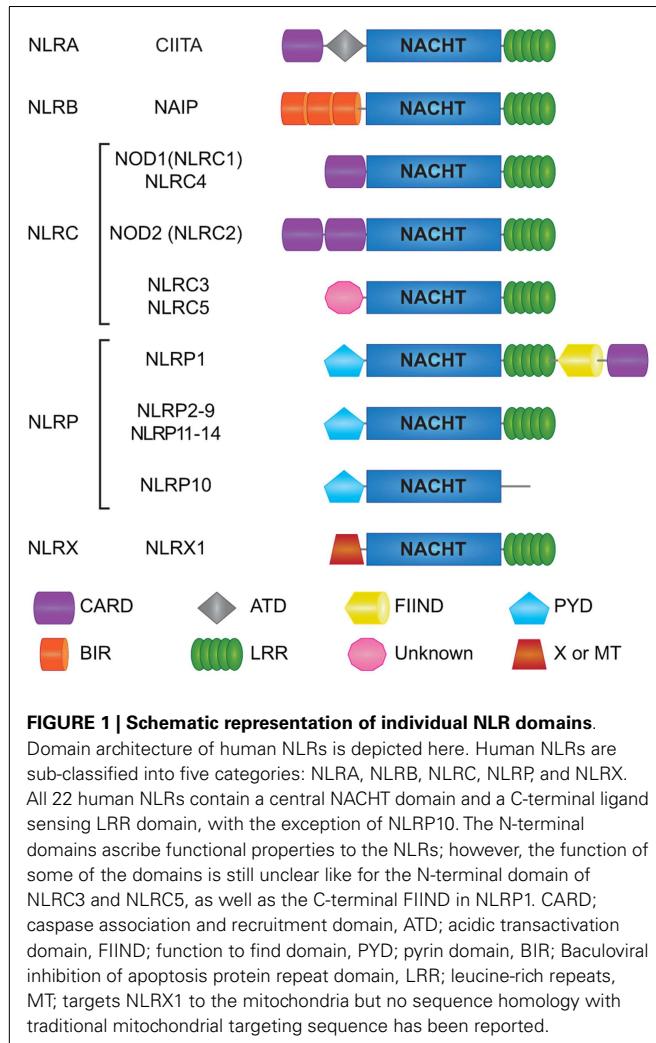
NOD-LIKE RECEPTORS IN CANCER

OVERVIEW OF NLRs

NOD-like receptors are a relatively recent addition to the PRR superfamily (40–42). All NLRs contain a central NACHT domain that facilitates oligomerization, and bear multiple leucine-rich repeats (LRRs) on their C-terminal for ligand sensing (5, 43). The

22 human NLRs can be distinguished into five subfamilies by their N-terminal effector domains that bestow unique functional characteristics to each NLR (43) (Figure 1). NLRs with an N-terminal acidic transactivation domain are termed NLRA (CIITA) and serve as transcriptional regulators of MHC class II antigen presentation (44). NLRB (NAIP) proteins have an N-terminal baculoviral inhibition of apoptosis repeat (BIR) domain and are largely recognized for their roles in host defense and cell survival. For instance, NAIP5 is known to induce host defense against bacterial infections by curtailing macrophage permissiveness to *Legionella pneumophila*, the causative agent of the Legionnaires' disease (45–47). N-terminal caspase activation and recruitment domain (CARD) distinguishes the NLRC subfamily (NLRC 1–5) and allows direct interaction between members of this family and other CARD carrying adaptor proteins. NOD1 (NLRC1) and NOD2 (NLRC2), the founding members of the NLRs, are key sensors of bacterial peptidoglycan (PGN) and are crucial for tissue homeostasis and host defense against bacterial pathogens (48). Notably, single-nucleotide polymorphisms (SNPs) in the NOD2 (CARD15) gene are among the most significant genetic risk factors associated with Crohn's disease (CD) susceptibility (49, 50), hence the rising interest in unraveling the functions of NOD1 and NOD2 receptors in microbial sensing, intestinal homeostasis, and disease. Members of the pyrin domain (PYD) containing NLRP subfamily (NLRP 1–14) are best known for their role in inducing the formation of the oligomeric inflammatory complex "Inflammasome" (51). NLRX1, the only described member of the NLRX subfamily contains an N-terminal mitochondria-targeting sequence required for its trafficking to the mitochondrial membrane (Figure 1). Mechanistically, NLRX1 was shown to down-regulate mitochondrial anti-viral signaling protein (MAVS)-mediated type I interferon (IFN) production (52), interfere with the TLR-TRAF6-NF- κ B pathways (53, 54), and enhance virus induced-autophagy (55, 56). On the other hand, NLRX1 was implicated in the generation of ROS induced by TNF α and Shigella infection magnifying the JNK and NF- κ B signaling (57). Interestingly, NLRX1-mediated ROS generation was involved in promoting *Chlamydia trachomatis* replication in epithelial cells (58). However, recent data from Soares et al. revealed that bone marrow macrophages (BMMs) and mouse embryonic fibroblasts (MEFs) from Wild type (WT) or *Nlrx1*^{-/-} mice respond equally to *in vitro* infection with Sendai virus or following *in vivo* challenge with influenza A virus and TLR3 ligand Poly I:C (59). Additionally, Rebsamen et al. reported no significant contribution of NLRX1 in RLR-MAVS signaling both *in vitro* and *in vivo* (60). Overall, the precise role of NLRX1 remains controversial and further research is required to validate its pro or anti-inflammatory properties.

Dysregulated apoptosis and autophagy pathways, as well as excessive chronic inflammation are major drivers of carcinogenesis. NLRs are innate immune sensors that actively communicate with a myriad of cell death regulators. Hence, these PRRs are well-positioned to influence tumor development and progression particularly at sites with high host-microbiome interactions like the gut. One of the mysteries of the innate immune system is how do NLRs sense molecular patterns from both commensal and pathogenic microorganisms and manage to tolerate one while help eradicate the other (5, 61). This disparity in NLR functions is particularly useful in the intestinal epithelia where host cells are in



constant contact with millions of microbes. Consequently, it came as little surprise when common variants in the NLR genes were correlated with the incidence of CD and susceptibility to cancers (50, 62–64). Due to these correlations, most of the studies have been focused on understanding the mechanisms by which NODs and inflammasome NLRs regulate intestinal inflammation and tumorigenesis.

NOD1 AND NOD2 IN CANCER

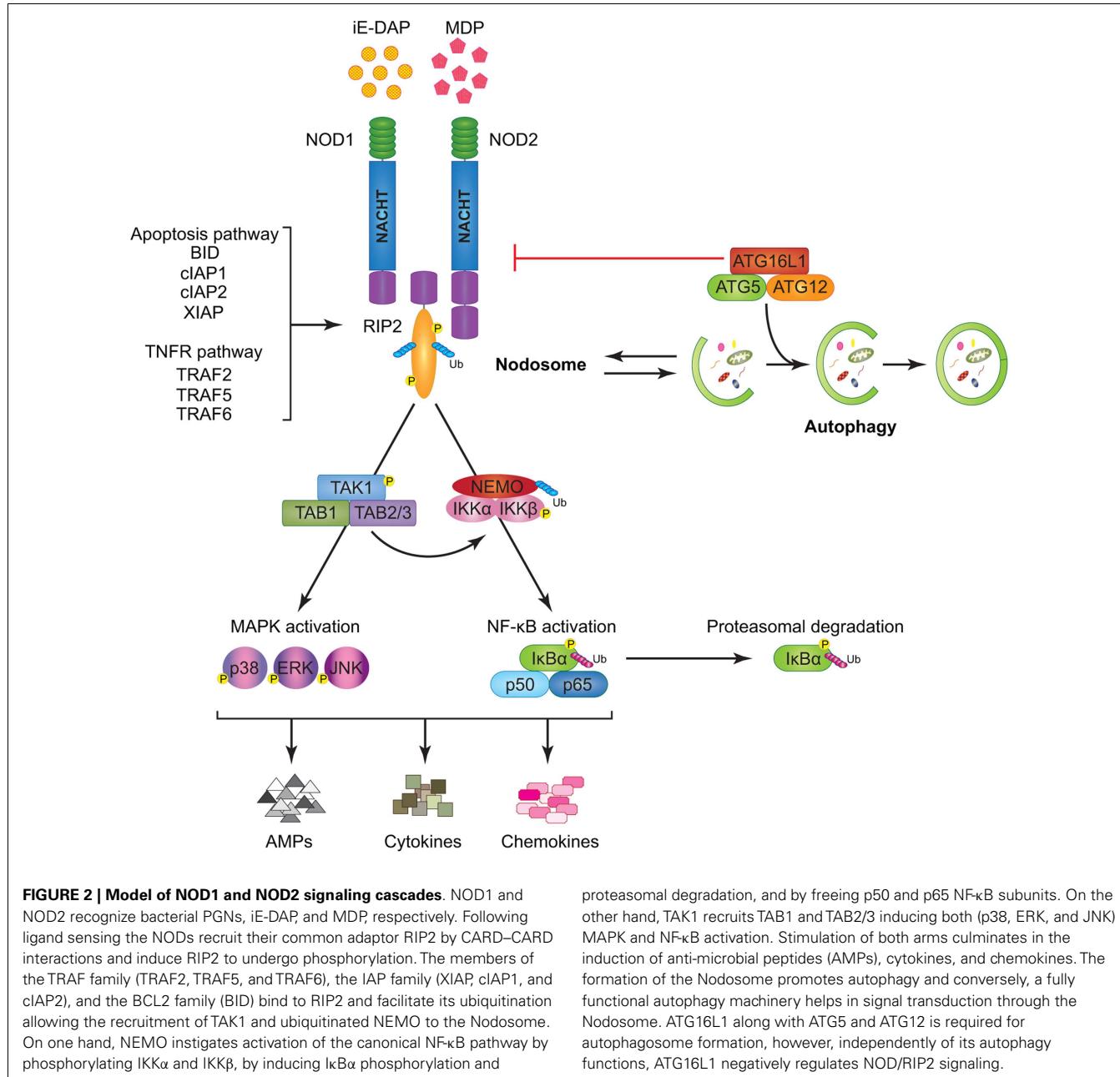
NOD-DEPENDENT SIGNALING CASCADES

NOD1 and NOD2 are cytosolic proteins that sense intracellular bacterial PGN and trigger signal transduction via NF- κ B and MAPK activation. NOD1 is expressed in both hematopoietic and non-hematopoietic cells and responds to intracellular gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP) mostly present on Gram-negative bacteria and only on some select Gram-positive bacteria, like *Listeria* and *Bacillus* species (65–67). Unlike NOD1, NOD2 expression is largely restricted to hematopoietic cells and certain specialized epithelial cells such as the small intestinal Paneth cells (68). NOD2 recognizes cytosolic muramyl dipeptide (MDP) found in the PGN of all bacteria (69). Besides

providing immunity against intracellular bacteria, NODs were revealed to be critical for host defense against non-invasive Gram-negative bacteria like *Helicobacter pylori*, following delivery of its PGN into the host cells through the bacterial type IV secretion system (70). Moreover, NOD1 and NOD2 ligands were also described to gain access to the cytosol by endocytosis with the help of transporter proteins like SLC15A3 and SLC15A4 (71–73). Notably, NOD1 and NOD2 have been reported to localize to the plasma membrane at the sites of infection; however, the biological relevance of this translocation remains elusive (74, 75). Interestingly, a recent report accentuated the importance of NOD proteins in monitoring the activation state of small Rho GTPases (e.g., RAC1, CDC42, and RHOA) and inducing unusual immune responses in the host in response to bacterial infection (76). Upon activation by their cognate ligands both NOD1 and NOD2 self-oligomerize, undergo a conformational change, and through homotypic CARD–CARD interactions allow the recruitment of the CARD containing adaptor Receptor-interacting protein kinase 2 (RIP2 or RIPK2) (41, 42, 77, 78) (Figure 2). This event facilitates the formation of a multi-protein signaling complex termed “Nodosome,” which leads to downstream NF- κ B and MAPK-mediated inflammatory and anti-microbial output. Indeed, cells or mice lacking RIP2 do not respond to NOD agonists and fail to produce pro-inflammatory and anti-microbial molecules (78–80). Initially, it was thought that NOD oligomerization initiated RIP2 aggregation and activation by “induced proximity” (81). While this model still stands true, over the years new body of research has contributed a wealth of data regarding specific sequence of events that leads to RIP2 activation. In contrast to the earlier studies (82–85), recent *in vitro* data using pharmacological inhibitors as well as *in vivo* evidence using a knock-in mouse with kinase-dead RIP2 (K47A) have highlighted the key role of the kinase activity of RIP2 in NOD-mediated immune responses (86, 87).

Lately, it was described that the pathways activated downstream of NOD proteins are closely related to those activated by death receptors, notably TNF receptor 1 (TNFR1). For instance, hierarchical recruitment of selective TNFR-associated factors (TRAF2, TRAF5, or TRAF6) facilitates Lys63 poly-ubiquitination and activation of RIP2 (88–90). Activated RIP2 facilitates ubiquitination of NEMO (also called IKK γ) leading to the recruitment of tumor growth factor β -activated kinase 1 (TAK1) and TAK1 binding proteins (TAB) 1, TAB2, or TAB3 (91, 92). Following this complex formation, IKKs (IKK α and IKK β) get phosphorylated eventually driving the phosphorylation and degradation of I κ B α and subsequent transcription of NF- κ B target genes (5, 89, 92) (Figure 2). RIP2 activation also constitutes a key event that links the NOD–RIP2 cascade with the p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) MAPK pathways (93).

In addition to TRAFs, members of the IAP family including X-linked IAP (XIAP) and cellular IAP1 (cIAP1) and cIAP2 were described to physically interact with RIP2 and facilitate NOD-mediated immunity (94–98). Both *in vitro* and *in vivo* studies suggest a strong role for cIAP1 and cIAP2 in promoting NOD signaling (Figure 2); however, the mechanism for such positive regulation is still not fully understood (94, 99–101). Similarly, XIAP was reported to recruit a linear ubiquitin chain assembly complex (LUBAC) for RIP2 ubiquitination and this step was proven



critical for downstream NF- κ B regulation (96, 97). Upon microbial sensing another E3 ubiquitin ligase, ITCH, also ubiquitinates RIP2, and it is speculated that ITCH-mediated ubiquitination acts like a molecular switch dictating the fate of the signaling circuit to NF- κ B or p38 and JNK activation (102). Pathogen-mediated NOD1 activation has also been shown to elicit protective immune responses via RIP2-TRAF3-IRF7-mediated transcription of IFN β (79). Overall, it is tempting to speculate that similar to pro-survival association of RIP1 with cIAP1 and cIAP2 (103), interactions between RIP2 and the IAPs may also lead to modulation of cellular apoptosis. However, neither NODs nor RIP2 has been demonstrated to exploit these associations to affect cell survival. Similarly, several studies have alluded to NODs as being regulators

of caspase-mediated apoptosis (82, 104, 105); yet, no direct link has so far been reported. Recently, the pro-apoptotic BH3 only BCL2 family protein BID (BH3 interacting-domain death agonist) was identified in a genome wide siRNA screen as a positive regulator of NOD signaling (101). BID was demonstrated to bind to RIP2 bridging both NOD and IKK complexes to specifically transduce NF- κ B and ERK signaling events (101). Notably, BID was phosphorylated upon activation with NOD agonists and these innate immune functions of BID were found to be independent of its pro-apoptotic processing by caspase-8 (101). The discovery involving a classical pro-apoptotic protein, such as BID, in NOD-RIP2 signaling strengthens the concept that inflammatory and cell death pathways do not function as discrete mechanisms but share

common adaptors. Such adaptors can exert multiple functions depending upon the nature of the stimuli (5, 106–108) (**Figure 2**). One recent study have reported that BID-deficient mice exhibit a normal NOD-mediated immunity (109), suggesting that further investigations are still needed to clearly decipher the implication of BID in NOD signaling.

Similar to *NOD2*, a SNP encoding a missense variant in the autophagy gene *ATG16L1* was strongly associated with the incidence of CD, raising a possible common role of both genes in host defense mechanisms (110, 111). Intriguingly, it has been described that NOD1 and NOD2 stimulation enhances autophagy, either directly by interacting with ATG16L1 (112) or indirectly (112–115). Conversely, pharmacological inhibition of both early and late autophagy has been proven to down-regulate MDP-mediated NF-κB and MAPK activation, suggesting that autophagocytic trafficking of MDP may be required for efficient NOD2 signaling (114). Surprisingly, ATG16L1 was recently shown to negatively regulate NOD1- and NOD2-mediated inflammatory signaling by interfering with RIP2 ubiquitination and recruitment to the Nodosome (116) (**Figure 2**). Taken together, this information suggests that different NLRs can have opposing regulatory effects on autophagy and cell death, yet the molecular triggers that dictate these actions are not fully understood.

NOD PROTEINS AND CANCER

Three mutations within the LRR region of the *NOD2* gene have been associated with increased CD susceptibility. Interestingly, these same mutations have also been found to directly interfere with NOD2's bacterial sensing faculties and downstream NF-κB activation (49, 50). Notably, such inactivation of NOD2 immunity has been indicated to enhance the risk of bacterial infections following chemotherapy in patients with acute myeloid leukemia (117). In addition, *NOD2* polymorphisms have been correlated with modifications in gastric mucosa and increased risk for *H. pylori* induced gastric cancer (118). Apart from intestinal disorders, mutations in *NOD2* have been linked with increased prevalence of early onset breast (119) and lung cancers (120, 121). However, how *NOD2* contributes to the initiation and the progression of cancer remains ill defined. Although no mutations in the *NOD1* gene have been so far associated with the incidence of intestinal inflammation or even colorectal cancer (CRC), murine models clearly designate a central anti-tumorigenic function for NOD1 in the pathophysiology of disease. For instance, *Nod1*^{−/−} mice have been described to be susceptible to dextran sulfate sodium (DSS), a sulfated polysaccharide highly toxic to enterocytes (122). Upon combination of a single hit of the carcinogen, azoxymethane (AOM), with DSS (123), NOD1-deficient mice were found to develop significantly more and larger colonic tumors as compared to WT mice (122). This experimental CRC model is particularly applicable when the focus is on understanding colitis-driven tumor initiation and progression. The *Apc*^{Min/+} mouse is a *N*-Ethyl-*N*-Nitrosourea (ENU) mutant model carrying the multiple intestinal neoplasia (Min^{+/+}) mutation and recapitulates many aspects of human hereditary or sporadic CRCs with mutations in the adenomatous polyposis coli (Apc) gene (124–127). Intriguingly, it has been reported that treatment with low doses of DSS leads to increased colonic tumors in

Apc^{Min/+}*Nod1*^{−/−} mice suggesting that NOD1 serves as a negative regulator of the tumor-promoting Wnt/β-catenin cascade (128, 129). Further analysis revealed that absence of NOD1 exacerbated NF-κB-mediated inflammation early during colitis causing gut barrier damage and prompted a second wave of microbiota driven inflammation and intestinal epithelial cell (IEC) proliferation, thus initiating tumor development. These conclusions are supported by the observation that antibiotic treatment of *Nod1*^{−/−} mice ameliorated DSS-induced CRC (122). While most investigations have been focused on the role of NOD1 in models of intestinal tumorigenesis, one report provided experimental evidence for the protective role of NOD1 in breast cancer (104). Herein, it was shown that NOD1-deficient MCF-7 breast cancer cells were resistant to iE-DAP and cycloheximide mediated cell death. Interestingly, SCID mice grafted with NOD1 overexpressing cells exhibited rapid tumor regression. In sharp contrast, mice grafted with NOD1-deficient MCF-7 cells displayed increased and continued tumor growth (104).

Like *Nod1*^{−/−} mice, NOD2-deficient mice have been revealed to be highly susceptible to DSS-induced colitis by inheritance of dysbiotic microbiota that markedly sensitizes mice to injury (130). Furthermore, *Nod2*^{−/−} mice have been found to display worse disease outcome with increased epithelial dysplasia, heightened tumor burden, and elevated expression of the pro-inflammatory cytokine IL-6 when subjected to AOM–DSS treatment. This transmissible phenotype was significantly ameliorated upon treatment with broad-spectrum antibiotics or using the neutralizing IL-6 receptor antibody (130). Altogether, these findings reinforce the idea that aberrant NOD signaling gives rise to dysbiosis that in an inflammatory setting ultimately causes mucosal injury and drives CRC. So far, the translational value of this knowledge is limited but with the recent technological advances in the microbiome research it is predicted that modulation of dysbiosis could be used as a therapeutic strategy for patients with CD as well as CRC.

Contrary to the protective role for NODs in intestinal tumorigenesis, increased expression of both NOD1 and NOD2 has been reported in the head and neck squamous cell carcinoma biopsies as compared to the healthy nasal biopsies. These findings implicate NODs in enhancing head and neck cancers; however, thus far no corroborating experimental evidence has been reported (131). Furthermore, iE-DAP stimulation of human pharyngeal squamous carcinoma cell lines (Detroit 562 and FaDu) has been determined to augment the production of β-defensins, which can serve as chemoattractants, thus fostering an inflammatory and pro-tumorigenic environment (131).

INFLAMMASOME NLRs IN CANCER

INFLAMMASOME NLRs: NLRP3-MEDIATED SIGNALING CASCADES

While NOD1 and NOD2 form the Nodosome, other NLRs assemble macromolecular inflammasome complexes. To date, various inflammasome platforms have been described (132), but the NLRP3 inflammasome is the most commonly studied. The reason behind this could be the initial discovery of mutations in the *NLRP3* (*CIAS1*) gene implicating this PYD containing protein in both familial cold auto-inflammatory syndrome (FCAS) and Muckle–Wells Syndrome (MWS) (133). Thus, the NLRP3 inflammasome will be described here as a prototype for these NLRs

(Figure 3). Classically, the inflammasome has been described to consist of an NLRP, the inflammatory protease caspase-1, and the apoptosis-associated speck like protein (ASC) (51). ASC contains an N-terminal PYD and a C-terminal CARD making it uniquely suited for bringing into close proximity the two key components, caspase-1 and NLRPs (134, 135). Upon activation, NLRP3 recruits ASC and caspase-1, which is a prerequisite for the cleavage and maturation of the inflammatory cytokines IL-1 β and IL-18 and consequent inflammatory cell death named pyroptosis (136–141). Lately, a more complex model for NLRP3-inflammasome activation has been proposed where two adaptors, ASC and mitochondrial MAVS, are required for optimal inflammasome triggering (142).

Owing to its widespread expression in numerous cell types such as neutrophils, monocytes, DCs, epithelial cells, and T cells (140, 143, 144), NLRP3 is exposed to a wide array of PAMPs and DAMPs that instigate the assembly and activation of the inflammasome [reviewed in Ref. (5, 132, 145–148)]. The NLRP3-inflammasome formation requires a two-step process (149). The priming step (or signal 1) involves TLR-NF- κ B-driven induction of inflammasome components, as basal expression of NLRP3 in resting cells is insufficient for effective inflammasome activation (149, 150). However, certain cells like the human blood monocytes and murine macrophages appear to activate the NLRP3 inflammasome in response to LPS stimulation alone (151, 152). It is noteworthy that a transcriptionally silent mechanism for TLR4-mediated inflammasome priming has been lately discovered (153, 154). This mechanism involves mitochondrial ROS (mtROS)-driven deubiquitination of NLRP3, suggesting that constitutive ubiquitination of NLRs may be a homeostatic mechanism to prevent overt inflammasome activity (154). The second activation step (or signal 2) promotes the NLRs to undergo homotypic oligomerization and assemble the inflammasome.

While several models have been proposed to define the signals behind NLRP3 activation, the precise mechanisms remain hitherto unresolved. Various bacterial pathogens induce potassium efflux and activate the NLRP3 inflammasome via the action of secreted pore-forming toxins (e.g., nigericin from *Streptomyces hygroscopicus*, listeriolysin O from *Listeria monocytogenes*, pneumolysin from *Streptococcus pneumoniae*, alpha-hemolysin, etc.) (138, 155, 156) (**Figure 3**). In addition, NLRP3 inflammasomes have been known to assemble in response to cytosolic bacterial and viral RNA both *in vivo* and *in vitro* (137, 157–160). Extracellular adenosine tri-phosphate (ATP) released from dying or damaged cells also causes NLRP3-inflammasome activation through either paracrine or autocrine sensing of ATP by the purinergic receptor P2X7 (138, 161–163). Besides, it has been defined that ATP released from phagocytosed dying cells acts similarly on P2X7 and prompts pannexin-1 (PANX1) channels to open, thus resulting in potassium (K $^{+}$) efflux and allowing other agonists to further engage and activate NLRP3 (164) (**Figure 3**).

Monosodium urate (MSU) and calcium pyrophosphate dehydrate crystals, alum, amyloid- β fibrils, as well as environmental pollutants like asbestos and silica strongly activate the NLRP3 inflammasome (139, 165–170). According to one model for this mode of activation, uptake of the crystalline and particulate matters into the cell causes lysosomal destabilization and release of

cathepsin B, which is sensed by NLRP3 (168, 169). Interestingly, however, opposing results were obtained when cathepsin B-deficient BMMs were used to test this hypothesis, as no differences in IL-1 β or caspase-1 cleavage were observed in response to several inflammasome activators such as hemozoin, MSU, or alum (171). Another model suggests that these activators prompt generation of mtROS and mitochondrial DNA, both of which are responsible for NLRP3-inflammasome activation (172–174). Evidently, pharmacological inhibition of mtROS production has been shown to prevent NLRP3-inflammasome formation indicating that ROS generation is an upstream event for NLRP3 activation (165, 166) (**Figure 3**). Liposomes have been found to induce mtROS and NLRP3-inflammasome activation by triggering calcium (Ca $^{2+}$) influx via the transient receptor potential melastatin 2 (TRPM2), although the exact mechanism linking ROS production to TRPM2 channel opening is still not well-characterized (175). On the other hand, the mitochondrial protein cardiolipin has been shown to directly bind and activate NLRP3 in a ROS-independent manner suggesting that ROS may not be the common denominator engaging the NLRP3 inflammasome (176). Recent advances have put forward additional mechanisms underlying NLRP3-inflammasome activation. In BMMs stimulated with PAMPs, extracellular calcium has been shown to activate the calcium sensing receptor (CASR) mediating signal transduction pathways that culminate in the release of calcium stores from the endoplasmic reticulum (ER), eventually activating the NLRP3 inflammasome (177–179). The diverse nature of the NLRP3-inflammasome agonists allude to the likelihood that, instead of directly sensing PAMPs and DAMPs, NLRP3 may be activated by converging pathways with a final common ligand for NLRP3. Guanylate binding protein 5 (GBP5) has been recently proposed as one such component that directly participates in NLRP3-inflammasome activation; however, further investigation is needed to decipher how the GBP5 is activated and why it is required for select inflammasome assembly (180). Finally, studies by Munoz-Planillo et al. suggest that potassium efflux may perhaps be the sole intracellular event necessary for NLRP3 activation in response to a wide array of stimuli arguing for a unifying model for the NLRP3-inflammasome activation (181) (**Figure 3**).

Production of mtROS often culminates in mitophagy, an autophagic clearance of dysfunctional mitochondria. It has been demonstrated that inhibition of mitophagy enhances NLRP3-caspase-1-mediated secretion of IL-1 β and IL-18 in response to LPS and ATP (172). In addition, deletion of ATG16L1 was found to promote IL-1 β release in response to ATP, MSU, or LPS alone (182). Moreover, it has been recently suggested that autophagy may restrict NLRP3 activity by directly sequestering and targeting inflammasome components for degradation (183, 184). Overall, it is reasonable to speculate that autophagy could serve as a mechanism for preventing excessive NLRP3-inflammasome activation (172, 173, 183–185).

Mitochondrial dysfunction plays a central role in regulating the mechanisms involved in both inflammasome and apoptosis pathways. Loss of mitochondrial membrane potential is a pivotal event in intrinsic apoptosis and is tightly regulated by the BCL2 family of proteins through a system of checks and balances (30). Interestingly, anti-apoptotic BCL2 and BCL-XL proteins have

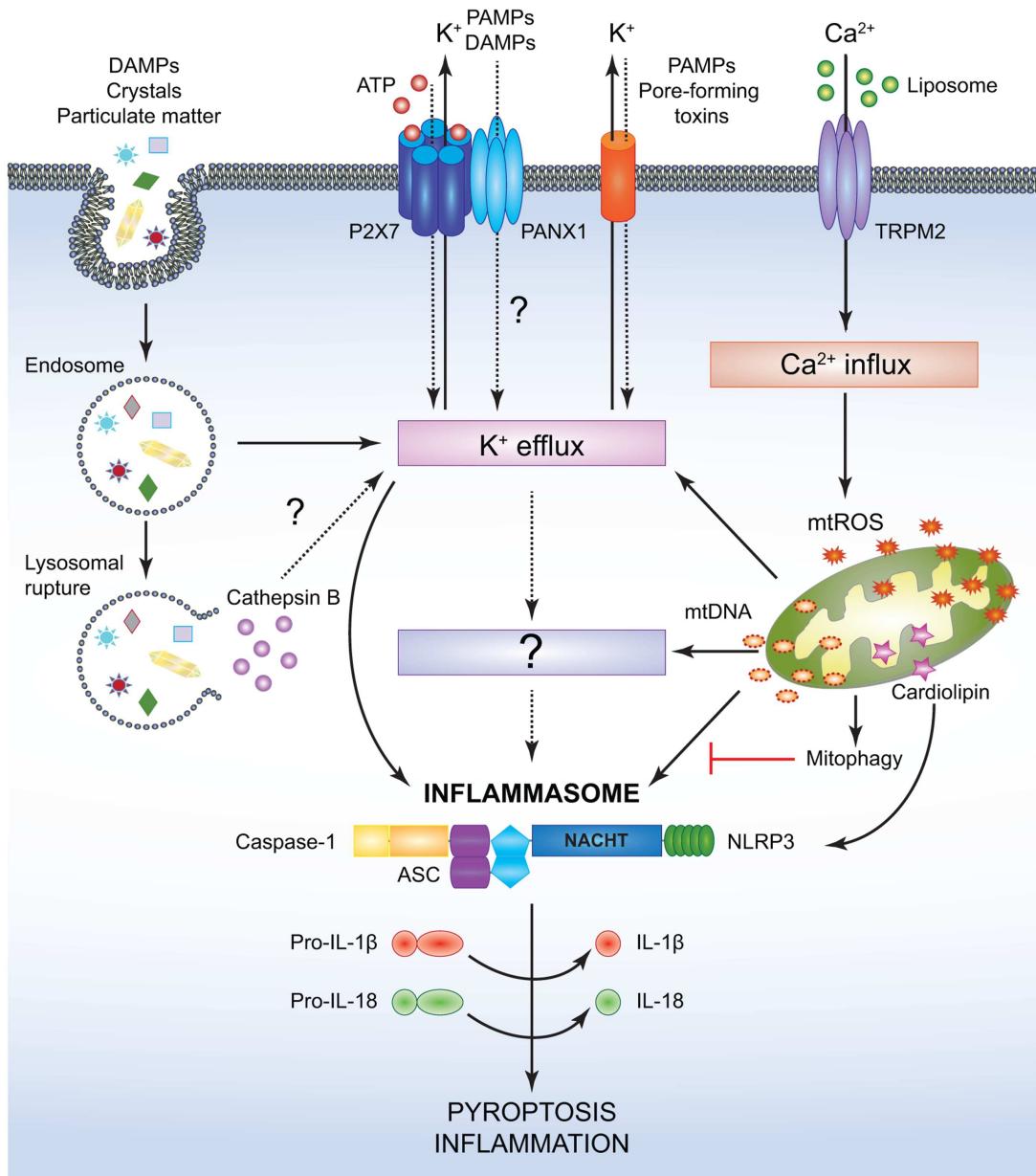


FIGURE 3 | Simplified mechanisms for the canonical NLRP3-inflammasome activation. Various PAMPs and DAMPs provide the signal 2 required to assemble and activate the NLRP3 inflammasome comprised of NLRP3, ASC, and caspase-1. Although the precise mechanism leading to NLRP3 activation is still controversial, it is speculated that K⁺ efflux may be the common cellular response that triggers inflammasome activation. However, this notion has not been fully verified and it is possible that an unidentified or intermediate adaptor may be required for transmitting signals between K⁺ efflux and the NLRP3 inflammasome. Crystals and particulate DAMPs enter the cell via endocytosis directly inducing K⁺ efflux and NLRP3-inflammasome formation. In addition, the endo-lysosomes carrying these DAMPs undergo lysosomal rupture and release cathepsin B, which acts as an intracellular DAMP and can induce K⁺ efflux. However, contradicting studies indicate that lysosomal rupture may cause K⁺ efflux and inflammasome activation even in the absence of cathepsin

B. ATP binds to the P2X7 receptor on the cell membrane and causes opening of the PANX1 channels allowing K⁺ efflux and influx of any PAMPs and DAMPs present in the extracellular space. PAMPs such as pore-forming toxins activate the NLRP3 inflammasome and facilitate K⁺ efflux. Liposomes instigate Ca²⁺ influx through opening of the TRPM2 channels. Accumulation of excessive Ca²⁺ in the cytosol causes mitochondrial dysfunction and release of mtROS and oxidized mtDNA, which may activate the NLRP3 inflammasome either directly or by inducing K⁺ efflux. Clearance of distressed mitochondria by mitophagy serves to evade such inflammasome activation. Mitochondrial Cardiolipin binds to NLRP3 and is required for the NLRP3-inflammasome activation. Following NLRP3-inflammasome assembly, caspase-1 undergoes proximity driven proteolytic cleavage and further processes pro-IL-18 and pro-IL-1β into their mature active forms. Activation of the NLRP3-caspase-1 axis results in inflammation and pyroptotic cell death.

been reported to directly interact with NLRP1 (CARD and PYD domain containing NLRP) to negatively regulate caspase-1 activation (186, 187). Similarly, BCL2 overexpression was shown to limit NLRP3-inflammasome activation (173, 174). In addition to BCL2 proteins, cIAP1, cIAP2, and XIAP have also been linked with inflammasome activation. Unlike their role in NOD signaling, initial studies have proposed that expression of these proteins might prevent caspase-1-dependent cell death (188). However, more recently cIAP1 and cIAP2 along with TRAF2 were found to enhance inflammasome activation seemingly by ubiquitinating and stabilizing caspase-1 and consequently prompting IL-1 β release (189). In another report, genetic ablation of cIAP1 or cIAP2 had no effect on NLRP3-inflammasome activation, but concurrent pharmacological degradation of XIAP, cIAP1, and cIAP2 using SMAC mimetics was shown to limit caspase-1 activation (190). Interestingly, further inquiries revealed that in the absence of XIAP, cIAP1, and cIAP2, cell death in response to LPS was primarily incited by RIP3 activation causing NLRP3-caspase-1- as well as caspase-8-dependent IL-1 β secretion (190). Lately, the concept of non-canonical inflammasome has been defined, which requires activation of caspase-11 in response to Gram-negative bacteria to facilitate either caspase-1-mediated IL-1 β secretion or caspase-1-independent pyroptosis (191–194). Interestingly, apoptosis mediators FADD and caspase-8 have been involved in canonical and non-canonical NLRP3-inflammasome signaling. Indeed, FADD and caspase-8 facilitate the priming in “signal 1” by instigating both, LPS-TLR-MyD88-triggered induction of pro-IL-1 β and NLRP3, as well as TLR-TRIF-mediated upregulation of pro-caspase-11 (195). Upon infection with *Citrobacter rodentium* or *Escherichia coli*, FADD and caspase-8 have been found to promote the “signal 2” by interacting with the NLRP3-inflammasome complex, thus influencing both canonical (caspase-1-dependent IL-1 β maturation) and non-canonical (caspase-11-dependent pyroptosis) inflammasomes (194, 195). Conversely, it has been exhibited that caspase-8-deficient murine DCs are hyper-responsive to LPS-induced NLRP3-inflammasome assembly and activation (196). Overall, these studies place caspase-11 and caspase-8 at the center of inflammasome activation; however, a general lack of consensus in the field makes it hard to aptly judge their contribution in inflammasome-induced inflammation.

INFLAMMASOME NLRs AND CANCER

NLRP3, previously associated with rare and severe auto-inflammatory disorders, has been lately implicated in CD susceptibility and correlated with decreased NLRP3 expression and IL-1 β production (62). Indeed, mice lacking NLRP3 have been shown to display exacerbated colonic inflammation upon DSS-induced colitis characterized by greater gut barrier damage, inflammatory immune cell infiltration, and cytokine production (197, 198). In accord, a central role has been ascribed for caspase-1 and ASC in intestinal epithelial repair after DSS-injury (199). Specifically, caspase-1, ASC, or NLRP3 deficiency in mice has been shown to be detrimental in DSS-induced intestinal inflammation, a mechanism attributed to the lack of IL-18 production by IECs (198, 199). Concomitantly, the increased colitogenic phenotype was completely reversed when mice were exogenously administered with the recombinant IL-18 cytokine (198, 199). The same lack

of inflammatory regulation was found to render *Nlrp3*^{-/-} and *Casp1*^{-/-} mice more susceptible to AOM-DSS carcinogenesis (197, 200). The heightened tumor growth in the caspase-1 deficient mice was accompanied with drastically low levels of colonic IL-18. Overall, NLRP3 was shown to be important for IL-18 secretion, which in turn through IFN γ production induces STAT1 (Signal transducers and activators of transcription 1) phosphorylation and thus promotes an anti-tumorigenic environment (200). Moreover, it has been shown that *Il18*^{-/-} or *Il18r*^{-/-} mice are more susceptible to DSS-induced colitis and CRC, mimicking the increased tumor burdens observed in NLRP3 and caspase-1 deficient mice (201). Recent findings have put forward a novel concept for the dual function of IL-18 in intestinal inflammation and colitis-driven CRC (202, 203). For instance, during acute injury IEC-derived IL-18 triggers repair and restitution of the ulcerated epithelial barrier, whereas under chronic inflammatory settings the excessive release of IL-18 both from IECs and lamina propria macrophages and DCs is deleterious (203, 204). A protective role for NLRP3 has also been described in hepatocellular carcinoma (HCC) (205). This correlation is primarily based on mRNA and protein expression data showing reduced levels of NLRP3 and other related inflammasome components seen in hepatic parenchymal cells derived from HCC tissue specimens as compared to non-cancerous liver sections (205). On the other hand, a gain of function SNP (Q705K) within the *NLRP3* gene has been associated with increased mortality in CRC patients (206). Significantly, the same SNP was also found to be more prevalent in patients with malignant melanoma (207). Human monocytic THP-1 cells overexpressing a mutant variant of NLRP3 bearing the Q705K SNP have been reported to greatly respond to the inflammasome agonist alum and to trigger the production of IL-1 β and IL-18, implying that overt NLRP3 activation could be detrimental for certain types of cancer (208). Similarly, another group implicated constitutive NLRP3-inflammasome signaling in the development and progression of melanomas (209).

Loss of function in the tumor suppressor gene p53 has been associated with a large number of sporadic cancers (36). One of the mechanisms for p53-induced clearance of potentially carcinogenic cells has been found to be via transcriptional up regulation of cell death activators (210). In light of this knowledge, the discovery of NLRC4 as a downstream transcriptional target of p53 was a promising evidence for the anti-tumorigenic functions of this NLR (211). Moreover, lack of NLRC4 inflammasome has been associated with the attenuation of p53-mediated cell death, indicative of a protective role of NLRC4 during tumor development (211). Several groups have investigated the role of NLRC4 in colitis and CRC. However, lack of consensus in the susceptibility of *Nlrc4*^{-/-} mice to DSS as well as AOM-DSS treatment makes it difficult to gage the protective effect of NLRC4 in these models (197, 212). It has been demonstrated that mice deficient in NLRC4 develop higher tumor burdens than WT mice when subjected to DSS-induced CRC (212). In addition, bone marrow chimera experiments verified that NLRC4 expression within the radioresistant compartment was the major driver of CRC protection (212). Surprisingly, similar colitic phenotypes have been observed between WT and *Nlrc4*^{-/-} mice following DSS administration, suggesting that tumor regulation by NLRC4 is mostly

cell intrinsic and not through down-regulation of inflammation (213). Given the unique capacity of NLRC4 to sense and differentiate between commensal and pathogenic microbes in the gut (214), it is surprising that the tumor restraining roles of NLRC4 have been ruled to be independent of its immune regulatory functions. One unifying theory addressing these discrepancies could be that anti-tumor functions of NLRC4 are attributed to the cells of non-hematopoietic origin, whereas intestinal mononuclear phagocytes are the primary source of NLRC4 for microbial sensing and pathogen clearance (213, 214). Overall, these assumptions warrant deeper inquiries to clearly elucidate the mechanisms by which NLRC4 exerts protective functions during CRC and to decipher the relevance of p53-mediated role of NLRC4 in tumorigenesis.

Akin to NLRP3, both NLRP6 and NLRP12 have been recently described to use ASC-caspase-1 molecular platforms and assemble inflammasomes. A first hint of NLRP6 being an inflammasome NLR was gleaned from *in vitro* experiments showing increased caspase-1 cleavage when ASC and NLRP6 were co-expressed (215). Further *in vivo* evidence emphasized a protective role for NLRP6 in intestinal inflammation and tumorigenesis as *Nlrp6*^{-/-} mice showed high susceptibility to DSS-induced colitis and AOM-DSS-induced CRC (216–218). Unlike NLRC4, dampening of inflammation is purported to be one of the primary mechanisms for NLRP6-mediated protection and tissue homeostasis. NLRP6 has been shown to promote a gut microbiome that limits chronic inflammation. In fact, it has been evidenced that *Nlrp6*^{-/-} mice display a distinct transmissible pro-colitogenic microbiome with increased prevalence of the bacterial genus *Prevotellaceae* (217). These mice presented a steady state colitic phenotype and an enhanced sensitivity to DSS colitis (217). Overall, a mechanism has been suggested wherein dysbiosis in the gut, caused by aberrant NLRP6 inflammasome signaling, drives excessive CCL5-mediated IL-6 production, barrier damage, and inflammation (217). In agreement with the findings in *Casp1*^{-/-} mice (199), NLRP6-deficient mice had impaired IL-18 production mainly from the intestinal epithelial compartment further diminishing the capacity of these mice to recover from colitis. Likewise, overt inflammation and lack of IL-18 in the *Nlrp6*^{-/-} mice has been associated with increased colonic tumor development (216), however, as seen for *Nlrp3*^{-/-} mice it is still unknown whether administration of IL-18 is capable of rescuing the susceptibility phenotype. Interestingly, gene expression profiling of colorectal tumors derived from WT and *Nlrp6*^{-/-} mice revealed an increased expression of paracrine factors of the Wnt and NOTCH signaling cascades, underscoring a novel function of NLRP6 in controlling intestinal proliferation (218). Sensing of damaged or dying cells by NLRP6 and NLRP3 inflammasomes has lately been hypothesized to prevent CRC through maintaining the balance between IL-22 and IL-22 binding protein (IL22-BP) (219). It has been speculated that sensing of DAMPs by both NLRs instigates IL-18-dependent down-regulation of the inhibitory molecule IL-22BP, thus allowing IL-22 to repair the injured tissue. However, dysregulated NLRP6 or NLRP3 signaling could potentially lead to inappropriate IL-22BP expression, thus creating a pro-tumorigenic environment caused by either excessive cell proliferation or lack of tissue repair (219). Although the dual function of IL-22 in CRC has been well-described, further

experimental validation is needed to pinpoint the exact mode by which NLRP3 or NLRP6 regulate IL-22/IL-22BP ratio during colon tumorigenesis.

NLRP12 was originally defined as an inflammasome NLR due to its co-localization with ASC and caspase-1, induction of IL-1 β and IL-18 secretion as well as NF- κ B activation (220, 221). SNPs within the *NLRP12* gene have been associated with increased susceptibility to atopic dermatitis and periodic fever syndromes accompanied mostly with caspase-1 activation and IL-1 β release (222–225). It has been observed that NLRP12 can negatively regulate both canonical and non-canonical NF- κ B pathways by targeting the IL-1R-associated kinase 1 (IRAK1) and NF- κ B inducing kinase (NIK) for proteasomal degradation (226–228). Two independent studies proposed that NLRP12 acts as a tumor suppressive molecule *ex vivo* and in *in vivo* animal models of colitis and colitis-induced CRC (229, 230). Mice lacking NLRP12 have been found to be more susceptible to DSS-injury with increased body weight loss, enhanced pathology scores coupled with massive infiltration of inflammatory cells and high inflammatory cytokine production (229, 230). Furthermore, AOM-DSS treatment of *Nlrp12*^{-/-} mice has been shown to further provoke colonic tumor development and progression (229, 230). In the first study, it was clearly demonstrated that lack of NLRP12 increases NIK-dependent non-canonical NF- κ B signaling and drives the regulation of cancer promoting genes like CXCL12 and CXCL13 (230). In the second report, the enhanced tumorigenicity in knockout mice was traced to excessive canonical NF- κ B activation due to lack of NLRP12 in hematopoietic cells. Indeed, enhanced LPS-induced canonical NF- κ B activation was exhibited in *Nlrp12*^{-/-} macrophages *ex vivo*, suggesting that microbial sensing and negative regulation of inflammation may account for NLRP12-mediated tumor suppression (229). Altogether, these results underscore the importance of anti-inflammatory signals provided by NLRP12 in maintaining colonic homeostasis and protecting from colitis and colon tumorigenesis.

THERAPEUTIC STRATEGIES AND CONCLUSION

It has been suggested that the strong immunomodulatory properties of NLRs could be exploited for mounting potent anti-tumorigenic responses. In fact, mice injected with B16 melanoma cells or EL4 thymoma cells expressing flagellin from *Salmonella typhimurium* were shown to display dramatic resistance to tumor establishment in NLRC4 dependent manner (231). In addition, immunization with flagellin expressing cancer cells lead to impressive antigen-specific CD4 and CD8 T cell responses via NLRC4 and NAIP5 signaling and bestowed anti-tumor immunity against a secondary inoculation with tumor cells (231). Similarly, activation of NODs, in particular NOD2, to elicit robust cell-based anti-tumor immunity has been under scrutiny for several years. Indeed, instillation of MDP in patients with lung cancer has been reported to enhance expression of inflammatory cytokines and neutrophils in the pleural fluid (232). Relatedly, it has been suggested that the local immune-modulatory activity of MDP helps improve prognosis in hamsters suffering from osteosarcoma (233) and significantly reduces tumor metastasis in several murine cancer models, such as B16-BL6 melanoma, colon 26-M#1 carcinoma, and L5178Y-ML25T T lymphoma (234, 235).

Overt activation of the NLRP3 inflammasome has been demonstrated to elicit cancer progression. For instance, in mouse models of methylcholanthrene (MCA, a highly potent carcinogen) induced fibrosarcoma, NLRP3 was demonstrated to promote cancer progression. Moreover, NLRP3 expression in myeloid cells was shown to interfere with the suppression of cancer metastasis by inhibiting recruitment of anti-tumor NK cells to the site of carcinogenesis (236). Besides interfering with natural tumor control, NLRP3 inflammasome-mediated IL-1 β has been described to attenuate anti-tumor effects of chemotherapeutic agents, gemcitabine (Gem), and 5-fluorouracil (5FU) (237). Mice lacking NLRP3 were far more receptive to thymoma regression upon treatment with Gem or 5FU as compared to WT mice. Furthermore, enhanced NLRP3-driven IL-1 β release was linked with the induction of T helper 17 (Th17) cells that promoted chemo-resistance in WT mice (237). Keeping these observations in view, several studies support the use of specific inhibitors, antagonists, and monoclonal antibodies against components of the inflammasome, e.g., caspase-1, IL-1 β , and IL-18, as therapeutic approaches beneficial for controlling inflammation and improving cancer prognosis (238).

An early phase clinical study suggests that administration of the IL-1R antagonist, Anakinra, alone or in combination with dexamethasone could potentially impede human multiple myeloma progression (239). Furthermore, it was demonstrated that IL-18 derived from tumor cells had the ability to subvert the NK cell-mediated tumor immunosurveillance and to promote tumor progression in a programmed death receptor 1 (PD1)-dependent manner (240, 241). These findings suggest the potential of using IL-18 as well as PD1 neutralization for cancer immunotherapy. Overall, selective attenuation of the activities of certain NLRs could potentially boost regression and improve responsiveness to chemotherapy. The variability in NLRP3- and IL-18-mediated effects in different cancers highlights the complexity in NLR circuits and suggests that any broad implications regarding NLR intervention in tumorigenesis should be carefully investigated.

Microbial environment, diet, mouse strain, tumor ontogeny, etc. are all part of the complex network that dictates how an NLR influences inflammation and tumorigenesis. Sensitivity to these factors has led to conflicting disease phenotypes in genetically modified mice lacking specific NLRs. Furthermore, NLR expression in hematopoietic or non-hematopoietic cellular compartments appears to have distinct influence on inflammatory regulation and tumorigenesis. Due to such discrepancies, it is still uncertain how dysregulation of these innate immune sensors incites inflammation that leads to carcinogenic transformation of cells. Although several mechanisms have been suggested like control of NF- κ B signaling, regulation of tissue repair factors, and IL-18 secretion, no unifying hypothesis exists. In addition, interaction of NLRs with different members of the TNFR pathway, BCL2 family of proteins, IAPs, apoptotic caspases, and autophagy regulators point toward more intricate mechanisms for NLR regulation than currently acknowledged. Future studies focusing on the biochemistry of interactions between cell death regulators and NLRs are required to delineate the co-integration of NLR-cell death mechanisms so as to facilitate implementation of NLR

modifying therapeutic strategies for inflammatory diseases and cancer.

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REFERENCES

1. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* (2002) **20**:197–216. doi:10.1146/annurev.immunol.20.083001.084359
2. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol* (2007) **81**:1–5. doi:10.1189/jlb.0306164
3. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* (2011) **34**:637–50. doi:10.1016/j.jimmuni.2011.05.006
4. Hansen JD, Vojtech LN, Laing KJ. Sensing disease and danger: a survey of vertebrate PRRs and their origins. *Dev Comp Immunol* (2011) **35**:886–97. doi:10.1016/j.dci.2011.01.008
5. Yeretssian G. Effector functions of NLRs in the intestine: innate sensing, cell death, and disease. *Immunol Res* (2012) **54**:25–36. doi:10.1007/s12026-012-8317-3
6. Wen H, Miao EA, Ting JP. Mechanisms of NOD-like receptor-associated inflammasome activation. *Immunity* (2013) **39**:432–41. doi:10.1016/j.jimmuni.2013.08.037
7. Goubau D, Deddouche S, Reis E, Sousa C. Cytosolic sensing of viruses. *Immunity* (2013) **38**:855–69. doi:10.1016/j.jimmuni.2013.05.007
8. Hardison SE, Brown GD. C-type lectin receptors orchestrate antifungal immunity. *Nat Immunol* (2012) **13**:817–22. doi:10.1038/ni.2369
9. Ratsimandresy RA, Dorfleutner A, Stehlík C. An update on PYRIN domain-containing pattern recognition receptors: from immunity to pathology. *Front Immunol* (2013) **4**:440. doi:10.3389/fimmu.2013.00440
10. Janowski AM, Kolb R, Zhang W, Sutterwala FS. Beneficial and detrimental roles of NLRs in carcinogenesis. *Front Immunol* (2013) **4**:370. doi:10.3389/fimmu.2013.00370
11. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* (2001) **357**:539–45. doi:10.1016/S0140-6736(00)04046-0
12. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* (2008) **454**:436–44. doi:10.1038/nature07205
13. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* (2010) **140**:883–99. doi:10.1016/j.cell.2010.01.025
14. de Martel C, Franceschi S. Infections and cancer: established associations and new hypotheses. *Crit Rev Oncol Hematol* (2009) **70**:183–94. doi:10.1016/j.critrevonc.2008.07.021
15. Takahashi H, Ogata H, Nishigaki R, Broide DH, Karin M. Tobacco smoke promotes lung tumorigenesis by triggering IKKbeta- and JNK1-dependent inflammation. *Cancer Cell* (2010) **17**:89–97. doi:10.1016/j.ccr.2009.12.008
16. Khasawneh J, Schulz MD, Walch A, Rozman J, Hrabe De Angelis M, Klingenspor M, et al. Inflammation and mitochondrial fatty acid beta-oxidation link obesity to early tumor promotion. *Proc Natl Acad Sci U S A* (2009) **106**:3354–9. doi:10.1073/pnas.0802864106
17. Park EJ, Lee JH, Yu GY, He G, Ali SR, Holzer RG, et al. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. *Cell* (2010) **140**:197–208. doi:10.1016/j.cell.2009.12.052
18. Ershler WB, Keller ET. Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. *Annu Rev Med* (2000) **51**:245–70. doi:10.1146/annurev.med.51.1.245
19. Rodier F, Coppe JP, Patil CK, Hoeijmakers WA, Munoz DP, Raza SR, et al. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol* (2009) **11**:973–9. doi:10.1038/ncb1909
20. Coussens LM, Werb Z. Inflammation and cancer. *Nature* (2002) **420**:860–7. doi:10.1038/nature01322
21. Easwaran H, Tsai HC, Baylin SB. Cancer epigenetics: tumor heterogeneity, plasticity of stem-like states, and drug resistance. *Mol Cell* (2014) **54**:716–27. doi:10.1016/j.molcel.2014.05.015
22. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) **144**:646–74. doi:10.1016/j.cell.2011.02.013

23. Strasser A, Cory S, Adams JM. Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. *EMBO J* (2011) **30**:3667–83. doi:10.1038/emboj.2011.307
24. Bai L, Smith DC, Wang S. Small-molecule SMAC mimetics as new cancer therapeutics. *Pharmacol Ther* (2014). doi:10.1016/j.pharmthera.2014.05.007
25. Suzuki Y, Nakabayashi Y, Nakata K, Reed JC, Takahashi R. X-linked inhibitor of apoptosis protein (XIAP) inhibits caspase-3 and -7 in distinct modes. *J Biol Chem* (2001) **276**:27058–63. doi:10.1074/jbc.M102415200
26. Busca A, Saxena M, Kryworuchko M, Kumar A. Anti-apoptotic genes in the survival of monocytic cells during infection. *Curr Genomics* (2009) **10**:306–17. doi:10.2174/13892029078920967
27. Gyrd-Hansen M, Meier P. IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. *Nat Rev Cancer* (2010) **10**:561–74. doi:10.1038/nrc2889
28. Saxena M, Busca A, Pandey S, Kryworuchko M, Kumar A. CpG protects human monocytic cells against HIV-Vpr-induced apoptosis by cellular inhibitor of apoptosis-2 through the calcium-activated JNK pathway in a TLR9-independent manner. *J Immunol* (2011) **187**:5865–78. doi:10.4049/jimmunol.1100115
29. Busca A, Saxena M, Kumar A. Critical role for antiapoptotic Bcl-xL and Mcl-1 in human macrophage survival and cellular IAP1/2 (cIAP1/2) in resistance to HIV-Vpr-induced apoptosis. *J Biol Chem* (2012) **287**:15118–33. doi:10.1074/jbc.M111.312660
30. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* (2008) **9**:47–59. doi:10.1038/nrm2308
31. Kelly PN, Strasser A. The role of Bcl-2 and its pro-survival relatives in tumourigenesis and cancer therapy. *Cell Death Differ* (2011) **18**:1414–24. doi:10.1038/cdd.2011.17
32. Sax JK, Fei P, Murphy ME, Bernhard E, Korsmeyer SJ, El-Deiry WS. BID regulation by p53 contributes to chemosensitivity. *Nat Cell Biol* (2002) **4**:842–9. doi:10.1038/ncb866
33. Michalak EM, Jansen ES, Happo L, Cragg MS, Tai L, Smyth GK, et al. Puma and to a lesser extent Noxa are suppressors of Myc-induced lymphomagenesis. *Cell Death Differ* (2009) **16**:684–96. doi:10.1038/cdd.2008.195
34. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* (1992) **356**:215–21. doi:10.1038/356215a0
35. Olivier M, Hollstein M, Hainaut P. TP53 mutations in human cancers: origins, consequences, and clinical use. *Cold Spring Harb Perspect Biol* (2010) **2**:a001008. doi:10.1101/cshperspect.a001008
36. Bieging KT, Mello SS, Attardi LD. Unravelling mechanisms of p53-mediated tumour suppression. *Nat Rev Cancer* (2014) **14**:359–70. doi:10.1038/nrc3711
37. Hager KM, Gu W. Understanding the non-canonical pathways involved in p53-mediated tumor suppression. *Carcinogenesis* (2014) **35**:740–6. doi:10.1093/carcin/bgt487
38. Chretien AS, Le Roy A, Vey N, Prebet T, Blaise D, Fauriat C, et al. Cancer-induced alterations of NK-mediated target recognition: current and investigational pharmacological strategies aiming at restoring NK-mediated anti-tumor activity. *Front Immunol* (2014) **5**:122. doi:10.3389/fimmu.2014.00122
39. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol* (2012) **12**:239–52. doi:10.1038/nri3174
40. Bertin J, Nir WI, Fischer CM, Tayber OV, Errada PR, Grant JR, et al. Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. *J Biol Chem* (1999) **274**:12955–8. doi:10.1074/jbc.274.19.12955
41. Inohara N, Koseki T, Del Peso L, Hu Y, Yee C, Chen S, et al. Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J Biol Chem* (1999) **274**:14560–7. doi:10.1074/jbc.274.21.14560
42. Ogura Y, Inohara N, Benito A, Chen FF, Yamaoka S, Nunez G. Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J Biol Chem* (2001) **276**:4812–8. doi:10.1074/jbc.M008072200
43. Ting JP, Lovering RC, Alnemri ES, Bertin J, Boss JM, Davis BK, et al. The NLR gene family: a standard nomenclature. *Immunity* (2008) **28**:285–7. doi:10.1016/j.jimmuni.2008.02.005
44. Nickerson K, Sisk TJ, Inohara N, Yee CS, Kennell J, Cho MC, et al. Dendritic cell-specific MHC class II transactivator contains a caspase recruitment domain that confers potent transactivation activity. *J Biol Chem* (2001) **276**:19089–93. doi:10.1074/jbc.M101295200
45. Diez E, Lee SH, Gauthier S, Yaraghi Z, Tremblay M, Vidal S, et al. Bircle is the gene within the Lgn1 locus associated with resistance to Legionella pneumophila. *Nat Genet* (2003) **33**:55–60. doi:10.1038/ng1065
46. Wright EK, Goodart SA, Grownay JD, Hadinoto V, Endrizzi MG, Long EM, et al. Naip5 affects host susceptibility to the intracellular pathogen Legionella pneumophila. *Curr Biol* (2003) **13**:27–36. doi:10.1016/S0960-9822(02)01359-3
47. Lightfield KL, Persson J, Brubaker SW, Witte CE, Von Moltke J, Dunipace EA, et al. Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. *Nat Immunol* (2008) **9**:1171–8. doi:10.1038/ni.1646
48. Philpott DJ, Sorbara MT, Robertson SJ, Croitoru K, Girardin SE. NOD proteins: regulators of inflammation in health and disease. *Nat Rev Immunol* (2014) **14**:9–23. doi:10.1038/nri3565
49. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezerad JP, Belaiche J, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* (2001) **411**:599–603. doi:10.1038/35079107
50. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* (2001) **411**:603–6. doi:10.1038/35079114
51. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* (2002) **10**:417–26. doi:10.1016/S1097-2765(02)00599-3
52. Moore CB, Bergstrahl DT, Duncan JA, Lei Y, Morrison TE, Zimmermann AG, et al. NLRX1 is a regulator of mitochondrial antiviral immunity. *Nature* (2008) **451**:573–7. doi:10.1038/nature06501
53. Allen IC, Moore CB, Schneider M, Lei Y, Davis BK, Scull MA, et al. NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-I-MAVS and TRAF6-NF-kappaB signaling pathways. *Immunity* (2011) **34**:854–65. doi:10.1016/j.jimmuni.2011.03.026
54. Xia X, Cui J, Wang HY, Zhu L, Matsueda S, Wang Q, et al. NLRX1 negatively regulates TLR-induced NF-kappaB signaling by targeting TRAF6 and IKK. *Immunity* (2011) **34**:843–53. doi:10.1016/j.jimmuni.2011.02.022
55. Lei Y, Wen H, Yu Y, Taxman DJ, Zhang L, Widman DG, et al. The mitochondrial proteins NLRX1 and TUFM form a complex that regulates type I interferon and autophagy. *Immunity* (2012) **36**:933–46. doi:10.1016/j.jimmuni.2012.03.025
56. Lei Y, Wen H, Ting JP. The NLR protein, NLRX1, and its partner, TUFM, reduce type I interferon, and enhance autophagy. *Autophagy* (2013) **9**:432–3. doi:10.4161/auto.23026
57. Tattoli I, Carneiro LA, Jehanno M, Magalhaes JG, Shu Y, Philpott DJ, et al. NLRX1 is a mitochondrial NOD-like receptor that amplifies NF-kappaB and JNK pathways by inducing reactive oxygen species production. *EMBO Rep* (2008) **9**:293–300. doi:10.1038/sj.embo.7401161
58. Abdul-Sater AA, Said-Sadier N, Lam VM, Singh B, Pettengill MA, Soares F, et al. Enhancement of reactive oxygen species production and chlamydial infection by the mitochondrial Nod-like family member NLRX1. *J Biol Chem* (2010) **285**:41637–45. doi:10.1074/jbc.M110.137885
59. Soares F, Tattoli I, Wortzman ME, Arnoult D, Philpott DJ, Girardin SE. NLRX1 does not inhibit MAVS-dependent antiviral signalling. *Innate Immun* (2013) **19**:438–48. doi:10.1177/1753425912467383
60. Rebsamen M, Vazquez J, Tardivel A, Guarda G, Curran J, Tschopp J. NLRX1/NOD5 deficiency does not affect MAVS signalling. *Cell Death Differ* (2011) **18**:1387. doi:10.1038/cdd.2011.64
61. Franchi L, Munoz-Planillo R, Nunez G. Sensing and reacting to microbes through the inflammasomes. *Nat Immunol* (2012) **13**:325–32. doi:10.1038/ni.2231
62. Villani AC, Lemire M, Fortin G, Louis E, Silverberg MS, Collette C, et al. Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. *Nat Genet* (2009) **41**:71–6. doi:10.1038/ng.285
63. Kutikhin AG. Role of NOD1/CARD4 and NOD2/CARD15 gene polymorphisms in cancer etiology. *Hum Immunol* (2011) **72**:955–68. doi:10.1016/j.humimm.2011.06.003
64. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* (2012) **491**:119–24. doi:10.1038/nature11582
65. Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan

- containing diaminopimelic acid. *Nat Immunol* (2003) **4**:702–7. doi:10.1038/ni945
66. Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jehanno M, Viala J, et al. Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* (2003) **300**:1584–7. doi:10.1126/science.1084677
 67. Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, et al. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J Biol Chem* (2003) **278**:41702–8. doi:10.1074/jbc.M307198200
 68. Ogura Y, Lala S, Xin W, Smith E, Dowds TA, Chen FF, et al. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut* (2003) **52**:1591–7. doi:10.1136/gut.52.11.1591
 69. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* (2003) **278**:8869–72. doi:10.1074/jbc.C200651200
 70. Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, et al. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat Immunol* (2004) **5**:1166–74. doi:10.1038/ni1131
 71. Lee J, Tattoli I, Wojtal KA, Vavricka SR, Philpott DJ, Girardin SE. pH-dependent internalization of muramyl peptides from early endosomes enables Nod1 and Nod2 signaling. *J Biol Chem* (2009) **284**:23818–29. doi:10.1074/jbc.M109.033670
 72. Marina-Garcia N, Franchi L, Kim YG, Hu Y, Smith DE, Boons GJ, et al. Clathrin- and dynamin-dependent endocytic pathway regulates muramyl dipeptide internalization and NOD2 activation. *J Immunol* (2009) **182**:4321–7. doi:10.4049/jimmunol.0802197
 73. Nakamura N, Lill JR, Phung Q, Jiang Z, Bakalarski C, De Maziere A, et al. Endosomes are specialized platforms for bacterial sensing and NOD2 signalling. *Nature* (2014) **509**:240–4. doi:10.1038/nature13133
 74. Fukazawa A, Alonso C, Kurachi K, Gupta S, Lesser CF, McCormick BA, et al. GEF-H1 mediated control of NOD1 dependent NF-kappaB activation by Shigella effectors. *PLoS Pathog* (2008) **4**:e1000228. doi:10.1371/journal.ppat.1000228
 75. Kufer TA, Kremmer E, Adam AC, Philpott DJ, Sansonetti PJ. The pattern-recognition molecule Nod1 is localized at the plasma membrane at sites of bacterial interaction. *Cell Microbiol* (2008) **10**:477–86.
 76. Keestra AM, Winter MG, Auburger JJ, Frassle SP, Xavier MN, Winter SE, et al. Manipulation of small Rho GTPases is a pathogen-induced process detected by NOD1. *Nature* (2013) **496**:233–7. doi:10.1038/nature12025
 77. Chin AI, Dempsey PW, Bruhn K, Miller JF, Xu Y, Cheng G. Involvement of receptor-interacting protein 2 in innate and adaptive immune responses. *Nature* (2002) **416**:190–4. doi:10.1038/416190a
 78. Park JH, Kim YG, McDonald C, Kanneganti TD, Hasegawa M, Body-Malapel M, et al. RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. *J Immunol* (2007) **178**:2380–6. doi:10.4049/jimmunol.178.4.2380
 79. Watanabe T, Asano N, Fichtner-Feigl S, Gorelick PL, Tsuji Y, Matsumoto Y, et al. NOD1 contributes to mouse host defense against *Helicobacter pylori* via induction of type I IFN and activation of the ISGF3 signaling pathway. *J Clin Invest* (2010) **120**:1645–62. doi:10.1172/JCI39481
 80. Magalhaes JG, Lee J, Geddes K, Rubino S, Philpott DJ, Girardin SE. Essential role of Rip2 in the modulation of innate and adaptive immunity triggered by Nod1 and Nod2 ligands. *Eur J Immunol* (2011) **41**:1445–55. doi:10.1002/eji.201040827
 81. Inohara N, Koseki T, Lin J, Del Peso L, Lucas PC, Chen FF, et al. An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. *J Biol Chem* (2000) **275**:27823–31.
 82. Inohara N, Del Peso L, Koseki T, Chen S, Nunez G. RICK, a novel protein kinase containing a caspase recruitment domain, interacts with CLARP and regulates CD95-mediated apoptosis. *J Biol Chem* (1998) **273**:12296–300. doi:10.1074/jbc.273.20.12296
 83. McCarthy JV, Ni J, Dixit VM. RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase. *J Biol Chem* (1998) **273**:16968–75. doi:10.1074/jbc.273.27.16968
 84. Thome M, Hofmann K, Burns K, Martinon F, Bodmer JL, Mattmann C, et al. Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr Biol* (1998) **8**:885–8. doi:10.1016/S0960-9822(07)00352-1
 85. Eickhoff J, Hanko M, Stein-Gerlach M, Kiang TP, Herzberger K, Habenberger P, et al. RICK activates a NF-kappaB-dependent anti-human cytomegalovirus response. *J Biol Chem* (2004) **279**:9642–52. doi:10.1074/jbc.M312893200
 86. Windheim M, Lang C, Peggie M, Plater LA, Cohen P. Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide. *Biochem J* (2007) **404**:179–90. doi:10.1042/BJ20061704
 87. Nembrini C, Kisielow J, Shamshiev AT, Tortola L, Coyle AJ, Kopf M, et al. The kinase activity of Rip2 determines its stability and consequently Nod1- and Nod2-mediated immune responses. *J Biol Chem* (2009) **284**:19183–8. doi:10.1074/jbc.M109.006353
 88. Yang Y, Yin C, Pandey A, Abbott D, Sasseci C, Kelliher MA. NOD2 pathway activation by MDP or Mycobacterium tuberculosis infection involves the stable polyubiquitination of Rip2. *J Biol Chem* (2007) **282**:36223–9. doi:10.1074/jbc.M703079200
 89. Hasegawa M, Fujimoto Y, Lucas PC, Nakano H, Fukase K, Nunez G, et al. A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. *EMBO J* (2008) **27**:373–83. doi:10.1038/sj.emboj.7601962
 90. Tigno-Aranjuez JT, Asara JM, Abbott DW. Inhibition of RIP2's tyrosine kinase activity limits NOD2-driven cytokine responses. *Genes Dev* (2010) **24**:2666–77. doi:10.1101/gad.1964410
 91. Abbott DW, Wilkins A, Asara JM, Cantley LC. The Crohn's disease protein, NOD2, requires RIP2 in order to induce ubiquitylation of a novel site on NEMO. *Curr Biol* (2004) **14**:2217–27. doi:10.1016/j.cub.2004.12.032
 92. Abbott DW, Yang Y, Hutt JE, Madhavarapu S, Kelliher MA, Cantley LC. Coordinated regulation of Toll-like receptor and NOD2 signaling by K63-linked polyubiquitin chains. *Mol Cell Biol* (2007) **27**:6012–25. doi:10.1128/MCB.00270-07
 93. Navas TA, Baldwin DT, Stewart TA. RIP2 is a Raf1-activated mitogen-activated protein kinase kinase. *J Biol Chem* (1999) **274**:33684–90. doi:10.1074/jbc.274.47.33684
 94. Bertrand MJ, Doiron K, Labbe K, Korneluk RG, Barker PA, Saleh M. Cellular inhibitors of apoptosis cIAP1 and cIAP2 are required for innate immunity signaling by the pattern recognition receptors NOD1 and NOD2. *Immunity* (2009) **30**:789–801. doi:10.1016/j.immuni.2009.04.011
 95. Krieg A, Correa RG, Garrison JB, Le Negrate G, Welsh K, Huang Z, et al. XIAP mediates NOD signaling via interaction with RIP2. *Proc Natl Acad Sci U S A* (2009) **106**:14524–9. doi:10.1073/pnas.0907131106
 96. Damgaard RB, Nachbur U, Yabal M, Wong WW, Fiil BK, Kastirr M, et al. The ubiquitin ligase XIAP recruits LUBAC for NOD2 signaling in inflammation and innate immunity. *Mol Cell* (2012) **46**:746–58. doi:10.1016/j.molcel.2012.04.014
 97. Tokunaga F, Iwai K. Linear ubiquitination: a novel NF-kappaB regulatory mechanism for inflammatory and immune responses by the LUBAC ubiquitin ligase complex. *Endocr J* (2012) **59**:641–52. doi:10.1507/endocrj.EJ12-0148
 98. Damgaard RB, Fiil BK, Speckmann C, Yabal M, Zur Stadt U, Bekker-Jensen S, et al. Disease-causing mutations in the XIAP BIR2 domain impair NOD2-dependent immune signalling. *EMBO Mol Med* (2013) **5**:1278–95. doi:10.1002/emmm.201303090
 99. Watanabe T, Asano N, Murray PJ, Ozato K, Tailor P, Fuss IJ, et al. Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. *J Clin Invest* (2008) **118**:545–59. doi:10.1172/JCI33145
 100. Reardon C, Mak TW. cIAP proteins: keystones in NOD receptor signal transduction. *Immunity* (2009) **30**:755–6. doi:10.1016/j.immuni.2009.06.005
 101. Yeretssian G, Correa RG, Doiron K, Fitzgerald P, Dillon CP, Green DR, et al. Non-apoptotic role of BID in inflammation and innate immunity. *Nature* (2011) **474**:96–9. doi:10.1038/nature09982
 102. Tao M, Scacheri PC, Marinis JM, Harhaj EW, Matesic LE, Abbott DW. ITCH K63-ubiquitinates the NOD2 binding protein, RIP2, to influence inflammatory signaling pathways. *Curr Biol* (2009) **19**:1255–63. doi:10.1016/j.cub.2009.06.038
 103. Bertrand MJ, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, et al. cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. *Mol Cell* (2008) **30**:689–700. doi:10.1016/j.molcel.2008.05.014
 104. da Silva Correia J, Miranda Y, Austin-Brown N, Hsu J, Mathison J, Xiang R, et al. Nod1-dependent control of tumor growth. *Proc Natl Acad Sci U S A* (2006) **103**:1840–5. doi:10.1073/pnas.0509228103
 105. da Silva Correia J, Miranda Y, Leonard N, Hsu J, Ulevitch RJ. Regulation of Nod1-mediated signaling pathways. *Cell Death Differ* (2007) **14**:830–9. doi:10.1038/sj.cdd.4402070
 106. Yeretssian G, Labbe K, Saleh M. Molecular regulation of inflammation and cell death. *Cytokine* (2008) **43**:380–90. doi:10.1016/j.cyto.2008.07.015

107. Galluzzi L, Kepp O, Trojel-Hansen C, Kroemer G. Non-apoptotic functions of apoptosis-regulatory proteins. *EMBO Rep* (2012) **13**:322–30. doi:10.1038/embor.2012.19
108. Yeretssian G, Correa RG, Doiron K, Fitzgerald P, Dillon CP, Green DR, et al. Is BID required for NOD signalling? reply. *Nature* (2012) **488**:E6–8. doi:10.1038/nature11367
109. Nachbur U, Vince JE, O'Reilly LA, Strasser A, Silke J. Is BID required for NOD signalling? *Nature* (2012) **488**:E4–6. doi:10.1038/nature11366
110. Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* (2007) **39**:207–11. doi:10.1038/ng1954
111. Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* (2007) **39**:596–604. doi:10.1038/ng2032
112. Travassos LH, Carneiro LA, Ramjeet M, Hussey S, Kim YG, Magalhaes JG, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol* (2010) **11**:55–62. doi:10.1038/ni.1823
113. Cooney R, Baker J, Brain O, Danis B, Pichulik T, Allan P, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* (2010) **16**:90–7. doi:10.1038/nm.2069
114. Homer CR, Richmond AL, Rebert NA, Achkar JP, McDonald C. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. *Gastroenterology* (2010) **139**:1630–41. doi:10.1053/j.gastro.2010.07.006
115. Homer CR, Kabi A, Marina-Garcia N, Sreekumar A, Nesvizhskii AI, Nickerson KP, et al. A dual role for receptor-interacting protein kinase 2 (RIP2) kinase activity in nucleotide-binding oligomerization domain 2 (NOD2)-dependent autophagy. *J Biol Chem* (2012) **287**:25565–76. doi:10.1074/jbc.M111.326835
116. Sorbara MT, Ellison LK, Ramjeet M, Travassos LH, Jones NL, Girardin SE, et al. The protein ATG16L1 suppresses inflammatory cytokines induced by the intracellular sensors Nod1 and Nod2 in an autophagy-independent manner. *Immunity* (2013) **39**:88–73. doi:10.1016/j.immuni.2013.10.013
117. Yomade O, Spies-Weisshart B, Glaser A, Schnetzke U, Hochhaus A, Scholl S. Impact of NOD2 polymorphisms on infectious complications following chemotherapy in patients with acute myeloid leukaemia. *Ann Hematol* (2013) **92**:1071–7. doi:10.1007/s00277-013-1734-0
118. Hnatyszyn A, Szalata M, Stanczyk J, Cichy W, Slomski R. Association of c.802C>T polymorphism of NOD2/CARD15 gene with the chronic gastritis and predisposition to cancer in H. pylori infected patients. *Exp Mol Pathol* (2010) **88**:388–93. doi:10.1016/j.yexmp.2010.03.003
119. Huzarski T, Lener M, Domagala W, Gronwald J, Byrski T, Kurzawski G, et al. The 3020insC allele of NOD2 predisposes to early-onset breast cancer. *Breast Cancer Res Treat* (2005) **89**:91–3. doi:10.1007/s10549-004-1250-y
120. Lener MR, Oszutowska D, Castaneda J, Kurzawski G, Suchy J, Nej-Wolosiak K, et al. Prevalence of the NOD2 3020insC mutation in aggregations of breast and lung cancer. *Breast Cancer Res Treat* (2006) **95**:141–5. doi:10.1007/s10549-005-9057-z
121. Rigoli L, Di Bella C, Fedele F, Procopio V, Amorini M, Lo Giudice G, et al. TLR4 and NOD2/CARD15 genetic polymorphisms and their possible role in gastric carcinogenesis. *Anticancer Res* (2010) **30**:513–7.
122. Chen GY, Shaw MH, Redondo G, Nunez G. The innate immune receptor Nod1 protects the intestine from inflammation-induced tumorigenesis. *Cancer Res* (2008) **68**:10060–7. doi:10.1158/0008-5472.CAN-08-2061
123. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci* (2003) **94**:965–73. doi:10.1111/j.1349-7006.2003.tb01386.x
124. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* (1990) **247**:322–4. doi:10.1126/science.229672
125. Itzkowitz S. Colon carcinogenesis in inflammatory bowel disease: applying molecular genetics to clinical practice. *J Clin Gastroenterol* (2003) **36**:S70–4. doi:10.1097/00004836-200305001-00012
126. Salinger AP, Justice MJ. Mouse mutagenesis using N-Ethyl-N-nitrosourea (ENU). *CSH Protoc* (2008) **2008**:pdb.prot4985. doi:10.1101/pdb.prot4985
127. Nassiri M, Kooshyar MM, Roudbar Z, Mahdavi M, Doosti M. Genes and SNPs associated with non-hereditary and hereditary colorectal cancer. *Asian Pac J Cancer Prev* (2013) **14**:5609–14. doi:10.7314/APJCP.2013.14.10.5609
128. Maruyama K, Ochiai A, Akimoto S, Nakamura S, Baba S, Moriya Y, et al. Cytoplasmic beta-catenin accumulation as a predictor of hematogenous metastasis in human colorectal cancer. *Oncology* (2000) **59**:302–9. doi:10.1159/000012187
129. Yamada Y, Mori H. Multistep carcinogenesis of the colon in Apc(Min/+) mouse. *Cancer Sci* (2007) **98**:6–10. doi:10.1111/j.1349-7006.2006.00348.x
130. Couturier-Maillard A, Secher T, Rehman A, Normand S, De Arcangelis A, Haesler R, et al. NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. *J Clin Invest* (2013) **123**:700–11. doi:10.1172/JCI62236
131. Millrud CR, Kvarnhammar AM, Tajti J, Munck-Wikland E, Uddman R, Cardell LO. Nod-like receptors in head and neck squamous cell carcinoma. *Acta Otolaryngol* (2013) **133**:1333–44. doi:10.3109/00016489.2013.831476
132. Schroder K, Tschopp J. The inflammasomes. *Cell* (2010) **140**:821–32. doi:10.1016/j.cell.2010.01.040
133. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet* (2001) **29**:301–5. doi:10.1038/ng1976
134. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* (2004) **430**:213–8. doi:10.1038/nature02664
135. Mariathasan S, Monack DM. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nat Rev Immunol* (2007) **7**:31–40. doi:10.1038/nri1997
136. Agostini L, Martinon F, Burns K, Mcdermott MF, Hawkins PN, Tschoopp J. NALP3 forms an IL-1 β -processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* (2004) **20**:319–25. doi:10.1016/j.jci.2004.09.046
137. Kanneganti TD, Ozoren N, Body-Malapel M, Amer A, Park JH, Franchi L, et al. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* (2006) **440**:233–6. doi:10.1038/nature04517
138. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* (2006) **440**:228–32. doi:10.1038/nature04515
139. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschoopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* (2006) **440**:237–41. doi:10.1038/nature04516
140. Sutterwala FS, Ogura Y, Szczepanik M, Lara-Tejero M, Lichtenberger GS, Grant EP, et al. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* (2006) **24**:317–27. doi:10.1016/j.jci.2006.02.004
141. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* (2009) **7**:99–109. doi:10.1038/nrmicro2070
142. Subramanian N, Natarajan K, Clatworthy MR, Wang Z, Germain RN. The adaptor MAVS promotes NLRP3 mitochondrial localization and inflammasome activation. *Cell* (2013) **153**:348–61. doi:10.1016/j.cell.2013.02.054
143. Kummer JA, Broekhuizen R, Everett H, Agostini L, Kuijk L, Martinon F, et al. Inflammasome components NALP1 and 3 show distinct but separate expression profiles in human tissues suggesting a site-specific role in the inflammatory response. *J Histochem Cytochem* (2007) **55**:443–52. doi:10.1369/jhc.6A7101.2006
144. Guarda G, Zenger M, Yazdi AS, Schroder K, Ferrero I, Menu P, et al. Differential expression of NLRP3 among hematopoietic cells. *J Immunol* (2011) **186**:2529–34. doi:10.4049/jimmunol.1002720
145. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol* (2011) **29**:707–35. doi:10.1146/annurev-immunol-031210-101405
146. Lamkanfi M, Dixit VM. Inflammasomes and their roles in health and disease. *Annu Rev Cell Dev Biol* (2012) **28**:137–61. doi:10.1146/annurev-cellbio-101011-155745
147. Muniz LR, Knosp C, Yeretssian G. Intestinal antimicrobial peptides during homeostasis, infection, and disease. *Front Immunol* (2012) **3**:310. doi:10.3389/fimmu.2012.00310

148. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. *Nat Rev Immunol* (2013) **13**:397–411. doi:10.1038/nri3452
149. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, Macdonald K, Speert D, et al. Cutting edge: NF- κ B activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* (2009) **183**:787–91. doi:10.4049/jimmunol.0901363
150. Ghonime MG, Shamaa OR, Das S, Eldomany RA, Fernandes-Alnemri T, Alnemri ES, et al. Inflammasome priming by lipopolysaccharide is dependent upon ERK signaling and proteasome function. *J Immunol* (2014) **192**:3881–8. doi:10.4049/jimmunol.1301974
151. Schumann RR, Belka C, Reuter D, Lamping N, Kirschning CJ, Weber JR, et al. Lipopolysaccharide activates caspase-1 (interleukin-1-converting enzyme) in cultured monocytic and endothelial cells. *Blood* (1998) **91**:577–84.
152. Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, Van Der Meer JH, et al. Differential requirement for the activation of the inflammasome for processing and release of IL-1 β in monocytes and macrophages. *Blood* (2009) **113**:2324–35. doi:10.1182/blood-2008-03-146720
153. Bauernfeind F, Bartok E, Rieger A, Franchi L, Nunez G, Hornung V. Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. *J Immunol* (2011) **187**:613–7. doi:10.4049/jimmunol.1100613
154. Juliana C, Fernandes-Alnemri T, Kang S, Farias A, Qin F, Alnemri ES. Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation. *J Biol Chem* (2012) **287**:36617–22. doi:10.1074/jbc.M112.407130
155. Craven RR, Gao X, Allen IC, Gris D, Bubeck Wardenburg J, Mcelvania-Tekippe E, et al. *Staphylococcus aureus* alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS One* (2009) **4**:e7446. doi:10.1371/journal.pone.0007446
156. McNeela EA, Burke A, Neill DR, Baxter C, Fernandes VE, Ferreira D, et al. Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4. *PLoS Pathog* (2010) **6**:e1001191. doi:10.1371/journal.ppat.1001191
157. Kanneganti TD, Body-Malapel M, Amer A, Park JH, Whitfield J, Franchi L, et al. Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J Biol Chem* (2006) **281**:36560–8. doi:10.1074/jbc.M607594200
158. Allen IC, Scull MA, Moore CB, Holl EK, Mcelvania-Tekippe E, Taxman DJ, et al. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* (2009) **30**:556–65. doi:10.1016/j.immuni.2009.02.005
159. Thomas PG, Dash P, Aldridge JR Jr, Ellebedy AH, Reynolds C, Funk AJ, et al. The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* (2009) **30**:566–75. doi:10.1016/j.immuni.2009.02.006
160. Rajan JV, Warren SE, Miao EA, Aderem A. Activation of the NLRP3 inflammasome by intracellular poly I:C. *FEBS Lett* (2010) **584**:4627–32. doi:10.1016/j.febslet.2010.10.036
161. Kanneganti TD, Lamkanfi M, Kim YG, Chen G, Park JH, Franchi L, et al. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* (2007) **26**:433–43. doi:10.1016/j.immuni.2007.03.008
162. Piccini A, Carta S, Tassi S, Lasiglie D, Fossati G, Rubartelli A. ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1 β and IL-18 secretion in an autocrine way. *Proc Natl Acad Sci U S A* (2008) **105**:8067–72. doi:10.1073/pnas.0709684105
163. Gombault A, Baron L, Couillin I. ATP release and purinergic signaling in NLRP3 inflammasome activation. *Front Immunol* (2012) **3**:414. doi:10.3389/fimmu.2012.00414
164. Ayna G, Krysko DV, Kaczmarek A, Petrovski G, Vandenabeele P, Fesus L. ATP release from dying autophagic cells and their phagocytosis are crucial for inflammasome activation in macrophages. *PLoS One* (2012) **7**:e40069. doi:10.1371/journal.pone.0040069
165. Cassel SL, Eisenbarth SC, Iyer SS, Sadler JJ, Colegio OR, Tephly LA, et al. The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A* (2008) **105**:9035–40. doi:10.1073/pnas.0803933105
166. Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschopp J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* (2008) **320**:674–7. doi:10.1126/science.1156995
167. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* (2008) **453**:1122–6. doi:10.1038/nature06939
168. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T, et al. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* (2008) **9**:857–65. doi:10.1038/ni.1636
169. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* (2008) **9**:847–56. doi:10.1038/ni.1631
170. Kingsbury SR, Conaghan PG, McDermott MF. The role of the NLRP3 inflammasome in gout. *J Inflamm Res* (2011) **4**:39–49. doi:10.2147/JIR.S11330
171. Dostert C, Guarda G, Romero JF, Menu P, Gross O, Tardivel A, et al. Malarial hemozoin is a Nalp3 inflammasome activating danger signal. *PLoS One* (2009) **4**:e6510. doi:10.1371/journal.pone.0006510
172. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* (2011) **12**:222–30. doi:10.1038/ni.1980
173. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* (2011) **469**:221–5. doi:10.1038/nature09663
174. Shimada K, Crother TR, Karlin J, Dagvadorj J, Chiba N, Chen S, et al. Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* (2012) **36**:401–14. doi:10.1016/j.immuni.2012.01.009
175. Zhong Z, Zhai Y, Liang S, Mori Y, Han R, Sutterwala FS, et al. TRPM2 links oxidative stress to NLRP3 inflammasome activation. *Nat Commun* (2013) **4**:1611. doi:10.1038/ncomms2608
176. Iyer SS, He Q, Janczy JR, Elliott EI, Zhong Z, Olivier AK, et al. Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* (2013) **39**:311–23. doi:10.1016/j.immuni.2013.08.001
177. Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, et al. The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca $^{2+}$ and cAMP. *Nature* (2012) **492**:123–7. doi:10.1038/nature11588
178. Murakami T, Ockinger J, Yu J, Byles V, Mccoll A, Hofer AM, et al. Critical role for calcium mobilization in activation of the NLRP3 inflammasome. *Proc Natl Acad Sci U S A* (2012) **109**:11282–7. doi:10.1073/pnas.1117765109
179. Rossol M, Pierer M, Raulien N, Quandt D, Meusch U, Rothe K, et al. Extracellular Ca $^{2+}$ is a danger signal activating the NLRP3 inflammasome through G protein-coupled calcium sensing receptors. *Nat Commun* (2012) **3**:1329. doi:10.1038/ncomms2339
180. Shenoy AR, Wellington DA, Kumar P, Kassa H, Booth CJ, Cresswell P, et al. GBP5 promotes NLRP3 inflammasome assembly and immunity in mammals. *Science* (2012) **336**:481–5. doi:10.1126/science.1217141
181. Munoz-Planillo R, Kuffa P, Martinez-Colon G, Smith BL, Rajendiran TM, Nunez G. K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* (2013) **38**:1142–53. doi:10.1016/j.immuni.2013.05.016
182. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1 β production. *Nature* (2008) **456**:264–8. doi:10.1038/nature07383
183. Harris J, Hartman M, Roche C, Zeng SG, O'Shea A, Sharp FA, et al. Autophagy controls IL-1 β secretion by targeting pro-IL-1 β for degradation. *J Biol Chem* (2011) **286**:9587–97. doi:10.1074/jbc.M110.202911
184. Shi CS, Shenderov K, Huang NN, Kabat J, Abu-Asab M, Fitzgerald KA, et al. Activation of autophagy by inflammatory signals limits IL-1 β production by targeting ubiquitinated inflammasomes for destruction. *Nat Immunol* (2012) **13**:255–63. doi:10.1038/ni.2215
185. Lupfer C, Thomas PG, Anand PK, Vogel P, Milasta S, Martinez J, et al. Receptor interacting protein kinase 2-mediated mitophagy regulates inflammasome activation during virus infection. *Nat Immunol* (2013) **14**:480–8. doi:10.1038/ni.2563
186. Bruyne JM, Bruyne-Sedano N, Luciano F, Zhai D, Balpai R, Xu C, et al. Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1. *Cell* (2007) **129**:45–56. doi:10.1016/j.cell.2007.01.045
187. Faustin B, Chen Y, Zhai D, Le Negrate G, Lartigue L, Satterthwait A, et al. Mechanism of Bcl-2 and Bcl-X(L) inhibition of NLRP1 inflammasome: loop domain-dependent suppression of ATP binding and oligomerization. *Proc Natl Acad Sci U S A* (2009) **106**:3935–40. doi:10.1073/pnas.0809414106

188. Hawkins CJ, Uren AG, Hacker G, Medcalf RL, Vaux DL. Inhibition of interleukin 1 beta-converting enzyme-mediated apoptosis of mammalian cells by baculovirus IAP. *Proc Natl Acad Sci U S A* (1996) **93**:13786–90. doi:10.1073/pnas.93.24.13786
189. Labbe K, McIntire CR, Doiron K, Leblanc PM, Saleh M. Cellular inhibitors of apoptosis proteins cIAP1 and cIAP2 are required for efficient caspase-1 activation by the inflammasome. *Immunity* (2011) **35**:897–907. doi:10.1016/j.immuni.2011.10.016
190. Vince JE, Wong WW, Gentle I, Lawlor KE, Allam R, O'Reilly L, et al. Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. *Immunity* (2012) **36**:215–27. doi:10.1016/j.immuni.2012.01.012
191. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, et al. Non-canonical inflammasome activation targets caspase-11. *Nature* (2011) **479**:117–21. doi:10.1038/nature10558
192. Gurung P, Malireddi RK, Anand PK, Demon D, Vande Walle L, Liu Z, et al. Toll or interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-beta (TRIF)-mediated caspase-11 protease production integrates Toll-like receptor 4 (TLR4) protein- and Nlrp3 inflammasome-mediated host defense against enteropathogens. *J Biol Chem* (2012) **287**:34474–83. doi:10.1074/jbc.M112.401406
193. Rathinam VA, Vanaja SK, Waggoner L, Sokolovska A, Becker C, Stuart LM, et al. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* (2012) **150**:606–19. doi:10.1016/j.cell.2012.07.007
194. Sagulenko V, Thygesen SJ, Sester DP, Idris A, Cridland JA, Vajjhala PR, et al. AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell Death Differ* (2013) **20**:1149–60. doi:10.1038/cdd.2013.37
195. Gurung P, Anand PK, Malireddi RK, Vande Walle L, Van Opdenbosch N, Dillon CP, et al. FADD and caspase-8 mediate priming and activation of the canonical and noncanonical Nlrp3 inflammasomes. *J Immunol* (2014) **192**:1835–46. doi:10.4049/jimmunol.1302839
196. Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D. Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity* (2013) **38**:27–40. doi:10.1016/j.immuni.2012.09.015
197. Allen IC, Tekippe EM, Woodford RM, Uronis JM, Holl EK, Rogers AB, et al. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med* (2010) **207**:1045–56. doi:10.1084/jem.20100050
198. Zaki MH, Boyd KL, Vogel P, Kastan MB, Lamkanfi M, Kanneganti TD. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity* (2010) **32**:379–91. doi:10.1016/j.immuni.2010.03.003
199. Dupaul-Chicoine J, Yeretssian G, Doiron K, Bergstrom KS, McIntire CR, Leblanc PM, et al. Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity* (2010) **32**:367–78. doi:10.1016/j.immuni.2010.02.012
200. Zaki MH, Vogel P, Body-Malapel M, Lamkanfi M, Kanneganti TD. IL-18 production downstream of the Nlrp3 inflammasome confers protection against colorectal tumor formation. *J Immunol* (2010) **185**:4912–20. doi:10.4049/jimmunol.1002046
201. Salcedo R, Worschach A, Cardone M, Jones Y, Gyulai Z, Dai RM, et al. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. *J Exp Med* (2010) **207**:1625–36. doi:10.1084/jem.20100199
202. Reuter BK, Pizarro TT. Commentary: the role of the IL-18 system and other members of the IL-1R/TLR superfamily in innate mucosal immunity and the pathogenesis of inflammatory bowel disease: friend or foe? *Eur J Immunol* (2004) **34**:2347–55. doi:10.1002/eji.200425351
203. Siegmund B. Interleukin-18 in intestinal inflammation: friend and foe? *Immunity* (2010) **32**:300–2. doi:10.1016/j.immuni.2010.03.010
204. Pastorelli L, De Salvo C, Mercado JR, Vecchi M, Pizarro TT. Central role of the gut epithelial barrier in the pathogenesis of chronic intestinal inflammation: lessons learned from animal models and human genetics. *Front Immunol* (2013) **4**:280. doi:10.3389/fimmu.2013.00280
205. Wei Q, Mu K, Li T, Zhang Y, Yang Z, Jia X, et al. Dereulation of the NLRP3 inflammasome in hepatic parenchymal cells during liver cancer progression. *Lab Invest* (2014) **94**:52–62. doi:10.1038/labinvest.2013.126
206. Ungerbeck J, Belenki D, Jawad Ul-Hassan A, Fredrikson M, Fransen K, Elander N, et al. Genetic variation and alterations of genes involved in NFkappaB/TNFAIP3- and NLRP3-inflammasome signaling affect susceptibility and outcome of colorectal cancer. *Carcinogenesis* (2012) **33**:2126–34. doi:10.1093/carcin/bgs256
207. Verma D, Sarndahl E, Andersson H, Eriksson P, Fredrikson M, Jonsson JI, et al. The Q705K polymorphism in NLRP3 is a gain-of-function alteration leading to excessive interleukin-1beta and IL-18 production. *PLoS One* (2012) **7**:e34977. doi:10.1371/journal.pone.0034977
208. Verma D, Bivik C, Farahani E, Synnerstad I, Fredrikson M, Enerback C, et al. Inflammasome polymorphisms confer susceptibility to sporadic malignant melanoma. *Pigment Cell Melanoma Res* (2012) **25**:506–13. doi:10.1111/j.1755-148X.2012.01008.x
209. Okamoto M, Liu W, Luo Y, Tanaka A, Cai X, Norris DA, et al. Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta. *J Biol Chem* (2010) **285**:6477–88. doi:10.1074/jbc.M109.064907
210. Nikulenkov F, Spinnler C, Li H, Tonelli C, Shi Y, Turunen M, et al. Insights into p53 transcriptional function via genome-wide chromatin occupancy and gene expression analysis. *Cell Death Differ* (2012) **19**:1992–2002. doi:10.1038/cdd.2012.89
211. Sadasivam S, Gupta S, Radha V, Batta K, Kundu TK, Swarup G. Caspase-1 activator Ipaf is a p53-inducible gene involved in apoptosis. *Oncogene* (2005) **24**:627–36. doi:10.1038/sj.onc.1208201
212. Hu B, Elinav E, Flavell RA. Inflammasome-mediated suppression of inflammation-induced colorectal cancer progression is mediated by direct regulation of epithelial cell proliferation. *Cell Cycle* (2011) **10**:1936–9. doi:10.4161/cc.10.12.16008
213. Hu B, Elinav E, Huber S, Booth CJ, Strowig T, Jin C, et al. Inflammation-induced tumorigenesis in the colon is regulated by caspase-1 and NLRCA. *Proc Natl Acad Sci U S A* (2010) **107**:21635–40. doi:10.1073/pnas.1016814108
214. Franchi L, Kamada N, Nakamura Y, Burberry A, Kuffa P, Suzuki S, et al. NLRCA-driven production of IL-1beta discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. *Nat Immunol* (2012) **13**:449–56. doi:10.1038/ni.2263
215. Grenier JM, Wang L, Manji GA, Huang WJ, Al-Garawi A, Kelly R, et al. Functional screening of five PYPAF family members identifies PYPAF5 as a novel regulator of NF-kappaB and caspase-1. *FEBS Lett* (2002) **530**:73–8. doi:10.1016/S0014-5793(02)03416-6
216. Chen GY, Liu M, Wang F, Bertin J, Nunez G. A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *J Immunol* (2011) **186**:7187–94. doi:10.4049/jimmunol.1100412
217. Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, Booth CJ, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* (2011) **145**:745–57. doi:10.1016/j.cell.2011.04.022
218. Normand S, Delaney-Crespin A, Bressonot A, Huot L, Grandjean T, Peyrin-Biroulet L, et al. Nod-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proc Natl Acad Sci U S A* (2011) **108**:9601–6. doi:10.1073/pnas.1100981108
219. Huber S, Gagliani N, Zenewicz LA, Huber FJ, Bosurgi L, Hu B, et al. IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine. *Nature* (2012) **491**:259–63. doi:10.1038/nature11535
220. Wang L, Manji GA, Grenier JM, Al-Garawi A, Merriam S, Lora JM, et al. PYPAF7, a novel PYRIN-containing Apaf1-like protein that regulates activation of NF-kappa B and caspase-1-dependent cytokine processing. *J Biol Chem* (2002) **277**:29874–80. doi:10.1074/jbc.M203915200
221. Vladimer GI, Weng D, Paquette SW, Vanaja SK, Rathinam VA, Aune MH, et al. The NLRP12 inflammasome recognizes *Yersinia pestis*. *Immunity* (2012) **37**:96–107. doi:10.1016/j.immuni.2012.07.006
222. Macaluso F, Nothnagel M, Parwez Q, Petrasch-Parwez E, Bechara FG, Epplen JT, et al. Polymorphisms in NACHT-LRR (NLR) genes in atopic dermatitis. *Exp Dermatol* (2007) **16**:692–8. doi:10.1111/j.1600-0625.2007.00589.x
223. Jeru I, Duquesnoy P, Fernandes-Alnemri T, Cochet E, Yu JW, Lackmy-Port-Lis M, et al. Mutations in NALP12 cause hereditary periodic fever syndromes. *Proc Natl Acad Sci U S A* (2008) **105**:1614–9. doi:10.1073/pnas.0708616105
224. Arthur JC, Lich JD, Ye Z, Allen IC, Gris D, Wilson JE, et al. Cutting edge: NLRP12 controls dendritic and myeloid cell migration to affect contact hypersensitivity. *J Immunol* (2010) **185**:4515–9. doi:10.4049/jimmunol.1002227
225. Borghini S, Tassi S, Chiesa S, Caroli F, Carta S, Caorsi R, et al. Clinical presentation and pathogenesis of cold-induced autoinflammatory disease in a family

- with recurrence of an NLRP12 mutation. *Arthritis Rheum* (2011) **63**:830–9. doi:10.1002/art.30170
226. Williams KL, Lich JD, Duncan JA, Reed W, Rallabhandi P, Moore C, et al. The CATERPILLER protein monarch-1 is an antagonist of toll-like receptor-, tumor necrosis factor alpha-, and *Mycobacterium tuberculosis*-induced pro-inflammatory signals. *J Biol Chem* (2005) **280**:39914–24. doi:10.1074/jbc.M502820200
227. Arthur JC, Lich JD, Aziz RK, Kotb M, Ting JP. Heat shock protein 90 associates with monarch-1 and regulates its ability to promote degradation of NF-kappaB-inducing kinase. *J Immunol* (2007) **179**:6291–6. doi:10.4049/jimmunol.179.9.6291
228. Lich JD, Williams KL, Moore CB, Arthur JC, Davis BK, Taxman DJ, et al. Monarch-1 suppresses non-canonical NF-kappaB activation and p52-dependent chemokine expression in monocytes. *J Immunol* (2007) **178**:1256–60. doi:10.4049/jimmunol.178.3.1256
229. Zaki MH, Vogel P, Malireddi RK, Body-Malapel M, Anand PK, Bertin J, et al. The NOD-like receptor NLRP12 attenuates colon inflammation and tumorigenesis. *Cancer Cell* (2011) **20**:649–60. doi:10.1016/j.ccr.2011.10.022
230. Allen IC, Wilson JE, Schneider M, Lich JD, Roberts RA, Arthur JC, et al. NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-kappaB signaling. *Immunity* (2012) **36**:742–54. doi:10.1016/j.immuni.2012.03.012
231. Garaude J, Kent A, Van Rooijen N, Blander JM. Simultaneous targeting of toll- and nod-like receptors induces effective tumor-specific immune responses. *Sci Transl Med* (2012) **4**:120ra116. doi:10.1126/scitranslmed.3002868
232. Yanagawa H, Haku T, Takeuchi E, Suzuki Y, Nokihara H, Sone S. Intrapleural therapy with MDP-Lys (L18), a synthetic derivative of muramyl dipeptide, against malignant pleurisy associated with lung cancer. *Lung Cancer* (2000) **27**:67–73. doi:10.1016/S0169-5002(99)00090-2
233. Nitta Y, Sugita T, Ikuta Y, Murakami T. Inhibitory effect of liposomal MDP-Lys on lung metastasis of transplantable osteosarcoma in hamster. *Oncol Res* (2000) **12**:25–31.
234. Yoo YC, Saiki I, Sato K, Azuma I. MDP-Lys(L18), a lipophilic derivative of muramyl dipeptide, inhibits the metastasis of haematogenous and non-haematogenous tumours in mice. *Vaccine* (1994) **12**:175–260. doi:10.1016/0264-410X(94)90057-4
235. Fujimura T, Yamasaki K, Hidaka T, Ito Y, Aiba S. A synthetic NOD2 agonist, muramyl dipeptide (MDP)-Lys (L18) and IFN-beta synergistically induce dendritic cell maturation with augmented IL-12 production and suppress melanoma growth. *J Dermatol Sci* (2011) **62**:107–15. doi:10.1016/j.jdermsci.2011.02.002
236. Chow MT, Scaneay J, Paget C, Wong CS, Duret H, Tschoopp J, et al. NLRP3 suppresses NK cell-mediated responses to carcinogen-induced tumors and metastases. *Cancer Res* (2012) **72**:5721–32. doi:10.1158/0008-5472.CAN-12-0509
237. Bruchard M, Mignot G, Derangere V, Chalmin F, Chevriaux A, Vegran F, et al. Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nat Med* (2013) **19**:57–64. doi:10.1038/nm.2999
238. Zitvogel L, Kepp O, Galluzzi L, Kroemer G. Inflammasomes in carcinogenesis and anticancer immune responses. *Nat Immunol* (2012) **13**:343–51. doi:10.1038/ni.2224
239. Lust JA, Lacy MQ, Zeldenrust SR, Dispensieri A, Gertz MA, Witzig TE, et al. Induction of a chronic disease state in patients with smoldering or indolent multiple myeloma by targeting interleukin 1 β -induced interleukin 6 production and the myeloma proliferative component. *Mayo Clin Proc* (2009) **84**:114–22. doi:10.4065/84.2.114
240. Terme M, Ullrich E, Aymeric L, Meinhardt K, Desbois M, Delahaye N, et al. IL-18 induces PD-1-dependent immunosuppression in cancer. *Cancer Res* (2011) **71**:5393–9. doi:10.1158/0008-5472.CAN-11-0993
241. Terme M, Ullrich E, Aymeric L, Meinhardt K, Coudert JD, Desbois M, et al. Cancer-induced immunosuppression: IL-18-elicited immunoablative NK cells. *Cancer Res* (2012) **72**:2757–67. doi:10.1158/0008-5472.CAN-11-3379

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Nod-like receptors: key molecular switches in the conundrum of cancer

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It is believed the immune system can contribute to oncogenic transformation especially in settings of chronic inflammation, be activated during immunosurveillance to destroy early neoplastic cells before they undergo malignant outgrowth, and finally, can assist growth of established tumors by preventing clearance, remodeling surrounding tissue, and promoting metastatic events. These seemingly opposing roles of the immune system at the different stages of cancer development must all be mediated by innate signaling mechanisms that regulate the overall state of immune activation. Recently, the cytosolic nod-like receptor (NLR) pathway of innate immunity has gained a lot of attention in the tumor immunology field due to its known involvement in promoting inflammation and immunity, and conversely, in regulating tissue repair processes. In this review, we present all the current evidence for NLR involvement in the different stages of neoplasia to understand how a single molecular pathway can contribute to conflicting immunological interactions with cancer.

Keywords: nod-like receptors, cancer, immunoediting, immunosurveillance, innate immunity, transformation

INTRODUCTION

The pervading conception of the immune system today depicts it simply as the body's means of warding off infection. In her *Anthropology of Immunology*, Martin eloquently describes "the body as nation state at war over its borders, containing internal surveillance systems (encompassed in the immune system) to monitor foreign intruders" (1). However, this "infection-centric" view does not consider profound facets of the immune system, now well established in the literature, and largely forgotten since the earliest immunologists predicted their existence. As early as the 1890s, Ilya Metchnikoff conceived of the theory of "physiological inflammation," in which the immune system, especially phagocytic cells, were essential for maintaining homeostasis within all tissues of the body (2). He postulated that phagocytic cells uphold the balance between competing cell types and organs as they arise within a multicellular organism, establishing a unified "organismal identity" (2). This did not ignore the role of phagocytes in fighting infection, but suggested a "wide functional spectrum, of which host defense against pathogens was only one aspect" (2). Included were roles in regulating tissue development, clearance of damaged tissue, promotion of wound repair after any insult, be it infectious or sterile, and resolution of unwarranted inflammatory processes.

There is no better example of a question of organismal identity, of the need for a restoration of homeostasis, or of cell types or tissues in competition with one another, than that of cancer. Because they are initially derived from self-tissue, transformed cells pose a dilemma – to destroy or repair? It seems the immune system is responsible for answering this question, and is now known to be intimately involved in the oncogenic process from the very emergence of the first transformed cells through to malignant disease (3–5). Due to the nature of the predicament at hand, the immune

system has been described to have conflicting roles depending on which stage of cancer progression is being studied (6). How the opposing immunological phenotypes in cancer are controlled is not well known, but nod-like receptors (NLRs) have been implicated in various stages of the disease process and have the required capacity to act as key regulators of physiological and pathological inflammation (7–9). NLRs are initiators of the inflammasome pathway, a cytosolic signaling apparatus that canonically activates caspase-1, and IL-1 β and IL-18 thereafter (10). NLRs can respond to both pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively), and the pathway has been shown to have important roles in mounting immune responses to both microbial pathogens and damaged self, as well as regulating tissue repair after damage (11, 12). Here, we will review the evidence for NLR involvement in the initial emergence of neoplastic lesions, in the control and destruction of transformed cells during a phase of immunosurveillance, and finally the immune shift to supporting growth of established disease. We will argue that the conflicting roles of the immune system during oncogenesis can be reconciled within the framework of Metchnikoff's theory of immune control of tissue homeostasis, and that NLRs and their downstream signaling elements serve as key molecular switches in this process.

EMERGENCE OF TRANSFORMATION

Schreiber and colleagues categorized immune interaction with cancer into three stages of immunoediting: elimination by immunosurveillance mechanisms; equilibrium, when cancer attains a latent balance between aberrant growth and destruction; and escape, when the tumor overcomes suppression as an edited malignancy (13). Although overlooked in the "Three E's model"

of immunoediting, the involvement of inflammatory processes in the initial emergence of cancer is well established within the literature. Chronic inflammation is a major risk factor for neoplasia in the clinic, working to both disrupt the microenvironment to favor neoplastic outgrowth, and contribute to genetic instability and altered turnover rates of stromal cells, promoting accelerated emergence of malignant clones (14). Many studies have now implicated the inflammasome pathway and the NLRs in this context, but with contrasting influences depending on the context and specifics under scrutiny.

A predominant model used to study NLR and inflammasome contributions to carcinogenesis is the AOM/DSS model (15). DSS causes damage to the colonic epithelium, while AOM causes G-to-A mutations in DNA of cells undergoing DNA replication. Deficiency in NLRP6, an NLR primarily expressed in colonic myofibroblasts, resulted in decreased repair of the intestinal epithelium following DSS treatment, but conversely, was associated with increased epithelial colonocyte proliferation and transcript expression of molecules involved in cell cycle progression (16). Another study showed prolonged colitis and epithelial destruction in *Nlrp6*^{-/-} mice after DSS treatment was related to alterations in commensal microbiota, and was phenocopied when mice were deficient in any of the NLRP6 inflammasome components ASC (a common adapter to many inflammasomes), and caspase-1 (17). The IL-1 β cytokine, cleaved into its biologically active form by activated caspase-1, has emerged as a key cytokine downstream of inflammasome activation that enables epithelial repair after damage, but also prevents cancer progression through its induction of the tumor suppressors STAT1 and IFN- γ (18). When treated with AOM/DSS, the resulting increased epithelial proliferation and exacerbated inflammation in *Nlrp6*^{-/-} mice led to accelerated outgrowth of colonic cancer (16). In addition to NLRP6, loss of NLR family members NOD1, NOD2, NLRP3, NLRC4, and NLRP12 has resulted in similar exacerbated colitis and accelerated rates of cancer (19–24). Together, results from these gut studies suggest NLRs and their associated inflammasome components are essential for controlling wound repair responses and preventing transformative events and unwarranted epithelial proliferation early in potentially neoplastic settings (20). Much work needs to be done to clarify the mechanisms of NLR regulation in these processes, especially their connection to regulation of epithelial regrowth.

Paradoxically, over-expression of NLR pathway components also drives cancer rather than suppresses its emergence. As might be predicted from the above evidence, the derepression of caspase-1 that occurs in *Casp12*^{-/-} mice results in accelerated recovery from colitis after DSS. However, after AOM/DSS, these mice have accelerated rather than decreased colorectal cancer development, a pathology linked to increased levels of inflammatory cytokine gene expression including *Il1b* (25). In a model of HCV infection, IL-1 β production downstream of NLRP3 by hepatic macrophages was linked to chronic hepatitis (26). Similarly, CCl₄ treated *Nlrp3*^{-/-} and *Asc*^{-/-} mice exhibited reduced levels of liver fibrosis, and wild-type hepatic stellate cells treated with monosodium urate crystals upregulated the *Tgfb* and *Colla* genes in an inflammasome-dependent manner (27). Thus in the liver, NLRs contribute to chronic inflammatory processes, both infectious and sterile, that

result in the hepatitis and fibrosis commonly found prior to hepatocellular carcinoma.

IL-1 β has many pleiotropic effects involved in inflammation, immunosuppression, cell proliferation and differentiation, tissue regeneration, tumor-promotion, and chemoresistance (28). In addition to its roles in hepatic carcinoma, the cytokine has been implicated in accelerating tumor development in mammary epithelial (29), gastric (30), and skin (31) cancer models, further establishing its role as an inflammatory instigator of oncogenesis. Drexler et al. were able to show both anti- and pro-tumorigenic effects of ASC in a single model of chemically induced skin carcinogenesis (31). ASC expression in infiltrating myeloid cells helped drive carcinogenesis, while ASC expression in keratinocytes suppressed epithelial cell proliferation and carcinogenesis (although in a caspase-1-independent manner). While the specific NLR implicated in these opposing roles of ASC was not identified, involvement of the inflammasome pathway was strongly implicated.

These studies all demonstrate opposing roles of the inflammasome in the early initiation of neoplastic disease. NLR activation can inhibit malignant transformation by controlling epithelial cell regeneration, but can also contribute to chronic inflammation that eventually results in carcinogenesis. The NLRs mediate a fine balance between inflammation and repair to maintain homeostasis in each tissue. If tipped in either direction, malignancy can result.

ELIMINATION OF TRANSFORMED CELLS

Once a transformed cell appears, it immediately presents a unique challenge to the immune system. Its uncontrolled proliferation threatens the evolutionarily defined healthy function of the tissue of its origin. Although derived from self, it no longer obeys the rules of organismal identity. From observations of homograft rejection, and increased cancer incidence in immunocompromised individuals, Lewis Thomas and Sir MacFarlane Burnett postulated the theory of immunosurveillance – the ability of the immune system to recognize and destroy abnormal self despite its ontogenic origins (32). Schreiber and others have built a strong case for the existence of adaptive immunosurveillance, and now evidence is emerging in spontaneous models of neoplasia (33–36).

Every adaptive response requires innate priming, thus innate immunity must be involved. Some studies have shown innate cell involvement (34, 37, 38), but thorough examinations of the molecular pathways that enable immune activation against tumor antigens are scarce. However, there are a few studies directly demonstrating NLRs can be involved in immunosurveillance. In an allograft model, Ghiringelli et al. show that chemotherapeutic killing of tumor cells causes a release of ATP that binds the P2RX7 purinergic receptor on dendritic cells (DCs), eventually leading to the activation of the NLRP3 inflammasome in these cells (37). By synergizing with HMGB1, released from dying tumor cells and signaling through toll-like receptor (TLR) 4, activated DC are licensed to prime an anti-tumor immune response in a caspase-1- and IL-1 β -dependent manner. Another study found that extracts from an anti-tumorigenic mushroom functioned by activating the same P2RX7/NLRP3 pathway in macrophages, but did not draw a direct link to altered tumor kinetics (39). Although these conclusions derive from experimental models, anthracycline-treated

breast cancer patients with mutations in the *P2rx7* gene were found to develop metastatic disease faster than those with normal *P2rx7* genes, suggesting the NLRP3-dependent pathway may be activated in humans with spontaneous disease (37). In addition to NLRP3, in 2012 we published on the ability of flagellin to synergistically activate TLR5 and the NLRC4 inflammasome, resulting in effective priming of CD4 and CD8 immunity against subcutaneously implanted allografts in mice (40). Besides priming of adaptive immunosurveillance, NLRs have been implicated in anti-tumor immunity through the link between IL-18 and increased NK cell activity against tumors (41–44). However, these latter findings were made in the presence of exogenous administration or expression of IL-18 above normal levels.

All these studies involve some artificial intervention that enhances NLR activity, but present a strong case for the ability of the pathway to influence immunosurveillance. It remains to be shown if the inflammasome pathway is involved in intrinsic immunosurveillance mechanisms, or is activated at this early stage of disease in any capacity. It is difficult to capture the elimination phase due to its transience and lack of overt disease phenotypes. Spontaneous models with a definable pre-malignant stage must be employed to further analyze which innate signaling pathways, and in which cell types, are naturally engaged to clear transformed cells before they cause disease. Selectively enhancing this engagement could greatly benefit therapeutic intervention. Additionally, these studies suggest a critical function of the inflammasome in priming adaptive immunity against transformed self-cells. It remains to be shown if this ability is mediated entirely through cytokine production, or if the inflammasome can influence T cell priming in a more direct manner. Conversely, it is possible there are strictly innate-mediated immunosurveillance or tumor-suppressing mechanisms engaged that help inhibit malignancy without priming T or NK cells (45). NLR involvement in these processes is unknown.

MAINTENANCE OF ESTABLISHED DISEASE

Malignant disease is the result of failed immunosurveillance mechanisms. The editing process selects for clones of the rapidly dividing and mutating transformed cell that are progressively less immunostimulatory (13). Eventually, the developing tumor attains a phenotype that no longer incites immune destruction and can grow uncontrolled. Furthermore, established tumors are known to usurp immune mechanisms to not only prevent destruction, but facilitate growth (46). Tumors have been described as wounds that will not heal due to their self origin, the stress they undergo as they rapidly expand, and their elicitation of reparative and protective immune functions (47, 48).

In light of this analogy, it is not surprising to find NLRs activated in malignant disease, in this context attempting to repair the “wound” to restore homeostasis and protect it from further immune destruction. A host of evidence supports various roles for NLR-activated IL-1 β in malignancy, notably in humanized models (49, 50). Okamoto et al. found that malignant human melanoma cells spontaneously activated their intrinsic NLRP3 inflammasome, resulting in caspase-1 cleavage and spontaneous secretion of IL-1 β (51). This secreted IL-1 β became increasingly autonomous with later stage disease, implicating it as an evolutionarily advantageous trait for the developing tumor. *In vitro*, the inflammasome

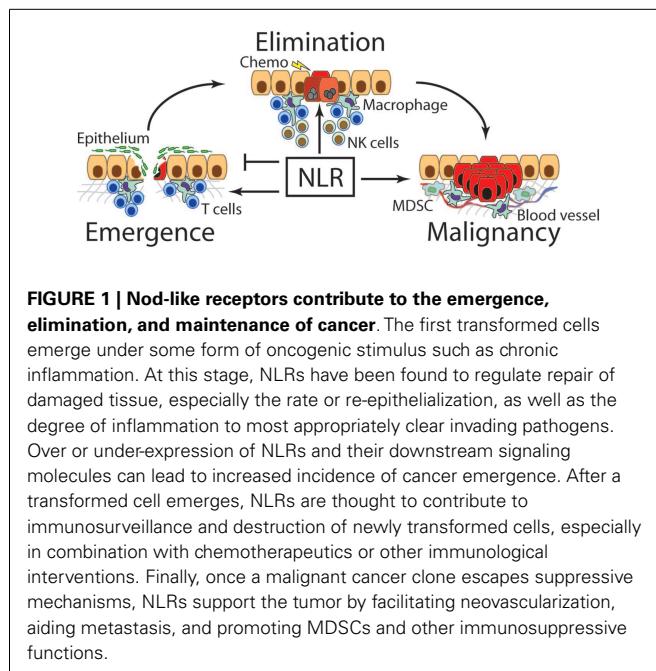
pathway and IL-1 β were shown to increase macrophage chemotaxis and angiogenesis, both features linked to worse prognosis in various cancers (52). Another study found that IL-1 β and caspase-1-deficient mice were much less susceptible to melanoma liver metastases by an injected allograft, improving their overall survival (53). *In vitro*, secreted factors from the melanoma cell line induced IL-18-dependent upregulation of VCAM-1 on hepatic sinusoidal endothelial cells, as well as IL-1 β secretion. In opposition to the results in the previous section, endogenous IL-18 from melanoma cells was also found to inhibit NK cell-mediated killing of melanoma cells by upregulating Fas ligand expression (54). Additionally, IL-18 was found to enhance immunosuppression of NK cells by inducing upregulation of the inhibitory molecule PD-1 (55).

Nod-like receptors are also implicated in the ability of myeloid-derived suppressor cells (MDSCs) to inhibit anti-tumor immuno-surveillance. Related to the gut studies in the first section, IL-1 β over-expression in the stomach was shown to induce inflammation and cancer (30). This was associated with an increase in MDSC numbers homing to the stomach in an IL-1R and NF- κ B-dependent fashion. In a model of DC-based vaccination against melanoma, van Deventer et al. demonstrated that *Nlrp3*^{-/-} mice had improved outcomes due to decreased numbers of MDSCs homing to the tumor site (56). However, they did not observe a change in MDSC function, such as the ability to suppress T cell responses. Finally, chemotherapy was found to trigger cathepsin B release within MDSCs, triggering NLRP3 within the same cells (57). The resultant IL-1 β production induced IL-17 secretion by CD4 T cells. Allograft tumor growth was slower in *Il17a*^{-/-}, *Il1r1*^{-/-}, *Nlrp3*^{-/-}, and *Casp1*^{-/-} mice after chemotherapy treatment, demonstrating all elements in this pathway play a part in tumor protection although the exact mechanism is unclear.

This evidence clearly implicates the NLRs and inflammasome pathway in tumor-promotion and defense. They directly facilitate tumor cell growth and metastasis, and help prevent any anti-tumor immune responses. It is curious to speculate how accurate the analogy of tumor to “unhealing wounds” is with regards to NLR involvement. Are NLRs engaged in the same way by malignant disease as they are by damaged tissues prior to malignant transformation, in both cases inducing repair and protective properties? Fitting with the tumor editing hypothesis, any pro-inflammatory DAMPs or other signals resulting from initial transformation that would trigger tumor clearance have in theory been selected away, leaving only those characteristic of damaged self in need of repair. Inflammasome involvement in such diverse functions as tissue repair, immune suppression, and inflammation warrants a search for more inflammasome-activated targets besides IL-1 β and IL-18 that could fine-tune downstream effector mechanisms. Are these two cytokines alone able to control such diverse effects, or are they working in collaboration with many other pathways, the overall milieu defining the result? Concerted efforts to consolidate information across tumor models and treatments, being mindful of cell-type specificity, will help clarify these points.

CONCLUSION

We have now seen how NLRs switch roles in every stage of cancer progression (Figure 1). In each, the NLRs can be conceptualized



as attempting to restore homeostasis. First, in situations where damage to self has occurred, the NLRs contribute both to fighting off infection and repairing the damaged epithelial layers. The latter implicates an ability of the NLR pathway to regulate growth of surrounding tissues, with a strong link to IL-18. These processes require perfect coordination to maintain equilibrium in the tissue. The fact that too much or too little NLR signaling in this type of setting can result in neoplasia betrays how essential this pathway is to maintaining balance and organismal integrity. Second, when the very idea of self is challenged by oncogenic mutations, again NLR signaling is observed. Presumably here in early pre-neoplastic situations, NLR activation functions as an innate defense against localized transformation events. When clinical pathology is observed, these endogenous protective functions of the NLR have failed. Therapeutic enhancement of this activation has been shown to be beneficial in mouse models, especially in concert with activation of other inflammatory pathways such as TLRs. Thus, development of therapies that employ NLRs could have great impact in the clinic, especially if used very early in neoplasia. Finally, after tumors become established and are immunologically indistinguishable from other self-tissues, NLR activation reverts to helping protect and maintain this neo-self, establishing a new, pathological state of homeostasis. Malignant disease is extremely hard to treat in part because of this unique pseudo-self phenotype and consequent immunoprotective state, reiterating the need for early intervention for successful treatment. Metchnikoff's prescient description of physiological inflammation is thus embodied within the recently discovered NLR pathway. Theories from this founding father of immunology can still help us conceptualize the perplexing and, in the case of NLRs and cancer, diametrically opposed functions of the immune system.

REFERENCES

- Martin E. Toward and anthropology of immunology: the body as nation state. *Med Anthropol Q* (1990) **4**:17. doi:10.1525/maq.1990.4.4.02a00030
- Tauber AI. Metchnikoff and the phagocytosis theory. *Nat Rev Mol Cell Biol* (2003) **4**:897–901. doi:10.1038/nrm1244
- Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* (2008) **454**:436–44. doi:10.1038/nature07205
- Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* (2010) **140**:883–99. doi:10.1016/j.cell.2010.01.025
- Del Prete A, Allavena P, Santoro G, Fumarulo R, Corsi MM, Mantovani A. Molecular pathways in cancer-related inflammation. *Biochem Med (Zagreb)* (2011) **21**:264–75. doi:10.1161/BM.2011.036
- de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* (2006) **6**:24–37. doi:10.1038/nrc1782
- Zitvogel L, Kepp O, Galluzzi L, Kroemer G. Inflammasomes in carcinogenesis and anticancer immune responses. *Nat Immunol* (2012) **13**:343–51. doi:10.1038/ni.2224
- Drexler SK, Yazdi AS. Complex roles of inflammasomes in carcinogenesis. *Cancer J* (2013) **19**:468–72. doi:10.1097/PPO.0000000000000004
- Kolb R, Liu GH, Janowski AM, Sutterwala FS, Zhang W. Inflammasomes in cancer: a double-edged sword. *Protein Cell* (2014) **5**:12–20. doi:10.1007/s13238-013-0001-4
- Ye Z, Ting JP. NLR, the nucleotide-binding domain leucine-rich repeat containing gene family. *Curr Opin Immunol* (2008) **20**:3–9. doi:10.1016/j.co.2008.01.003
- Ting JP, Lovering RC, Alnemri ES, Bertin J, Boss JM, Davis BK, et al. The NLR gene family: a standard nomenclature. *Immunity* (2008) **28**:285–7. doi:10.1016/j.immuni.2008.02.005
- Nunes T, de Souza HS. Inflammasome in intestinal inflammation and cancer. *Mediators Inflamm* (2013) **2013**:654963. doi:10.1155/2013/654963
- Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol* (2004) **22**:329–60. doi:10.1146/annurev.immunol.22.012703.104803
- Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* (2001) **357**:539–45. doi:10.1016/S0140-6736(00)04046-0
- De Robertis M, Massi E, Poeta ML, Carotti S, Morini S, Cecchetelli L, et al. The AOM/DSS murine model for the study of colon carcinogenesis: from pathways to diagnosis and therapy studies. *J Carcinog* (2011) **10**:9. doi:10.4103/1477-3163.78279
- Normand S, Delanoye-Crespin A, Bressenot A, Huot L, Grandjean T, Peyrin-Biroulet L, et al. NOD-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proc Natl Acad Sci U S A* (2011) **108**:9601–6. doi:10.1073/pnas.1100981108
- Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, Booth CJ, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* (2011) **145**:745–57. doi:10.1016/j.cell.2011.04.022
- Zaki MH, Vogel P, Body-Malapel M, Lamkanfi M, Kanneganti TD. IL-18 production downstream of the Nlrp3 inflammasome confers protection against colorectal tumor formation. *J Immunol* (2010) **185**:4912–20. doi:10.4049/jimmunol.1002046
- Allen IC, Tekippe EM, Woodford RM, Uronis JM, Holl EK, Rogers AB, et al. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med* (2010) **207**:1045–56. doi:10.1084/jem.20100050
- Hu B, Elinav E, Flavell RA. Inflammasome-mediated suppression of inflammation-induced colorectal cancer progression is mediated by direct regulation of epithelial cell proliferation. *Cell Cycle* (2011) **10**:1936–9. doi:10.4161/cc.10.12.16008
- Werts C, Rubino S, Ling A, Girardin SE, Philpott DJ. NOD-like receptors in intestinal homeostasis, inflammation, and cancer. *J Leukoc Biol* (2011) **90**:471–82. doi:10.1189/jlb.0411183
- Zaki MH, Lamkanfi M, Kanneganti TD. The Nlrp3 inflammasome: contributions to intestinal homeostasis. *Trends Immunol* (2011) **32**:171–9. doi:10.1016/j.it.2011.02.002
- Zaki MH, Vogel P, Malireddi RK, Body-Malapel M, Anand PK, Bertin J, et al. The NOD-like receptor NLRP12 attenuates colon inflammation and tumorigenesis. *Cancer Cell* (2011) **20**:649–60. doi:10.1016/j.ccr.2011.10.022

24. Allen IC, Wilson JE, Schneider M, Lich JD, Roberts RA, Arthur JC, et al. NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-kappaB signaling. *Immunity* (2012) **36**:742–54. doi:10.1016/j.jimmuni.2012.03.012
25. Dupaul-Chicoine J, Yeretssian G, Doiron K, Bergstrom KS, McIntire CR, Leblanc PM, et al. Control of intestinal homeostasis, colitis, and colitis-associated colorectal cancer by the inflammatory caspases. *Immunity* (2010) **32**:367–78. doi:10.1016/j.jimmuni.2010.02.012
26. Nagash AA, Ramos HJ, Crochet N, Lau DT, Doehle B, Papic N, et al. IL-1beta production through the NLRP3 inflammasome by hepatic macrophages links hepatitis C virus infection with liver inflammation and disease. *PLoS Pathog* (2013) **9**:e1003330. doi:10.1371/journal.ppat.1003330
27. Watanabe A, Sohail MA, Gomez DA, Hashmi A, Nagata J, Sutterwala FS, et al. Inflammasome-mediated regulation of hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* (2009) **296**:G1248–57. doi:10.1152/ajpgi.90223.2008
28. Dunn JH, Ellis LZ, Fujita M. Inflammasomes as molecular mediators of inflammation and cancer: potential role in melanoma. *Cancer Lett* (2012) **314**:24–33. doi:10.1016/j.canlet.2011.10.001
29. Reed JR, Leon RP, Hall MK, Schwertfeger KL. Interleukin-1beta and fibroblast growth factor receptor 1 cooperate to induce cyclooxygenase-2 during early mammary tumorigenesis. *Breast Cancer Res* (2009) **11**:R21. doi:10.1186/bcr2246
30. Tu S, Bhagat G, Cui G, Takaishi S, Kurt-Jones EA, Rickman B, et al. Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. *Cancer Cell* (2008) **14**:408–19. doi:10.1016/j.ccr.2008.10.011
31. Drexler SK, Bonsignore L, Masin M, Tardivel A, Jackstadt R, Hermeking H, et al. Tissue-specific opposing functions of the inflammasome adaptor ASC in the regulation of epithelial skin carcinogenesis. *Proc Natl Acad Sci U S A* (2012) **109**:18384–9. doi:10.1073/pnas.1209171109
32. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* (2002) **3**:991–8. doi:10.1038/ni1102-991
33. Smyth MJ, Thia KY, Street SE, Macgregor D, Godfrey DI, Trapani JA. Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *J Exp Med* (2000) **192**:755–60. doi:10.1084/jem.192.5.755
34. Street SE, Hayakawa Y, Zhan Y, Lew AM, Macgregor D, Jamieson AM, et al. Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells. *J Exp Med* (2004) **199**:879–84. doi:10.1084/jem.20031981
35. Bui JD, Schreiber RD. Cancer immuno-surveillance, immunoediting and inflammation: independent or interdependent processes? *Curr Opin Immunol* (2007) **19**:203–8. doi:10.1016/j.coim.2007.02.001
36. Croxford JL, Tang ML, Pan MF, Huang CW, Kamran N, Phua CM, et al. ATM-dependent spontaneous regression of early Emu-myc-induced murine B-cell leukemia depends on natural killer and T cells. *Blood* (2013) **121**:2512–21. doi:10.1182/blood-2012-08-449025
37. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med* (2009) **15**:1170–8. doi:10.1038/nm.2028
38. Asano K, Nabeyama A, Miyake Y, Qiu CH, Kurita A, Tomura M, et al. CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. *Immunity* (2011) **34**:85–95. doi:10.1016/j.jimmuni.2010.12.011
39. Huang TT, Ojcius DM, Young JD, Wu YH, Ko YF, Wong TY, et al. The anti-tumorigenic mushroom *Agaricus blazei* Murill enhances IL-1beta production and activates the NLRP3 inflammasome in human macrophages. *PLoS One* (2012) **7**:e41383. doi:10.1371/journal.pone.0041383
40. Garaude J, Kent A, Van Rooijen N, Blander JM. Simultaneous targeting of toll- and NOD-like receptors induces effective tumor-specific immune responses. *Sci Transl Med* (2012) **4**:120ra116. doi:10.1126/scitranslmed.3002868
41. Kikuchi T, Akasaki Y, Joki T, Abe T, Kurimoto M, Ohno T. Antitumor activity of interleukin-18 on mouse glioma cells. *J Immunother* (2000) **23**:184–9. doi:10.1097/00002371-200003000-00002
42. Hashimoto W, Tanaka F, Robbins PD, Taniguchi M, Okamura H, Lotze MT, et al. Natural killer, but not natural killer T, cells play a necessary role in the promotion of an innate antitumor response induced by IL-18. *Int J Cancer* (2003) **103**:508–13. doi:10.1002/ijc.10844
43. Nishio S, Yamada N, Ohyama H, Yamanegi K, Nakasho K, Hata M, et al. Enhanced suppression of pulmonary metastasis of malignant melanoma cells by combined administration of alpha-galactosylceramide and interleukin-18. *Cancer Sci* (2008) **99**:113–20. doi:10.1111/j.1349-7006.2007.00636.x
44. Zheng JN, Pei DS, Mao LJ, Liu XY, Sun FH, Zhang BF, et al. Oncolytic adenovirus expressing interleukin-18 induces significant antitumor effects against melanoma in mice through inhibition of angiogenesis. *Cancer Gene Ther* (2010) **17**:28–36. doi:10.1038/cgt.2009.38
45. Teng MW, Swann JB, Koebel CM, Schreiber RD, Smyth MJ. Immune-mediated dormancy: an equilibrium with cancer. *J Leukoc Biol* (2008) **84**:988–93. doi:10.1189/jlb.1107774
46. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) **144**:646–74. doi:10.1016/j.cell.2011.02.013
47. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* (1986) **315**:1650–9. doi:10.1056/NEJM198612253152606
48. Schafer M, Werner S. Cancer as an overhealing wound: an old hypothesis revisited. *Nat Rev Mol Cell Biol* (2008) **9**:628–38. doi:10.1038/nrm2455
49. Tyler DS, Francis GM, Frederick M, Tran AH, Ordóñez NG, Smith JL, et al. Interleukin-1 production in tumor cells of human melanoma surgical specimens. *J Interferon Cytokine Res* (1995) **15**:331–40. doi:10.1089/jir.1995.15.331
50. Elaraj DM, Weinreich DM, Varghese S, Puhlmann M, Hewitt SM, Carroll NM, et al. The role of interleukin 1 in growth and metastasis of human cancer xenografts. *Clin Cancer Res* (2006) **12**:1088–96. doi:10.1158/1078-0432.CCR-05-1603
51. Okamoto M, Liu W, Luo Y, Tanaka A, Cai X, Norris DA, et al. Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1 beta. *J Biol Chem* (2010) **285**:6477–88. doi:10.1074/jbc.M109.064907
52. Knowles H, Leek R, Harris AL. Macrophage infiltration and angiogenesis in human malignancy. *Novartis Found Symp* (2004) **256**:189–200. doi:10.1002/0470856734.ch14
53. Vidal-Vanaclocha F, Fantuzzi G, Mendoza L, Fuentes AM, Anasagasti MJ, Martin J, et al. IL-18 regulates IL-1beta-dependent hepatic melanoma metastasis via vascular cell adhesion molecule-1. *Proc Natl Acad Sci U S A* (2000) **97**:734–9. doi:10.1073/pnas.97.2.734
54. Cho D, Song H, Kim YM, Houh D, Hur DY, Park H, et al. Endogenous interleukin-18 modulates immune escape of murine melanoma cells by regulating the expression of Fas ligand and reactive oxygen intermediates. *Cancer Res* (2000) **60**:2703–9.
55. Terme M, Ullrich E, Aymeric L, Meinhardt K, Desbois M, Delahaye N, et al. IL-18 induces PD-1-dependent immunosuppression in cancer. *Cancer Res* (2011) **71**:5393–9. doi:10.1158/0008-5472.CAN-11-0993
56. van Deventer HW, Burgents JE, Wu QP, Woodford RM, Brickey WJ, Allen IC, et al. The inflammasome component NLRP3 impairs antitumor vaccine by enhancing the accumulation of tumor-associated myeloid-derived suppressor cells. *Cancer Res* (2010) **70**:10161–9. doi:10.1158/0008-5472.CAN-10-1921
57. Bruchard M, Mignot G, Derangere V, Chalmin F, Chevriaux A, Vegran F, et al. Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth. *Nat Med* (2013) **19**:57–64. doi:10.1038/nm.2999

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Non-inflammasome forming NLRs in inflammation and tumorigenesis

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Aberrant inflammation is an enabling characteristic of tumorigenesis. Thus, signaling cascades that alter inflammatory activation and resolution are of specific relevance to disease pathogenesis. Pattern recognition receptors (PRRs) are essential mediators of the host immune response and have emerged as critical elements affecting multiple facets of tumor pathobiology. The nucleotide-binding domain and leucine-rich repeat containing (NLR) proteins are intracellular PRRs that sense microbial and non-microbial products. Members of the NLR family can be divided into functional sub-groups based on their ability to either positively or negatively regulate the host immune response. Recent studies have identified a novel sub-group of non-inflammasome forming NLRs that negatively regulate diverse biological pathways associated with both inflammation and tumorigenesis. Understanding the mechanisms underlying the function of these unique NLRs will assist in the rationale design of future therapeutic strategies targeting a wide spectrum of inflammatory diseases and cancer. Here, we will discuss recent findings associated with this novel NLR sub-group and mechanisms by which these PRRs may function to alter cancer pathogenesis.

Keywords: Nod-like receptors, NLRP12, NLRX1, NLRC3, NF- κ B, TRAF, cancer, pattern recognition receptors

INTRODUCTION

The intimate association between inflammation and cancer was first noted over 150 years ago by Rudolf Virchow (1, 2). Indeed today, aberrant inflammation is considered both an emerging hallmark of tumorigenesis and an enabling characteristic of cancer (3). Tumorigenesis is a multistep process and inflammation functions at multiple levels to both antagonize and enhance tumor initiation and progression (3). During the early stages of tumorigenesis, an inflammatory microenvironment serves as an enabling characteristic to activate diverse signaling pathways and drive the progression of pre-malignant and malignant lesions toward cancer (3–5). In later stages, cancer cells typically acquire a diverse repertoire of defense mechanisms that allow the cells to both passively and actively evade immune surveillance and elimination (3, 6, 7). This immune system subversion is an emerging hallmark of cancer and serves to remove the most effective barriers employed by the host to defend against neoplasia, late-stage tumor, and micro-metastasis progression (3).

Pattern recognition receptors (PRRs) are an essential component of the host immune system and significantly contribute to cancer pathobiology. There are 4 major families of PRRs that have been implicated in tumorigenesis, including the toll-like receptors (TLRs), the nucleotide-binding domain and leucine-rich repeat containing (NLR) family of sensors, C-type lectin receptors (CLRs), and RIG-I-like receptors (RLRs) (8). These receptor families function to initiate inflammatory signaling cascades following the direct or indirect recognition of pathogens, damage and stress through sensing highly conserved pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns

(DAMPs). In addition to their roles in facilitating the immune response, PRRs also play fundamental roles in the regulation of proliferation, cell survival and death, reactive oxygen species generation, angiogenesis, and tissue remodeling and repair (8). In the context of cancer, PRRs drive the immune response following exposure to potentially carcinogenic pathogens, environmental exposures to mutagenic agents and insults, and cancer-associated cellular damage and stress (9–16). In general, increased PRR signaling creates an enriched, pro-inflammatory microenvironment that is favorable for tumor initiation and progression (17). Thus, we find that PRRs are stuck in a “Goldilocks Conundrum.” Robust PRR activation is critical in driving the host immune response following PAMP and DAMP exposure; whereas, an overzealous and persistent immune response driven by PRR activation can cause significant collateral damage to the host tissue that ultimately results in chronic inflammation and cancer.

To date, the majority of studies evaluating PRR signaling in cancer have focused on members of the TLR family. However, new and emerging findings have revealed a significant role for members of the NLR family in contributing either directly or indirectly to a variety of hallmarks associated with cancer, including inflammation, cell death, tumor growth, angiogenesis, invasion, and metastasis (18–26). There are at least 23 distinct NLR and NLR-like proteins that have been identified in humans and 34 family members identified in mice (23, 27–29). The NLR proteins function as cytosolic receptors and sensors to detect intracellular PAMPs and DAMPs. Since their discovery, a variety of names have been used to describe the members of this gene family and their respective proteins. For example, these PRRs

have been previously referred to as CATERPILLERs, NOD-like receptors, NACHT-leucine-rich repeats (LRR), and NBD-LRR proteins (28). This resulted in a lack of consistency in the field and resulted in the currently accepted and standardized nomenclature defining the NLRs as the NLR gene family (28). These proteins contain a highly conserved tripartite domain structure (28). The N-terminal domain of the protein is comprised of a variable, but limited number of effector domains that can include combinations of acidic transactivation domains (NLRA proteins), baculoviral inhibitory repeat (BIR)-like domains (NLRB proteins), caspase recruitment domains (NLRC proteins), and pyrin domains (NLRP proteins) (28). These N-terminal domains function to recruit adaptor, intermediary, or effector molecules that drive downstream signaling. The core of the protein is comprised of a conserved NACHT nucleotide-binding domain, which facilitates oligomerization (28). The C-terminal domain of the protein contains multiple LRR elements, which are essential for ligand sensing (28). Each LRR element is typically 28–29 residues in length and each NLR may contain up to 33 individual LRR elements (30, 31).

INFLAMMASOME FORMING NLRs IN CANCER

One of the most fundamental roles of the NLR family is to regulate pro-inflammatory cytokines and chemokines that drive the host innate immune response to pathogens and environmental insults. Key to this response is the proper regulation of IL-1 β and IL-18, which are both potent pro-inflammatory cytokines that affect diverse aspects of health and disease (32–37). Both of these cytokines are generated in an immature pro-form that requires post-translational cleavage for activation. A functional sub-group of NLRs has been identified as driving this process through the formation of a multi-protein complex termed the inflammasome (32, 35, 36). Upon activation, the NLR is thought to undergo a conformational change that allows the recruitment and binding of adaptor and effector proteins and inflammasome formation (35). The inflammasome is composed of an NLR that recognizes a specific repertoire of PAMPs and DAMPs, the adaptor protein ASC, and pro-Caspase-1 (32). These sub-units continue to multiplex, ultimately resulting in the maturation and activation of Caspase-1, which subsequently drives the cleavage and activation of IL-1 β and IL-18. These inflammasome forming NLRs are by far the best characterized and most highly studied members of the NLR family. To date, at least 6 NLR and NLR-like proteins have been strongly implicated in inflammasome formation, including NLRP1, NLRP3, NLRP6, NLRC4, NLRC5, and the PYHIN family member AIM2 (NLR-like) (32–37). Inflammasome forming NLRs significantly regulate the tumor microenvironment by modulating cytokine production. For example, many of the inflammasome forming NLRs have been shown to significantly attenuate inflammation and tumorigenesis in mouse models of colitis-associated colorectal cancer (CAC) by regulating IL-18 production (18, 19, 21, 22, 38–40). In addition to being a potent pro-inflammatory cytokine, IL-18 is also secreted by epithelial cells to stimulate regeneration and repair and improve barrier function in the colon, thus loss of this cytokine in NLR inflammasome deficient mice enhances tumorigenesis (41). Beyond colon cancer, NLR inflammasome activation may

also play important roles in many other types of cancer, including breast cancer, skin cancers, and virus-associated hepatocellular carcinoma (25, 26, 42–47).

NON-INFLAMMASOME FORMING NLRs THAT NEGATIVELY REGULATE INFLAMMATION

While the inflammasome forming NLRs are the best characterized members of this PRR family, recent studies have identified a functional sub-group of NLRs that negatively regulate inflammation (48–54). This sub-group is currently composed of three NLR family members, NLRP12, NLRX1, and NLRC3 (Figure 1). NLRP12 was one of the first NLR proteins to be described and is the best characterized member of this functional NLR sub-group. NLRP12 was previously known as monarch-1 and PYPAF7 and was originally suggested to form an inflammasome with ASC in overexpression systems (55, 56). In these overexpression studies, transient transfection of NLRP12 and ASC was also shown to induce the transcription of an NF- κ B reporter construct (56). Thus, these early *in vitro* studies initially suggested that NLRP12 was an inflammasome forming NLR and a positive regulator of NF- κ B signaling. These findings are also consistent with human data that has identified mutations in NLRP12 linked to a spectrum of hereditary periodic fever syndromes. The disorders associated with *NLRP12* mutations are characterized by redox alterations and enhanced secretion of IL-1 β , which are similar to the characteristics associated with the family of diseases linked to gain-of-function mutations in the *NLRP3* gene (57–59). Interestingly, these diseases are associated with increased caspase-1 activity, are sensitive to therapeutics targeting IL-1 β (anakinra), and appear to be independent of NF- κ B activation (57–59). However, the ability of NLRP12 to form a functional inflammasome under physiological situations and in the context of human disease appears to occur only under highly specific conditions and is an area of current investigation (60, 61). Indeed, several studies have evaluated NLRP12 inflammasome formation *ex vivo* and using *Nlrp12*^{-/-} mice under a variety of conditions and have directly shown that this NLR does not regulate IL-1 β /IL-18 maturation (62–69). The prevailing literature associated with NLRP12 indicates that this protein functions as a negative regulator of inflammation by modulating canonical and non-canonical NF- κ B signaling (48, 49, 62, 65, 66, 68, 70–73). NLRP12 negatively regulates non-canonical NF- κ B signaling through its association with TRAF3 and NF- κ B inducing kinase (NIK) (49, 68). This interaction leads to the degradation of NIK and subsequent attenuation of p100 cleavage to p52 (Figure 1). Similarly, NLRP12 attenuates canonical NF- κ B signaling through the inhibition of IRAK-1 phosphorylation (48, 66, 71) (Figure 1). In addition to directly mediating the NF- κ B cascade, NLRP12 has also been shown to attenuate ERK signaling, though the exact mechanism has yet to be fully resolved (66, 68). Thus, while some conflicting data has been reported, most issues can be resolved by considering the technical limitations of the assays used to define the respective mechanisms and the specific models being evaluated.

NLRX1 was originally characterized in 2008, and was shown to negatively regulate the host anti-viral immune response (51). NLRX1 is unique among the NLRs due to its mitochondrial localization and its relatively undefined N-terminal domain. Similar

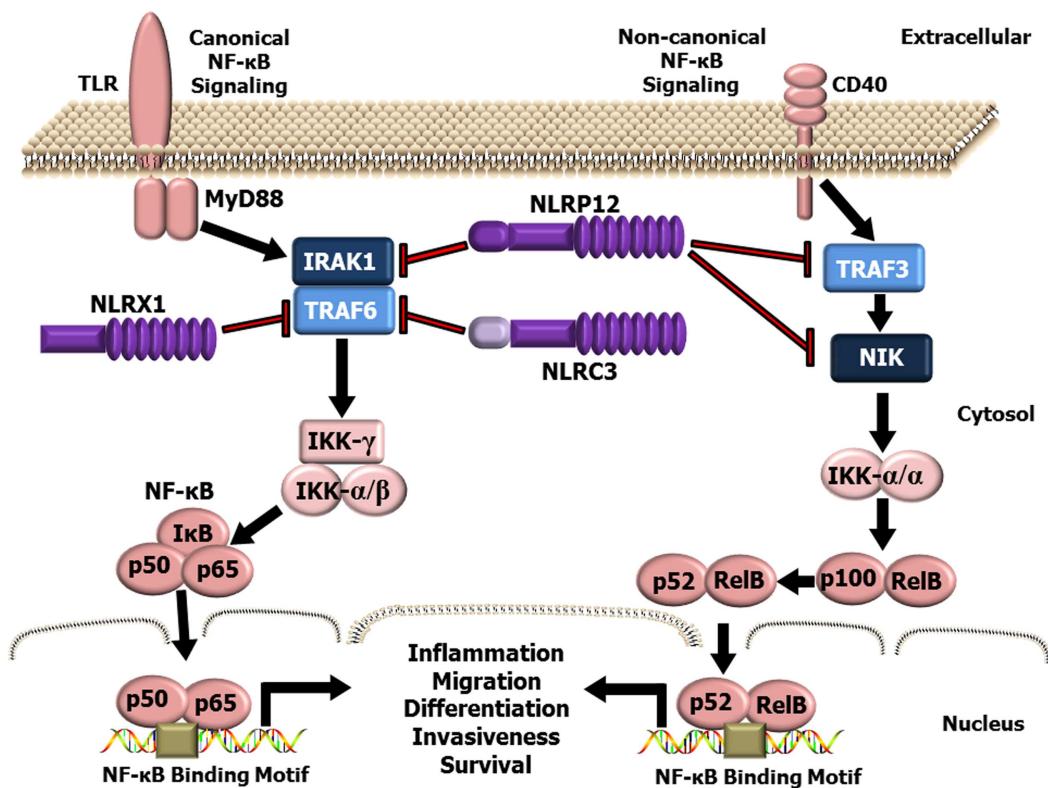


FIGURE 1 | Schematic illustrating NLR attenuation of canonical and non-canonical NF- κ B signaling. NF- κ B is a master regulator of gene transcription and contributes to several hallmarks of cancer. NLRX1, NLRP12, and NLRC3 negatively regulate NF- κ B signaling at multiple levels. NLRX1 interacts with and inhibits TRAF6 and the IKK complex resulting in the attenuation of NF- κ B signaling following TLR

stimulation. Likewise, NLRC3 was also shown to interact with TRAF6 and attenuate NF- κ B signaling through a similar mechanism. NLRP12, has been shown to attenuate both the canonical NF- κ B signaling pathway through modulating the phosphorylation of IRAK-1 and the non-canonical NF- κ B pathway through interactions with TRAF3 and NIK.

to NLRP12, NLRX1 negatively regulates canonical NF- κ B signaling (50, 52) (Figure 1). NLRX1 associates with TRAF6 and I κ B kinase (IKK) through an activation signal-dependent mechanism (50). Following stimulation, NLRX1 is rapidly ubiquitinated and disassociates from TRAF6 to bind the IKK complex and inhibit subsequent canonical NF- κ B activation (50). In addition to attenuating NF- κ B signaling, NLRX1 also negatively regulates type-I interferon (IFN-I) signaling through inhibiting the interaction between the PRR Rig-I and the mitochondrial anti-viral signaling (MAVS) protein following virus exposure (50–52, 74, 75) (Figure 2). NLRX1 also functions as a positive regulator of autophagy following virus exposure through interacting with the protein TUFM and the mitochondrial immune signaling complex (MISC), which also includes ATG5, ATG12, and ATG16L1 (74, 75) (Figure 2). Interestingly, autophagy also functions as a negative regulator of IFN-I signaling and provides an additional route for the negative regulatory properties of NLRX1. In addition to regulating NF- κ B and IFN-I signaling, subsequent studies have also shown that NLRX1 functions as a positive regulator of ROS production in epithelial cells following *Chlamydia trachomatis* infection, likely through interactions with the UQCRC2 protein (76, 77) (Figure 2). Thus, it is clear that NLRX1 regulation is quite

complex and appears to occur through cell type, temporal and signal-dependent mechanisms.

NLRC3 is the most recently characterized member of this functional sub-group and has been shown to negatively regulate NF- κ B and IFN-I signaling (54, 78). NLRC3 was originally identified as a negative regulator of T cell function, in part through delaying the degradation of I κ B α (78). Subsequent studies have since revealed that NLRC3 attenuates TLR signaling through interacting with and modulating TRAF6 activity and inhibiting canonical NF- κ B signaling (54). NLRC3 has also been recently shown to fine tune the host innate immune response to intracellular DNA, DNA viruses, and c-di-GMP (53). NLRC3 impedes STING-TANK-binding kinase 1 (TBK1) interactions and inhibits STING trafficking, which results in an attenuation of subsequent downstream activation of IFN-I genes (53).

While NLRP12, NLRX1, and NLRC3 each influence a variety of signaling pathways, the convergence on NF- κ B signaling appears to be a common strategy among the NLRs in this functional sub-group to attenuate inflammation (Figure 1). Additional mechanistic studies have revealed prevalent NLR-TRAF interactions in these models and support the emerging hypothesis that these NLRs function to inhibit NF- κ B signaling through the

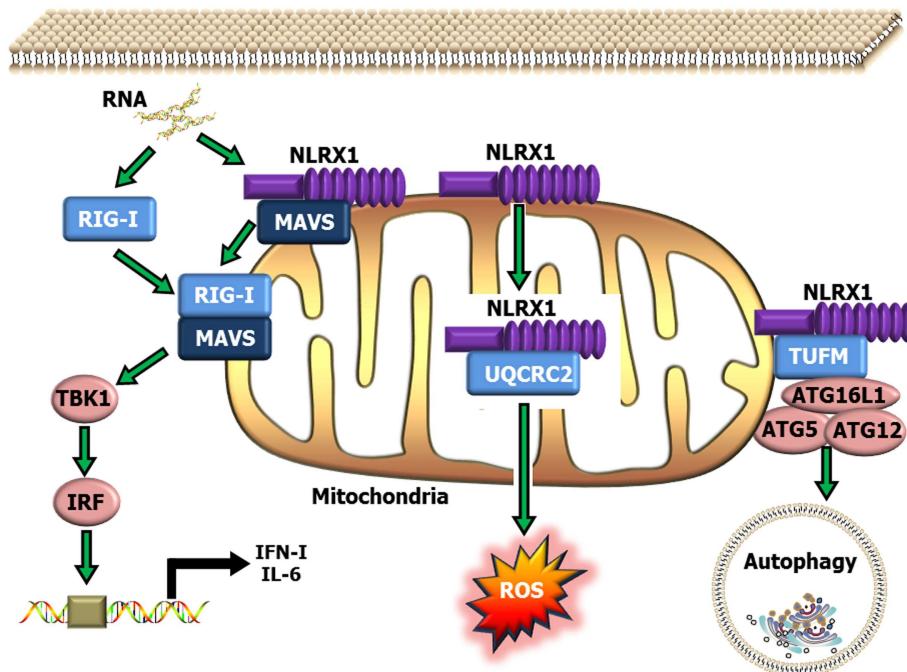


FIGURE 2 | Schematic illustrating NLRX1 regulation of type-I interferon, ROS and autophagy signaling. NLRX1 is localized to the mitochondria, where it has been shown to bind with MAVS and prevent the interaction between MAVS and RIG-I during the host anti-viral response. This interaction significantly attenuates MAVS activation of IRF3 and IRF7 and results in reduced IFN and IL-6 signaling. NLRX1 has also been shown to function as a positive regulator of autophagy through its interactions with the mitochondrial

protein TUFM, and the mitochondrial immune signaling complex (MISC), which includes Atg5–Atg12 and ATG16L1. This complex has been shown to be important in promoting virus-induced autophagy and concurrently attenuating IFN signaling. In addition to its role in attenuating host anti-viral signaling, NLRX1 has also been shown to significantly augment ROS generation from the mitochondria through interactions with UQCRC2 following infection with specific species of bacteria.

formation of a multi-protein “TRAFasome” complex (54). Dysregulated NF- κ B signaling and the additional pathways modulated by these NLRs are critical features in cancer initiation and progression. Thus, the NLRs that modulate these signaling cascades are highly relevant to cancer pathobiology and additional mechanistic insight will be critical for developing future therapeutic strategies.

NEGATIVE REGULATORY NLRs IN CANCER PATHOBIOLOGY

While several studies have characterized the contribution of the NLRP3, NLRC4, and NLRP6 inflammasomes in tumorigenesis, significantly less is known regarding the role of NLRs that negatively regulate inflammation. Initial studies have focused on NLRP12. In the context of cancer, somatic mutations in human *NLRP12* have been detected in several large scale screening studies evaluating a variety of cancer sub-types, including glioblastoma, breast cancer, lung squamous cell carcinoma, melanoma, prostate adenocarcinoma, and colon adenocarcinoma (<http://cancergenome.nih.gov/>). However, broader linkage with specific populations, causation, and mechanism for each mutation has not yet been established. In mice, NLRP12 has been shown to attenuate colorectal cancer. Using the AOM/DSS model of CAC, *Nlrp12*^{-/-} mice were shown to develop increased inflammation and tumorigenesis (66, 68). Colon histopathology revealed significant epithelial cell damage and loss of barrier integrity in these

animals, which resulted in increased pro-inflammatory cytokine and chemokine production (66, 68). These animals eventually develop extensive pre-cancerous lesions, which result in significantly increased areas of hyperplasia, dysplasia, and adenocarcinoma (66, 68). These studies revealed that NLRP12 attenuates inflammation and tumorigenesis through negatively regulating NF- κ B and ERK signaling (66, 68).

While the overall results of each study are quite complementary, it should be noted that a few mechanistic differences were proposed. In one study, the increased tumorigenesis was attributed to an increase in canonical NF- κ B signaling (66). NF- κ B signaling was evaluated *in vivo* and in macrophages isolated from wild type and *Nlrp12*^{-/-} mice following PAMP stimulation and a significant increase in the levels of p-p105, Rel-A, and p65 activity was observed (66). Furthermore, loss of NLRP12 was shown to significantly increase the transcription of a variety of pro-inflammatory mediators associated with canonical NF- κ B signaling and colon tumorigenesis, including *Il-6*, *Tnf- α* , and *Cox2* (66). These findings are consistent with earlier *in vitro* studies, which demonstrated that NLRP12 functions as an antagonist of TLR and TNFR-induced pro-inflammatory signals, in part through inhibiting IRAK-1 hyper-phosphorylation (48). In the second study, NLRP12 was shown to attenuate colon tumorigenesis through negatively regulating non-canonical NF- κ B signaling. While some markers of canonical NF- κ B signaling were found to be transiently increased

in the absence of NLRP12, this study revealed a significant increase in NIK activation and p100 to p52 cleavage in primary cells and in colon tissues isolated from *Nlrp12*^{-/-} mice during disease progression (68). These data are highly consistent with previous *in vitro* studies associating NLRP12 activity with NIK suppression and attenuation of non-canonical NF-κB signaling (49, 79). Loss of NLRP12 resulted in a significant increase in *Cxcl12* and *Cxcl13* expression in the colons from *Nlrp12*^{-/-} mice (68). These chemokines are highly associated with non-canonical NF-κB activation and cancer (49, 68, 80–82). CXCL12 (SDF-1) and CXCL13 (BLC) and their respective receptors CXCR4 and CXCR5 have been implicated in tumor growth, metastasis, and are critical for the regulation of the tumor microenvironment in multiple cancer sub-types as a component of the tumor “Immunome” (3, 83–85). Regulation of the NF-κB signaling pathway is highly complex. The apparent discrepancies between these two studies can be reconciled by previous findings, which show that non-canonical NF-κB signaling can influence both the canonical pathway and MAPK signaling (86, 87). It is also highly likely that NLRP12 regulates canonical and non-canonical NF-κB signaling through currently undefined cell type, temporal and/or stimuli-specific mechanisms.

To date, neither NLRX1 nor NLRC3 have been directly evaluated in the context of cancer. As previously stated, both of these NLRs negatively regulate NF-κB signaling and would be expected to attenuate tumorigenesis through mechanisms similar to those described for NLRP12. However, each also regulates pathways other than NF-κB that could dramatically influence cancer pathobiology. For example, NLRX1 has been shown to additionally regulate ROS production and autophagy. The dysregulation of oxidative stress signaling is a well-established and important element of tumor development (88). Similarly, autophagy is thought to have a dual function in cancer, where it can attenuate tumor initiation by suppressing tissue damage and inflammation signaling or it can function as a tumor promoter to sustain metabolism, growth, and survival through metabolite recycling (89, 90). Thus, it is highly likely that NLRX1 will contribute to tumorigenesis; however, it is difficult to speculate which of its many biologic functions will have a greater influence on disease pathogenesis.

CONCLUSION

The recent characterization of this unique sub-group of NLRs that function to attenuate inflammation emphasizes the point that a significant number of the identified NLR proteins in humans have yet to be adequately characterized. Identifying the unique regulatory and signaling pathways modulated by these NLRs is an essential step toward ultimately developing effective therapeutics targeting these proteins and the pathways they modulate. Characterizing unidentified ligands, cell type and temporal regulatory mechanisms, and redundant functions of these NLR family members will significantly improve our understanding of the contribution of these proteins in maintaining immune system homeostasis. It is also clear that NLRs significantly impact cancer pathobiology, beyond colorectal cancer. Additional studies are necessary to better define the contribution of both inflammasome forming NLRs and non-inflammasome forming NLRs in modulating the hallmarks of cancer.

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REFERENCES

- Trinchieri G. Cancer and inflammation: an old intuition with rapidly evolving new concepts. *Annu Rev Immunol* (2012) **30**:677–706. doi:10.1146/annurev-immunol-020711-075008
- Heidland A, Klassen A, Rutkowski P, Bahner U. The contribution of Rudolf Virchow to the concept of inflammation: what is still of importance? *J Nephrol* (2006) **19**(Suppl 10):S102–9.
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) **144**:646–74. doi:10.1016/j.cell.2011.02.013
- Cook J, Hagemann T. Tumour-associated macrophages and cancer. *Curr Opin Pharmacol* (2013) **13**:595–601. doi:10.1016/j.coph.2013.05.017
- Dutsch-Wicherek M, Kazmierczak W. Creation of a suppressive microenvironment by macrophages and cancer-associated fibroblasts. *Front Biosci (Landmark Ed)* (2013) **18**:1003–16. doi:10.2741/4159
- Marcus A, Gowen BG, Thompson TW, Iannello A, Ardolino M, Deng W, et al. Recognition of tumors by the innate immune system and natural killer cells. *Adv Immunol* (2014) **122**:91–128. doi:10.1016/B978-0-12-800267-4.00003-1
- Biragyn A, Longo DL. Neoplastic “Black Ops”: cancer’s subversive tactics in overcoming host defenses. *Semin Cancer Biol* (2012) **22**:50–9. doi:10.1016/j.semancer.2012.01.005
- Kutikhin AG, Yuzhalin AE. Inherited variation in pattern recognition receptors and cancer: dangerous liaisons? *Cancer Manag Res* (2012) **4**:31–8. doi:10.2147/CMAR.S28688
- Dostert C, Petrilli V, Van Bruggen R, Steele C, Mossman BT, Tschoch J. Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* (2008) **320**:674–7. doi:10.1126/science.1156995
- Cassel SL, Eisenbarth SC, Iyer SS, Sadler JJ, Colegio OR, Tephly LA, et al. The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A* (2008) **105**:9035–40. doi:10.1073/pnas.0803933105
- Feldmeyer L, Keller M, Niklaus G, Hohl D, Werner S, Beer HD. The inflammasome mediates UVB-induced activation and secretion of interleukin-1beta by keratinocytes. *Curr Biol* (2007) **17**:1140–5. doi:10.1016/j.cub.2007.05.074
- Hong S, Hwang I, Lee YS, Park S, Lee WK, Fernandes-Alnemri T, et al. Restoration of ASC expression sensitizes colorectal cancer cells to genotoxic stress-induced caspase-independent cell death. *Cancer Lett* (2013) **331**:183–91. doi:10.1016/j.canlet.2012.12.020
- Gregory SM, Davis BK, West JA, Taxman DJ, Matsuzawa S, Reed JC, et al. Discovery of a viral NLR homolog that inhibits the inflammasome. *Science* (2011) **331**:330–4. doi:10.1126/science.1199478
- Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, et al. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat Immunol* (2004) **5**:1166–74. doi:10.1038/ni1131
- Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem* (1999) **274**:17406–9. doi:10.1074/jbc.274.25.17406
- Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via toll-like receptor 2. *J Immunol* (1999) **163**:1–5.
- Kipanyula MJ, Seke Etet PF, Vecchio L, Farahna M, Nukenine EN, Nwabo Kamdje AH. Signaling pathways bridging microbial-triggered inflammation and cancer. *Cell Signal* (2013) **25**:403–16. doi:10.1016/j.cellsig.2012.10.014
- Zaki MH, Boyd KL, Vogel P, Kastan MB, Lamkanfi M, Kanneganti TD. The NLRP3 inflammasome protects against loss of epithelial integrity and mortality during experimental colitis. *Immunity* (2010) **32**:379–91. doi:10.1016/j.immuni.2010.03.003
- Allen IC, TeKippe EM, Woodford RM, Uronis JM, Holl EK, Rogers AB, et al. The NLRP3 inflammasome functions as a negative regulator of tumorigenesis during colitis-associated cancer. *J Exp Med* (2010) **207**:1045–56. doi:10.1084/jem.20100050
- Bauer C, Duewell P, Mayer C, Lehr HA, Fitzgerald KA, Dauer M, et al. Colitis induced in mice with dextran sulfate sodium (DSS) is mediated by the NLRP3 inflammasome. *Gut* (2010) **59**:1192–9. doi:10.1136/gut.2009.197822

21. Hu B, Elinav E, Huber S, Booth CJ, Strowig T, Jin C, et al. Inflammation-induced tumorigenesis in the colon is regulated by caspase-1 and NLRC4. *Proc Natl Acad Sci U S A* (2010) **107**:21635–40. doi:10.1073/pnas.1016814108
22. Hu B, Elinav E, Huber S, Strowig T, Hao L, Hafemann A, et al. Microbiota-induced activation of epithelial IL-6 signaling links inflammasome-driven inflammation with transmissible cancer. *Proc Natl Acad Sci U S A* (2013) **110**:9862–7. doi:10.1073/pnas.1307575110
23. Schroder K, Tschopp J. The inflammasomes. *Cell* (2010) **140**:821–32. doi:10.1016/j.cell.2010.01.040
24. Liu W, Luo Y, Dunn JH, Norris DA, Dinarello CA, Fujita M. Dual role of apoptosis-associated speck-like protein containing a CARD (ASC) in tumorigenesis of human melanoma. *J Invest Dermatol* (2013) **133**:518–27. doi:10.1038/jid.2012.317
25. Drexler SK, Bonsignore L, Masin M, Tardivel A, Jackstadt R, Herremans H, et al. Tissue-specific opposing functions of the inflammasome adaptor ASC in the regulation of epithelial skin carcinogenesis. *Proc Natl Acad Sci U S A* (2012) **109**:18384–9. doi:10.1073/pnas.1209171109
26. Okamoto M, Liu W, Luo Y, Tanaka A, Cai X, Norris DA, et al. Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta. *J Biol Chem* (2010) **285**:6477–88. doi:10.1074/jbc.M109.064907
27. Ting JP, Davis BK. CATERPILLER: a novel gene family important in immunity, cell death, and diseases. *Annu Rev Immunol* (2005) **23**:387–414. doi:10.1146/annurev.immunol.23.021704.115616
28. Ting JP, Lovering RC, Alnemri ES, Bertin J, Boss JM, Davis BK, et al. The NLR gene family: a standard nomenclature. *Immunity* (2008) **28**:285–7. doi:10.1016/j.immuni.2008.02.005
29. Chen GY. Role of Nlrp6 and Nlrp12 in the maintenance of intestinal homeostasis. *Eur J Immunol* (2014) **44**:321–7. doi:10.1002/eji.201344135
30. Kobe B, Kajava AV. The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol* (2001) **11**:725–32. doi:10.1016/S0959-440X(01)00266-4
31. Motyan JA, Bagossi P, Benko S, Tozser J. A molecular model of the full-length human NOD-like receptor family CARD domain containing 5 (NLRC5) protein. *BMC Bioinformatics* (2013) **14**:275. doi:10.1186/1471-2105-14-275
32. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* (2004) **20**:319–25. doi:10.1016/S1074-7613(04)00046-9
33. Włodarska M, Thaiss CA, Nowarski R, Henao-Mejia J, Zhang JP, Brown EM, et al. NLRP6 inflammasome orchestrates the colonic host-microbial interface by regulating goblet cell mucus secretion. *Cell* (2014) **156**:1045–59. doi:10.1016/j.cell.2014.01.026
34. Davis BK, Roberts RA, Huang MT, Willingham SB, Conti BJ, Brickey WJ, et al. Cutting edge: NLRC5-dependent activation of the inflammasome. *J Immunol* (2011) **186**:1333–7. doi:10.4049/jimmunol.1003111
35. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* (2002) **10**:417–26. doi:10.1016/S1097-2765(02)00599-3
36. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* (2004) **430**:213–8. doi:10.1038/nature02664
37. Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, et al. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* (2009) **458**:514–8. doi:10.1038/nature07725
38. Zaki MH, Vogel P, Body-Malapel M, Lamkanfi M, Kanneganti TD. IL-18 production downstream of the Nlrp3 inflammasome confers protection against colorectal tumor formation. *J Immunol* (2010) **185**:4912–20. doi:10.4049/jimmunol.1002046
39. Normand S, Delanoye-Crespin A, Bressenot A, Huot L, Grandjean T, Peyrin-Biroulet L, et al. Nod-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proc Natl Acad Sci U S A* (2011) **108**:9601–6. doi:10.1073/pnas.1100981108
40. Chen GY, Liu M, Wang F, Bertin J, Nunez G. A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *J Immunol* (2011) **186**:7187–94. doi:10.4049/jimmunol.1100412
41. Elinav E, Strowig T, Kau AL, Henao-Mejia J, Thaiss CA, Booth CJ, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell* (2011) **145**:745–57. doi:10.1016/j.cell.2011.04.022
42. Burdette D, Haskett A, Presser L, McRae S, Iqbal J, Waris G. Hepatitis C virus activates interleukin-1beta via caspase-1-inflammasome complex. *J Gen Virol* (2012) **93**:235–46. doi:10.1099/vir.0.034033-0
43. Dunn JH, Ellis LZ, Fujita M. Inflammasomes as molecular mediators of inflammation and cancer: potential role in melanoma. *Cancer Lett* (2012) **314**:24–33. doi:10.1016/j.canlet.2011.10.001
44. Jin L, Yuan RQ, Fuchs A, Yao Y, Joseph A, Schwall R, et al. Expression of interleukin-1beta in human breast carcinoma. *Cancer* (1997) **80**:421–34. doi:10.1002/(SICI)1097-0142(19970801)80:3<421::AID-CNCR10>3.0.CO;2-Z
45. Negash AA, Ramos HJ, Crochet N, Lau DT, Doeble B, Papic N, et al. IL-1beta production through the NLRP3 inflammasome by hepatic macrophages links hepatitis C virus infection with liver inflammation and disease. *PLoS Pathog* (2013) **9**:e1003330. doi:10.1371/journal.ppat.1003330
46. Pantschenko AG, Pushkar I, Anderson KH, Wang Y, Miller LJ, Kurtzman SH, et al. The interleukin-1 family of cytokines and receptors in human breast cancer: implications for tumor progression. *Int J Oncol* (2003) **23**:269–84. doi:10.3892/ijo.23.2.269
47. Snoussi K, Strosberg AD, Bouaouina N, Ben Ahmed S, Chouchane L. Genetic variation in pro-inflammatory cytokines (interleukin-1beta, interleukin-1alpha and interleukin-6) associated with the aggressive forms, survival, and relapse prediction of breast carcinoma. *Eur Cytokine Netw* (2005) **16**:253–60.
48. Williams KL, Lich JD, Duncan JA, Reed W, Rallabhandi P, Moore C, et al. The CATERPILLER protein monarch-1 is an antagonist of toll-like receptor-, tumor necrosis factor alpha-, and *Mycobacterium tuberculosis*-induced pro-inflammatory signals. *J Biol Chem* (2005) **280**:39914–24. doi:10.1074/jbc.M502820200
49. Lich JD, Williams KL, Moore CB, Arthur JC, Davis BK, Taxman DJ, et al. Monarch-1 suppresses non-canonical NF-kappaB activation and p52-dependent chemokine expression in monocytes. *J Immunol* (2007) **178**:1256–60.
50. Xia X, Cui J, Wang HY, Zhu L, Matsueda S, Wang Q, et al. NLRX1 negatively regulates TLR-induced NF-kappaB signaling by targeting TRAF6 and IKK. *Immunity* (2011) **34**:843–53. doi:10.1016/j.immuni.2011.02.022
51. Moore CB, Bergstrahl DT, Duncan JA, Lei Y, Morrison TE, Zimmermann AG, et al. NLRX1 is a regulator of mitochondrial antiviral immunity. *Nature* (2008) **451**:573–7. doi:10.1038/nature06501
52. Allen IC, Moore CB, Schneider M, Lei Y, Davis BK, Scull MA, et al. NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-I-MAVS and TRAF6-NF-kappaB signaling pathways. *Immunity* (2011) **34**:854–65. doi:10.1016/j.immuni.2011.03.026
53. Zhang L, Mo J, Swanson KV, Wen H, Petruccelli A, Gregory SM, et al. NLRC3, a member of the NLR family of proteins, is a negative regulator of innate immune signaling induced by the DNA sensor STING. *Immunity* (2014) **40**(3):329–41. doi:10.1016/j.immuni.2014.01.010
54. Schneider M, Zimmermann AG, Roberts RA, Zhang L, Swanson KV, Wen H, et al. The innate immune sensor NLRC3 attenuates toll-like receptor signaling via modification of the signaling adaptor TRAF6 and transcription factor NF-kappaB. *Nat Immunol* (2012) **13**:823–31. doi:10.1038/ni.2378
55. Williams KL, Taxman DJ, Linhoff MW, Reed W, Ting JP. Cutting edge: monarch-1: a pyrin/nucleotide-binding domain/leucine-rich repeat protein that controls classical and nonclassical MHC class I genes. *J Immunol* (2003) **170**:5354–8.
56. Wang L, Manji GA, Grenier JM, Al-Garawi A, Merriam S, Lora JM, et al. PYPAF7, a novel PYRIN-containing Apaf1-like protein that regulates activation of NF-kappa B and caspase-1-dependent cytokine processing. *J Biol Chem* (2002) **277**:29874–80. doi:10.1074/jbc.M203915200
57. Jeru I, Duquesnoy P, Fernandes-Alnemri T, Cochet E, Yu JW, Lackmy-Port-Lis M, et al. Mutations in NALP12 cause hereditary periodic fever syndromes. *Proc Natl Acad Sci U S A* (2008) **105**:1614–9. doi:10.1073/pnas.0708616105
58. Borghini S, Tassi S, Chiesa S, Caroli F, Carta S, Caorsi R, et al. Clinical presentation and pathogenesis of cold-induced autoinflammatory disease in a family with recurrence of an NLRP12 mutation. *Arthritis Rheum* (2011) **63**:830–9. doi:10.1002/art.30170
59. Jeru I, Hentgen V, Normand S, Duquesnoy P, Cochet E, Delwail A, et al. Role of interleukin-1beta in NLRP12-associated autoinflammatory disorders and

- resistance to anti-interleukin-1 therapy. *Arthritis Rheum* (2011) **63**:2142–8. doi:10.1002/art.30378
60. Ataide MA, Andrade WA, Zamboni DS, Wang D, Souza Mdo C, Franklin BS, et al. Malaria-induced NLRP12/NLRP3-dependent caspase-1 activation mediates inflammation and hypersensitivity to bacterial superinfection. *PLoS Pathog* (2014) **10**:e1003885. doi:10.1371/journal.ppat.1003885
61. Vladimer GI, Weng D, Paquette SW, Vanaja SK, Rathinam VA, Aune MH, et al. The NLRP12 inflammasome recognizes *Yersinia pestis*. *Immunity* (2012) **37**:96–107. doi:10.1016/j.immuni.2012.07.006
62. Arthur JC, Lich JD, Ye Z, Allen IC, Gris D, Wilson JE, et al. Cutting edge: NLRP12 controls dendritic and myeloid cell migration to affect contact hypersensitivity. *J Immunol* (2010) **185**:4515–9. doi:10.4049/jimmunol.1002227
63. Meixenberger K, Pache F, Eitel J, Schmeck B, Hippensiel S, Slevogt H, et al. *Listeria monocytogenes*-infected human peripheral blood mononuclear cells produce IL-1 β , depending on listeriolysin O and NLRP3. *J Immunol* (2010) **184**:922–30. doi:10.4049/jimmunol.0901346
64. Tsuchiya K, Hara H, Kawamura I, Nomura T, Yamamoto T, Daim S, et al. Involvement of absent in melanoma 2 in inflammasome activation in macrophages infected with *Listeria monocytogenes*. *J Immunol* (2010) **185**:1186–95. doi:10.4049/jimmunol.1001058
65. Pinheiro AS, Eibl C, Ekman-Vural Z, Schwarzenbacher R, Peti W. The NLRP12 pyrin domain: structure, dynamics, and functional insights. *J Mol Biol* (2011) **413**:790–803. doi:10.1016/j.jmb.2011.09.024
66. Zaki MH, Vogel P, Malireddi RK, Body-Malapel M, Anand PK, Bertin J, et al. The NOD-like receptor NLRP12 attenuates colon inflammation and tumorigenesis. *Cancer Cell* (2011) **20**:649–60. doi:10.1016/j.ccr.2011.10.022
67. Allen IC, Lich JD, Arthur JC, Jania CM, Roberts RA, Callaway JB, et al. Characterization of NLRP12 during the development of allergic airway disease in mice. *PLoS One* (2012) **7**:e30612. doi:10.1371/journal.pone.0030612
68. Allen IC, Wilson JE, Schneider M, Lich JD, Roberts RA, Arthur JC, et al. NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-kappaB signaling. *Immunity* (2012) **36**:742–54. doi:10.1016/j.immuni.2012.03.012
69. Allen IC, McElvania-Tekippe E, Wilson JE, Lich JD, Arthur JC, Sullivan JT, et al. Characterization of NLRP12 during the *in vivo* host immune response to *Klebsiella pneumoniae* and *Mycobacterium tuberculosis*. *PLoS One* (2013) **8**:e60842. doi:10.1371/journal.pone.0060842
70. Arthur JC, Lich JD, Aziz RK, Kotb M, Ting JP. Heat shock protein 90 associates with monarch-1 and regulates its ability to promote degradation of NF-kappaB-inducing kinase. *J Immunol* (2007) **179**:6291–6.
71. Ye Z, Lich JD, Moore CB, Duncan JA, Williams KL, Ting JP. ATP binding by monarch-1/NLRP12 is critical for its inhibitory function. *Mol Cell Biol* (2008) **28**:1841–50. doi:10.1128/MCB.01468-07
72. Wagner RN, Proell M, Kufer TA, Schwarzenbacher R. Evaluation of Nod-like receptor (NLR) effector domain interactions. *PLoS One* (2009) **4**:e4931. doi:10.1371/journal.pone.0004931
73. Zaki MH, Man SM, Vogel P, Lamkanfi M, Kanneganti TD. *Salmonella* exploits NLRP12-dependent innate immune signaling to suppress host defenses during infection. *Proc Natl Acad Sci U S A* (2014) **111**:385–90. doi:10.1073/pnas.1317643111
74. Lei Y, Wen H, Yu Y, Taxman DJ, Zhang L, Widman DG, et al. The mitochondrial proteins NLRX1 and TUFM form a complex that regulates type I interferon and autophagy. *Immunity* (2012) **36**:933–46. doi:10.1016/j.immuni.2012.03.025
75. Lei Y, Wen H, Ting JP. The NLR protein, NLRX1, and its partner, TUFM, reduce type I interferon, and enhance autophagy. *Autophagy* (2013) **9**:432–3. doi:10.4161/auto.23026
76. Abdul-Sater AA, Said-Sadier N, Lam VM, Singh B, Pettengill MA, Soares F, et al. Enhancement of reactive oxygen species production and chlamydial infection by the mitochondrial Nod-like family member NLRX1. *J Biol Chem* (2010) **285**:41637–45. doi:10.1074/jbc.M110.137885
77. Arnoult D, Soares F, Tattoli I, Castanier C, Philpott DJ, Girardin SE. An N-terminal addressing sequence targets NLRX1 to the mitochondrial matrix. *J Cell Sci* (2009) **122**:3161–8. doi:10.1242/jcs.051193
78. Conti BJ, Davis BK, Zhang J, O'Connor W Jr, Williams KL, Ting JP. CATER-PILLER 16.2 (CLR16.2), a novel NBD/LRR family member that negatively regulates T cell function. *J Biol Chem* (2005) **280**:18375–85. doi:10.1074/jbc.M413169200
79. Bonizzi G, Bebien M, Otero DC, Johnson-Vroom KE, Cao Y, Vu D, et al. Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. *EMBO J* (2004) **23**:4202–10. doi:10.1038/sj.emboj.7600391
80. Madge LA, May MJ. Classical NF-kappaB activation negatively regulates non-canonical NF-kappaB-dependent CXCL12 expression. *J Biol Chem* (2010) **285**:38069–77. doi:10.1074/jbc.M110.147207
81. Kew RR, Penzo M, Habil DM, Marcu KB. The IKKalpha-dependent NF-kappaB p52/RelB noncanonical pathway is essential to sustain a CXCL12 autocrine loop in cells migrating in response to HMGB1. *J Immunol* (2012) **188**:2380–6. doi:10.4049/jimmunol.1102454
82. Tando T, Ishizaka A, Watanabe H, Ito T, Iida S, Haraguchi T, et al. Requiem protein links RelB/p52 and the Brm-type SWI/SNF complex in a noncanonical NF-kappaB pathway. *J Biol Chem* (2010) **285**:21951–60. doi:10.1074/jbc.M109.087783
83. Domanska UM, Kruizinga RC, Nagengast WB, Timmer-Bosscha H, Huls G, de Vries EG, et al. A review on CXCR4/CXCL12 axis in oncology: no place to hide. *Eur J Cancer* (2013) **49**:219–30. doi:10.1016/j.ejca.2012.05.005
84. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf AC, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* (2013) **39**:782–95. doi:10.1016/j.immuni.2013.10.003
85. Restifo NP. A “big data” view of the tumor “immunome”. *Immunity* (2013) **39**:631–2. doi:10.1016/j.immuni.2013.10.002
86. Dhawan P, Richmond A. A novel NF-kappa B-inducing kinase-MAPK signaling pathway up-regulates NF-kappa B activity in melanoma cells. *J Biol Chem* (2002) **277**:7920–8. doi:10.1074/jbc.M112210200
87. Zarnegar B, Yamazaki S, He JQ, Cheng G. Control of canonical NF-kappaB activation through the NIK-IKK complex pathway. *Proc Natl Acad Sci U S A* (2008) **105**:3503–8. doi:10.1073/pnas.0707959105
88. Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. *Nat Rev Drug Discov* (2013) **12**:931–47. doi:10.1038/nrd4002
89. White E. Deconvoluting the context-dependent role for autophagy in cancer. *Nat Rev Cancer* (2012) **12**:401–10. doi:10.1038/nrc3262
90. Guo JY, Xia B, White E. Autophagy-mediated tumor promotion. *Cell* (2013) **155**:1216–9. doi:10.1016/j.cell.2013.11.019

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Emerging concepts about NAIP/NLRC4 inflammasomes

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Neuronal apoptosis inhibitory protein (NAIP)/NOD-like receptor (NLR) containing a caspase activating and recruitment domain (CARD) 4 (NLRC4) inflammasome complexes are activated in response to proteins from virulent bacteria that reach the cell cytosol. Specific NAIP proteins bind to the agonists and then physically associate with NLRC4 to form an inflammasome complex able to recruit and activate pro-caspase-1. NAIP5 and NAIP6 sense flagellin, component of flagella from motile bacteria, whereas NAIP1 and NAIP2 detect needle and rod components from bacterial type III secretion systems, respectively. Active caspase-1 mediates the maturation and secretion of the pro-inflammatory cytokines, IL-1 β and IL-18, and is responsible for the induction of pyroptosis, a pro-inflammatory form of cell death. In addition to these well-known effector mechanisms, novel roles have been described for NAIP/NLRC4 inflammasomes, such as phagosomal maturation, activation of inducible nitric oxide synthase, regulation of autophagy, secretion of inflammatory mediators, antibody production, activation of T cells, among others. These effector mechanisms mediated by NAIP/NLRC4 inflammasomes have been extensively studied in the context of resistance of infections and the potential of their agonists has been exploited in therapeutic strategies to non-infectious pathologies, such as tumor protection. Thus, this review will discuss current knowledge about the activation of NAIP/NLRC4 inflammasomes and their effector mechanisms.

Keywords: NAIP, NLRC4, flagellin, caspase-1, inflammasomes, lysosomes, cell death

INTRODUCTION

Inflammasomes are multiprotein platforms containing specialized cytosolic sensors for a wide range of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) that are able to activate the inflammatory caspase-1 and caspase-11 (caspase-4 in humans) in a manner dependent or independent of adaptor molecules (1–4). Inflammasomes are composed of a cytosolic receptor from the nucleotide-binding domain-leucine-rich repeat (NBD-LRR) [also named NOD-like receptors (NLR)] or the pyrin and HIN domain-containing protein (PYHIN) families; the adaptor molecule ASC [apoptosis-associated speck-like protein containing a caspase activating and recruitment domain (CARD)]; and pro-caspase-1 or pro-caspase-11. AIM2 is the only member of the PYHIN family described to form inflammasomes. AIM2 is composed of two domains: a C-terminal HIN200 domain and an N-terminal pyrin (PYD) domain. The members of the NLR family contain three domains: a central NBD that is responsible for protein oligomerization and common to all members; a C-terminal region composed of LRR sequences that are supposed to sense PAMPs or DAMPs; and an N-terminal portion that is responsible for the specificity of their molecular interactions and, therefore, their effector functions. The NLR proteins can be classified into NLRBs [NLR containing the baculovirus inhibitory (BIR) domain], NLRCs (NLRs containing the CARD domain), and NLRPs (NLRs containing the PYD domain) (5).

NOD-like receptor proteins are maintained in an autoinhibited state under physiological conditions. After agonist recognition, they undergo a conformational rearrangement, triggering the NBD domains. Then, these proteins expose the effector domain to allow the assembly of oligomeric complexes. The NLRs that lack the CARD domain to recruit and activate pro-caspases-1 and 11 require the assistance of the adapter molecule ASC, which contains the PYD and CARD domains for binding caspases (6, 7). The NLRC members can directly recruit pro-caspase-1 through homotypical interactions between CARD domains, or they can recruit the adaptor ASC to activate caspase-1 (2). The canonical effector mechanisms mediated by caspase-1 are the maturation and secretion of IL-1 β and IL-18 and the induction of pyroptosis, a pro-inflammatory form of cell death. Furthermore, caspase-11 seems to be able to induce pyroptosis (8).

After a decade of inflammasome discovery (9), little is known about the molecular complex formed by most members of the NLR family. AIM2, NLRP3, and NLRC4 are the best-characterized inflammasome complexes. The importance of these complexes to control bacterial, viral, fungal, and protozoan infections and their influence in inflammatory processes are gaining prominence in the literature, although their precise activation mechanisms remain to be elucidated. Here, we focus on NLRC4 inflammasomes, the recent advances in the understanding of their assembly and the consequences of their activation to the immune response.

ASSEMBLY AND ACTIVATION OF NAIP/NLRc4 INFLAMMASOMES

The first reports about the recognition of cytosolic flagellin, the monomeric subunit from flagella present in motile bacteria, demonstrated that the neuronal apoptosis inhibitory protein (NAIP)-5 was responsible for the detection of cytosolic flagellin from *L. pneumophila* and for the restriction of infection (10, 11). In the same year, studies with *S. typhimurium* revealed that another member of the NLR family, NLRC4, was also able to detect cytosolic flagellin (12, 13). NLRC4 was first described in 2001 as a mammalian protein homologous to CED4 of *C. elegans*, whose function is to recruit and activate caspases through its CARD domain (14, 15). Because of the ability to activate caspase-1, previously known as interleukin-1-converting enzyme (ICE), NLRC4 was first named IPAF (ICE-protease-activating factor). Although the involvement of NLRC4 in the control of infections was previously reported, their agonists remained a mystery until 2006.

Flagellin is one of the best-characterized agonists of the innate immune system. Extracellular flagellin is recognized by TLR5 (16) but it can be delivered to the cell cytosol through the secretion systems present in virulent bacteria strains, such as the *S. typhimurium* type III secretion system (T3SS SPI-1) and *L. pneumophila* type IV (T4SS). In the cell cytosol, flagellin induces the formation of the NAIP5/NLRC4 inflammasome, leading to the subsequent activation of caspase-1 (17, 18, 23). Notably, the activation NAIP5/NLRC4 inflammasomes by cytosolic flagellin occurs independently of TLR5 (20), and these two receptors recognize distinct regions of flagellin (16). TLR5 senses a region present in the D1 domain of the protein, whereas the amino acid sequences recognized by NAIP5/NLRC4 inflammasomes are in the D0 domain of the molecule (18, 23, 19, 21, 22).

Previous studies have pointed to the involvement of NAIP5 in controlling *L. pneumophila* flagellated bacteria (24, 25) and to the involvement of NLRC4 in caspase-1 activation and the induction of macrophage death (14, 15), although the role of flagellin in these processes was unidentified at that time. The simultaneous demonstration of cytosolic flagellin recognition by NAIP5 and NLRC4 prompted a model that proposed the existence of two distinct inflammasomes that recognize slight differences in the structure of flagellin (10–13). In 2008, with the advent of NAIP5-deficient mice, Lightfield and collaborators confirmed that NAIP5 is required for NLRC4-containing inflammasome activation in response to *L. pneumophila* infection in a flagellin-dependent manner; however, the NLRC4-mediated macrophage responses against *S. typhimurium* were only partially dependent on NAIP5 (21). A subsequent work from the same group demonstrated that the differential requirement for NAIP5 in response to *S. typhimurium* and *L. pneumophila* infection is not due to intrinsic differences between distinct flagellins, as a genetically engineered *L. pneumophila* developed to express the *S. typhimurium* flagellin also activated the NLRC4 inflammasome in a manner strictly dependent on NAIP5 (17). These data indicated that another agonist from *S. typhimurium* could activate NLRC4 independent of the presence of NAIP5. In fact, these studies confirm that NLRC4 responds to the *S. typhimurium* PrgJ protein independently of NAIP5, thus explaining why NLRC4-mediated

responses to *S. typhimurium* are only partially dependent on NAIP5.

The inflammasome structure formed by these proteins was unveiled only recently when two independent groups proposed a model for NAIP5/NLRC4 inflammasome assembly (18, 23). Using the transfection of inflammasome components and microbial molecules in HEK 293T cells or followed by biochemical assays, the authors demonstrated the ability of flagellin from different bacterial species to bind NAIP5. This interaction was dependent on the three leucine residues of the C-terminal portion of flagellin, confirming prior data (17). Furthermore, after the recognition of flagellin, a physical association between NAIP5 and NLRC4 was demonstrated, resulting in the formation of an oligomeric complex. Reconstitution experiments using truncated receptor variants showed that NAIPs are upstream of NLRC4 and suggest that they interact via the NBD domain. Notably, NAIP6 worked similarly to NAIP5, as it induced the oligomerization of NLRC4 in response to flagellin, and this could explain the response of NAIP5^{-/-} cells to high concentrations of flagellin. NAIP1 and NAIP2 also recruit NLRC4 in response to the bacterial needle and inner rod proteins of T3SS, respectively (18, 23). Therefore, NAIP proteins seem to be the universal sensors of cytosolic flagellin and secretory complex proteins, whereas NLRC4 acts as an adapter molecule and is responsible for the recruitment and activation of caspase-1. It is noteworthy that there is only one functional NAIP found in humans, which is not activated by flagellin but is able to detect needle proteins of T3SS, similar to NAIP1 (18).

Despite these recent contributions to the understanding of NAIP/NLRC4 assembly, the molecular requirements of bacterial proteins for the formation of the inflammasome complex still requires further clarification. Lightfield et al. (21) originally demonstrated that the final 35 amino acids of the C-terminal portion of the flagellin molecule are essential for the activation of NAIP5. Moreover, the replacement of three leucine residues by alanine in this region abrogated the potential of flagellin to activate NAIP5. However, these studies were based on constructs containing only the C-terminal portion of the flagellin structure. A recent study using whole flagellin with or without these regions have shown that although the three leucine residues were essential for the detection of the C-terminus, their involvement seems to be less important for full-length flagellin recognition, as whole flagellin containing three alanines instead of three leucines still induces cell death and inflammasome complex formation, although fewer complexes are formed (22). Surprisingly, although the absence of the N-terminal domain does not affect the ability of whole flagellin to interact with NAIP5, constructs containing only N-terminus also retain the ability to activate NAIP5/NLRC4. Thus, the molecular interaction between flagellin and NAIP5/6 still requires clarification. Moreover, although flagellin was found inside the NAIP5/NLRC4 complex, as demonstrated by immunoprecipitation (19, 26) and yeast two-hybrid (18) assays, providing a basis for the model of direct interaction between flagellin and NAIP5, our group recently demonstrated the ability of cytosolic flagellin to activate a lysosomal pathway and the requirement of cathepsin B for NLRC4-dependent IL-1 β secretion and pyroptosis (27). These observations raise the possibility that NAIP5/NLRC4 can also be activated by cytosolic alterations induced by the

presence of flagellin, as proposed for the activation of the NLRP3 inflammasome (28).

Challenging prior models that hypothesized that LRR domains are responsible for the detection of NLR agonists, a recent study found that these domains are dispensable for the ligand specificity of NAIPs (26). By using a series of chimeric proteins in which the N-terminal domains of NAIP5 or NAIP6 were fused to the C-terminal domains of NAIP2 or vice-versa, the authors demonstrated that NAIP proteins lost the ability to oligomerize with NLRC4 only when NOD domain-associated α -helical domains were absent, suggesting that ligand specificity maps to this region. Interestingly, a similar region in NLRC4 was recently associated with its autoinhibition (29), whereas LRR domain from NAIPs was shown to be required for the maintenance of this protein in an autoinhibited conformation (19). Despite, these unsolved pieces of the puzzle, it has been demonstrated that the interaction of NAIPs with their ligands and the association of NLRC4 with NAIPs induce conformational changes in these molecules that enable their oligomerization and activation (22, 30). Predicted models for the NAIP/NLRC4 inflammasome suggest that these complexes contain an excess of NLRC4 for each NAIP protein (22, 26) and that NLRC4 molecules are able to recruit and activate caspase-1 either directly or through an ASC adapter. The association of pro-caspase-1 with an inflammasomes-containing ASC allows its autoproteolytic cleavage to become an enzymatically active heterodimer capable of processing pro-IL-1 β and pro-IL-18 into mature cytokines (2). In contrast, an ASC-independent complex activates caspase-1 without autoproteolysis, which is sufficient for caspase-1 to target a distinct subset of substrates critical for the induction of pyroptosis.

CANONICAL EFFECTOR MECHANISMS INDUCED BY NAIP/NLRC4 INFLAMMASOMES

PYROPTOSIS

The NAIP5/NLRC4 inflammasome is perhaps the best-studied inflammasome complex with regard to resistance to infections. Their involvement has been reported against infections such as *S. typhimurium* (31, 32), *L. pneumophila* (25), *P. aeruginosa* (33, 34), *Y. pestis* (35), *S. flexneri* (36), and *A. veronii* (37). NAIP/NLRC4-mediated responses are related to the restriction of bacterial growth due to the active caspase-1-mediated canonical and non-canonical effector mechanisms, highlighting the importance of this inflammasome as a host defense mechanism against a large number of bacterial infections. The best elucidated effector mechanisms involved in the control of infections mediated by caspase-1 are the secretion of inflammatory cytokines IL-1 β and IL-18 and the induction of pyroptosis (38).

The term pyroptosis (from the Greek “pyro” meaning fire or fever, and “ptosis” to a fault) was coined in 2001 to describe a pro-inflammatory programmed cell death during *S. typhimurium* infection (39). Morphological and biochemical changes displayed by *S. typhimurium*-infected dying cells were more closely related to those found in classic necrosis compared with those observed during apoptosis, including the following: (1) diffuse DNA fragmentation with no chromatin condensation; (2) early loss of membrane integrity observed by the simultaneous uptake of annexin V with an impermeable membrane dye; (3) lactate

dehydrogenase (LDH) release, suggesting a loss of intracellular content; and (4) independence of any apoptotic caspase. Although cells dying by pyroptosis displayed features of necrosis with an inflammatory outcome, the authors found that this process was highly regulated by active caspase-1, as the addition of inhibitors of caspase-1 (z-YVAD-fmk) abolished *S. typhimurium*-induced cell death.

The induction of pyroptosis by pathogenic bacteria depends on an active secretion system that translocates bacterial proteins into the cell cytosol, such as the T3SS (SPI-1) of *S. typhimurium* and type IV (T4SS) of *L. pneumophila* (12, 13, 40–42). Mutant *L. pneumophila* (43) or *P. aeruginosa* (34, 44) lacking flagellin fail to activate caspase-1 and, therefore, are not able to induce pyroptosis and IL-1 β secretion in infected macrophages. Accordingly, the transfection of purified flagellin from *L. pneumophila* and *S. typhimurium* directly into the cell cytosol is sufficient to trigger caspase-1-dependent pore formation, pyroptosis, and IL-1 β secretion (45, 46). Importantly, infection with the non-flagellated bacteria *S. flexneri* also induces NLRC4-mediated pyroptosis, most likely in response to the inner rod component of T3SS (36).

Although the molecular mechanisms that regulate pyroptosis remain to be elucidated, the model of *S. typhimurium* infection has given us important knowledge about this form of cell death. The cell lysis observed during pyroptosis seems to result from a highly regulated process of pore formation in the plasma membrane (45, 46). Pores dissipate cellular ionic gradients but allow the retention of larger cytoplasmic constituents, leading to increased liquid osmotic pressure and water influx. These events are followed by cell swelling and subsequent osmotic lysis with the release of intracellular contents, which are potentially inflammatory (45, 46). Caspase-1-dependent DNA cleavage also occurs during pyroptosis (45, 47). However, the DNA cleavage observed during *S. typhimurium*-induced pyroptosis is independent of caspase-activated DNase (CAD) (45, 47), unlike what is observed during apoptosis, in which the proteolysis of inhibitor of CAD (ICAD) by apoptotic caspases mediates the release of CAD to the nucleus, where it cleaves DNA between nucleosomes. Therefore, pyroptotic cells do not display the typical pattern of oligonucleosomal fragmentation observed during apoptosis, a fact that can be used to distinguish between these two processes of cell death (48).

There is good evidence implicating pyroptosis as an important host defense mechanism mediated by NAIP/NLRC4 that clears intracellular pathogens *in vitro*. The death of infected macrophages by pyroptosis seems to correlate with a rapid loss of the replicative niche and high bacterial loads are recovered from macrophages deficient in components of inflammasomes or infected with mutant bacterial strains that fail to trigger their activation [reviewed by Bortoluci and Medzhitov (1) and Bergsbaken et al. (49)]. Moreover, a study conducted *in vivo* demonstrated that the NLRC4-dependent flagellin-mediated lysis of bacteria-containing macrophages not only results in the early loss of the intracellular replication niche but also creates an inflammatory milieu with the recruitment of effector cells to the infection site, which are involved in pathogen clearance (32). Although the possible targets of caspase-1 and caspase-11 mobilized during pyroptosis

remain unidentified, the studies involving NAIP/NLRC4 hugely contribute to the idea that this inflammatory form of cell death is an important effector mechanism against infections.

IL-1 β AND IL-18 SECRETION

IL-1 was the first identified cytokine and has been related to several inflammatory processes. IL-1 plays a role in virtually all cells and organs, ranging from fever and resistance to microorganisms to the activation of the hypothalamus–pituitary–adrenal axis (HPA) (50–56). IL-18 was first described in 1989 as a potent IFN- γ -inducing factor and an important component of polarized type-1 T helper cells (Th1) and type-1 macrophages (M1) responses, cells with a pro-inflammatory profile (57–59). Macrophages, monocytes, lymphocytes, keratinocytes, microglia, neutrophils, dendritic cells, and other cells are described as important sources of IL-1 β and IL-18 (60–64). IL-18 and IL-1 β have similar processing; they are both synthesized in an inactive form that requires processing by active caspase-1 to become biologically active (61, 65, 66). Although extensively studied, the mechanism responsible for IL-1 β and IL-18 release has not been fully elucidated. These cytokines can be passively released during cell lysis; however, there is recent evidence supporting the existence of active mechanisms involved in the secretion of IL-1 β and IL-18, such as caspase-1-induced membrane pores, vesicle shedding and lysosomal exocytosis (45, 49).

Although the precise effector mechanisms of IL-1 β and IL-18 remain to be elucidated, these cytokines have been reported to be important mediators induced by NAIP/NLRC4 to host resistance to bacterial infections (67). In addition to the effects of IL-1 β and IL-18 in the activation and recruitment of innate immune cells, these cytokines have important roles in the activation and differentiation of T lymphocytes (52). IL-1 β and IL-18 have been shown to drive the establishment of T CD4 $^{+}$ adaptive responses in mice and in humans and are responsible for the differentiation of Th17 and Th1, respectively (68–70). However, little is known about the involvement of IL-1 β and IL-18 in NAIP/NLRC4-induced adaptive immune responses. Kupz et al. demonstrated that IL-18, when produced by the activation of NLRC4 during infection by *S. typhimurium*, is required for the activation of non-cognate CD8 $^{+}$ T cells and the production of IFN- γ (71), supporting a role for this cytokine in the induction of cellular responses.

Additional evidence of the role of NAIP/NLRC4 in the activation of T cells came from an experimental vaccination with irradiated flagellin-expressing tumor cells. Authors demonstrated that the immunization of mice with flagellin-fused tumor cells induced tumor-specific CD4 $^{+}$ and CD8 $^{+}$ T cell responses and prevented parental tumor growth. Despite the well-known role of TLR5, the recognition of flagellin by the NAIP5/NLRC4 inflammasome was also required for the induction of a protective CD8 $^{+}$ T cell response and tumor suppression. Although the NAIP5/NLRC4 inflammasome-mediated IL-1 β secretion in response to the injection of flagellin-modified tumor cells, it is unclear whether the involvement of this cytokine was necessary for the success of this immunotherapy. The role of IL-1 β and IL-18 in tumorigenesis remains controversial. There is strong evidence supporting pro-tumorigenic properties of these cytokines via the induction

of chronic inflammation. Although the induction of Tregs and Th17 could impair the immune response against tumor cells, it is reasonable to consider that the activation of Th1 and cytotoxic CD8 T cells by IL-1 β and IL-18 may be beneficial to the host (72, 73).

EMERGING EFFECTOR MECHANISMS MEDIATED BY THE NAIP/NLRC4 INFLAMMASOME

HUMORAL EFFECTOR MECHANISMS

In addition to the well-characterized functions of NAIP/NLRC4 inflammasomes described above, non-canonical effector mechanisms have emerged. Recent data describe a range of effector functions mediated by NAIP/NLRC4 inflammasomes that operate independently of IL-1 β , IL-18 and pyroptosis. The NAIP5/NLRC4 inflammasome has been implicated in the activation of phospholipase A2 (cPLA2) with a consequent production of lipid mediators, such as prostaglandins and leukotrienes (74). Authors demonstrated that systemic cytosolic flagellin stimulation leads to an “eicosanoid storm” that initiates inflammation and the loss of vascular fluids, resulting in a very fast death in mice. Of note, these effects are mediated by NAIP5/NLRC4 and occur independently of IL-1 β /IL-18 or pyroptosis.

Inflammasomes have also been implicated in the active secretion of endogenous molecules known as DAMPs, challenging the idea that these molecules are only passively released during the process of cell lysis (75). IL-1 α is an alarmin, whose release has been recently linked to inflammasomes. Both IL-1 β and IL-1 α present some common features, such as belonging to the same family, synthesis in the cytoplasm and secretion by an unconventional pathway independent of the endoplasmic reticulum and Golgi complex (55); additionally, they are released simultaneously by various stimuli, and they act on the same receptor, IL-1R1, thus sharing some biological functions (52). However, despite these similarities, there are some important differences in the production, secretion, and function of these cytokines. Unprocessed forms of both IL-1 α and IL-1 β are thought to be produced in response to TLR ligands, but they have distinct activities. Unlike IL-1 β , which needs to be processed by caspase-1 to become biologically active (65), the uncleaved form of IL-1 α is able to engage IL-1R1 (60, 76), although its full activity seems to require cleavage by calpain (77). Although IL-1 α is not a substrate for caspase-1, there are some reports that have demonstrated that macrophages from caspase-1-deficient mice release less IL-1 α (27, 78–80), suggesting the involvement of inflammasomes.

The mechanism by which caspase-1 mediates IL-1 α secretion is still a matter of debate. A recent study demonstrated that the requirement of inflammasomes for IL-1 α secretion depends on the nature of agonists (81). Caspase-1 has been described as a shuttle that facilitates the secretion of leaderless proteins, such as IL-1 α (80). However, it is not clear whether active caspase-1 is the shuttle itself or whether it activates another machinery that is dependent on its activity, e.g., IL-1 β (82) or IL-1R2 (77), as has been proposed for the secretion of IL-1 α in response to NLRP3 agonists. The involvement of NLRC4 inflammasomes in IL-1 α secretion is poorly understood. In one previous study, infection by *S. typhimurium* resulted in NLRC4- and caspase-1-dependent

secretion of IL-1 α (81). Interestingly, in contrast with most of the NLRP3 agonists, the secretion of IL-1 α in response to *S. typhimurium* was completely independent of ASC, indicating a differential requirement for this adaptor molecule in cytokine secretion in response to NLRc4 agonists, as IL-1 β is entirely dependent on ASC (2). However, Barry et al. showed that IL-1 α initiates the inflammatory response driven by *L. pneumophila* independent of caspase-1 and NLRc4 (83). We recently reported that the activation of macrophages with purified flagellin inserted into lipidic vesicles induced IL-1 α secretion in a manner partially dependent on caspase-1 and cathepsin B (27). Therefore, the reasons for the discrepancies in the literature and the precise mechanisms involved in the cross talk between IL-1 α and NLRc4/caspase-1 axis remain to be addressed.

Another factor whose secretion has been linked to inflammasomes is the “High Mobility group box-1” (HMGB-1). HMGB-1 is a nuclear protein involved in the regulation of nucleosome function and DNA transcription that functions as an inflammatory mediator when released to the extracellular milieu (84). Lamkanfi et al. reported a critical role for HMGB-1 secreted through the NLRP3/ASC/caspase-1 axis in LPS-induced endotoxic shock (85). Interestingly, macrophages infected with *S. typhimurium* released significant amounts of HMGB-1 in a NLRc4 and caspase-1-dependent manner but independently of ASC, which is similar to previous reports of IL-1 α secretion (81). During pyroptosis induced by a variety of stimuli, including *S. typhimurium* infection, HMGB-1 did not undergo caspase-1-mediated processing before its secretion, but extracellular HMGB-1 was hyperacetylated at the nuclear localization sequences (NLSSs) (86). Because this translational modification is essential for HMGB-1 translocation from the nucleus to the cytoplasm (87, 88), HMGB-1 release upon inflammasome activation seems to be a coordinated process. More recently, Nystrom et al. (89) reported that NLRc4-mediated pyroptosis is the prevalent factor in the regulation of HMGB-1 secretion, leading to the release of the chemoattractant acetylated HMGB-1 isoform without requiring TLR-derived priming. Although the mechanisms by which inflammasome components can regulate DAMPs secretion still need to be better understood, DAMPs are already considered important therapeutic targets because of their role in host resistance against infection and their involvement in inflammatory disorders.

With respect to antibodies production NLRc4, NAIP5, and caspase-1 have been reported to have a redundant role with TLR5 in the induction of total IgG (90) or IgG1 (91) against flagellin or co-administered OVA and an additive effect to TLR5 in the induction of IgG2a (91). In the absence of MyD88, in which TLR5, IL-1 β , IL-1 α , and IL-18 signaling is compromised, the production of antibodies induced by flagellin was reduced but not abolished, and a large amount of antibodies was still produced (91). The same results were obtained with TLR5/caspase-1 double-knockout mice (91), supporting previous data that demonstrated that no significant difference was observed in specific anti-flagellin IgG titers in mice deficient for IL-18 (92) or IL-1R (93). These reports suggest that some yet-undiscovered mechanism that acts in addition to TLR5 and inflammasome-mediated cytokines could be involved in the adjuvant properties of flagellin, requiring new investigations into this agonist.

CELLULAR EFFECTOR MECHANISMS

In addition to inflammatory mediators and cell death processes, some cellular effector mechanisms mediated by NLRc4 have emerged. Previous studies from our group described a requirement of NAIP5, NLRc4, and caspase-1 for the activation of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) secretion in response to cytosolic flagellin (94). Interestingly, cytosolic flagellin-induced iNOS activation is preserved in the absence of MYD88, ruling out the participation of TLR5, IL-1 β , and IL-18. Moreover, NO secretion through the NAIP5/NLRc4-caspase-1 axis in response to flagellin is involved in the control of *L. pneumophila* (94) and *S. typhimurium* (unpublished data from our group) by macrophages, pointing to this pathway as an additional effector mechanism mediated by NAIP5/NLRc4.

Autophagy is another effector mechanism used by NAIP5/NLRc4 to control *L. pneumophila*. In the presence of NAIP5, NLRc4 macrophages present a rapid turnover of LC3 $^+$ *L. pneumophila*-containing vesicles, preventing the establishment of secondary infections (95). This response is mediated by the detection of flagellin, and the inhibition of autophagy in macrophages infected with flagellin-sufficient *L. pneumophila* increased the rate of pyroptosis in these cells. These data confirm a previous study that demonstrated that NLRc4 plays a role in the regulation of autophagy by binding Beclin-1 in steady-state conditions (96). Because the initiation of autophagy seems to precede the induction of pyroptosis, autophagy can be considered a pathway through which macrophages raise the threshold of contaminants necessary to result in the loss of cell by inflammatory cell death. NAIP5/NLRc4 can also restrict flagellin-competent *L. pneumophila* replication by promoting the delivery of *L. pneumophila*-containing phagosomes (LCP) to lysosomes for degradation (43, 97). In the absence of NAIP5/NLRc4/caspase-1, LCP avoids fusion with lysosomes, which allows the pathogen to exponentially replicate inside macrophages. This effect is dependent on caspase-1-mediated caspase-7 processing and does not require IL-1 β /IL-18 and the classical apoptosis pathway involving caspase-8 and -9 (98). These data corroborate a previous report that demonstrated a requirement of NLRc4, caspase-1, and ASC for caspase-7 processing during infection with flagellin-competent *S. ty* *typhimurium* (99). NLRc4 and ASC-dependent caspase-8 proteolysis was also reported during *S. typhimurium* infection (100). Interestingly, caspase-8 contributes to *Salmonella*-induced IL-1 β production, but it is dispensable for inducing pyroptosis, whereas caspase-1 processes pro-IL-1 β and coordinates pyroptosis. These data highlight the fact that inflammasomes are dynamic complexes that are able to recruit distinct members of the caspase family to induce diverse effector functions in response to *Salmonella* infection.

Similar to what has been demonstrated during apoptosis (101, 102) and necrosis (103), the cleavage of PARP1 (also called ARTD1) was also observed during pyroptosis induced by *S. typhimurium* (104). PARP1 processing in *S. typhimurium*-infected macrophages was abrogated in *Nlrc4* $^{-/-}$ but not in *Nlrc4* $^{+/+}$ cells, consistent with the role of the NAIP5/NLRc4 inflammasome in the induction of pyroptosis during *S. typhimurium* infection (12, 31, 105). PARP1 is a nuclear chromatin-associated multifunctional enzyme that catalyzes the polymerization of ADP-ribose units from donor NAD $^+$ molecules (106, 107). Although it has been

historically studied in the context of genotoxic stress signaling and consequent apoptosis, PARP1 has been related to chromatin structure regulation, transcription, and chromosomal organization (108, 109). Previous reports showed that inflammasomes are able to use PARP1 to induce the transcription of NF- κ B-dependent target genes independently of any type of programmed cell death (110). Upon LPS stimulation, caspase-7 is activated by caspase-1, which is translocated to the nucleus to induce PARP1 cleavage at the promoters of a subset of NF- κ B-dependent target genes that are negatively regulated by PARP1. Mutating the PARP1 cleavage site D214 renders PARP1 uncleavable and inhibits PARP1 release from chromatin and, therefore, chromatin decondensation, thereby restraining the expression of cleavage-dependent NF- κ B target genes, such as *il-6*, *cfs2*, and *lif*, but not *ip-10* (110). Preliminary and unpublished data from our group suggest the involvement of caspase-1-dependent PARP1 cleavage in iNOS gene expression upon cytosolic flagellin stimulation, as iNOS expression is significantly reduced in macrophages that harbor non-cleavable PARP1 (D214N). This is important evidence of the involvement of inflammasomes in epigenetic regulation and gene expression, although many of these outputs require further evaluation.

An important process of lysosomal exocytosis occurs during pyroptosis. Bergsbaken and Cookson (111) demonstrated that caspase-1-mediated pore formation induced during *S. typhimurium* infection promotes an influx of extracellular calcium, which is critical for lysosomal exocytosis. The release of lysosomal proteases with known antimicrobial activity contributes to the control of extracellular bacteria. In addition to the effect

of lysosomal contents in the extracellular compartment, recent data from our group demonstrated that cytosolic flagellin is also able to activate a lysosomal pathway that culminates in an inflammasome-independent inflammatory form of cell death. This inflammasome-independent cell death induced by cytosolic flagellin is regulated by cathepsins B and D and is temporally correlated with the restriction of *S. typhimurium* infection by macrophages (27). Together, these data indicate a cross talk between lysosomes and NAIP/NLRc4 inflammasomes that impact the control of bacterial infections and opens new avenues for the development of inflammasome-based therapeutic strategies for non-infectious pathologies such as tumors. In fact, lysosomes have been considered important targets for the development of anti-tumor drugs (112). Lysosomes from cancer cells appear to be less stable than normal cells, which has given rise to the development of therapies based on lysosomotropic detergents. In this sense, flagellin could be an alternative that in addition to the induction of lysosomal cell death, is able to mediate several effector mechanisms as described throughout this review (Figure 1).

CONCLUSION AND FUTURE DIRECTIONS

More than 10 years after their discovery (14, 15), the molecular mechanisms involved in the activation of NAIP/NLRc4 began to be elucidated (18, 19, 26). From two distinct inflammasome complexes, NAIPs emerged as universal sensors for cytosolic bacterial proteins, whereas NLRc4 became an adaptor molecule responsible for the recruitment and activation of caspase-1. At the same time, in addition to NAIP5, novel NAIPs members were described,

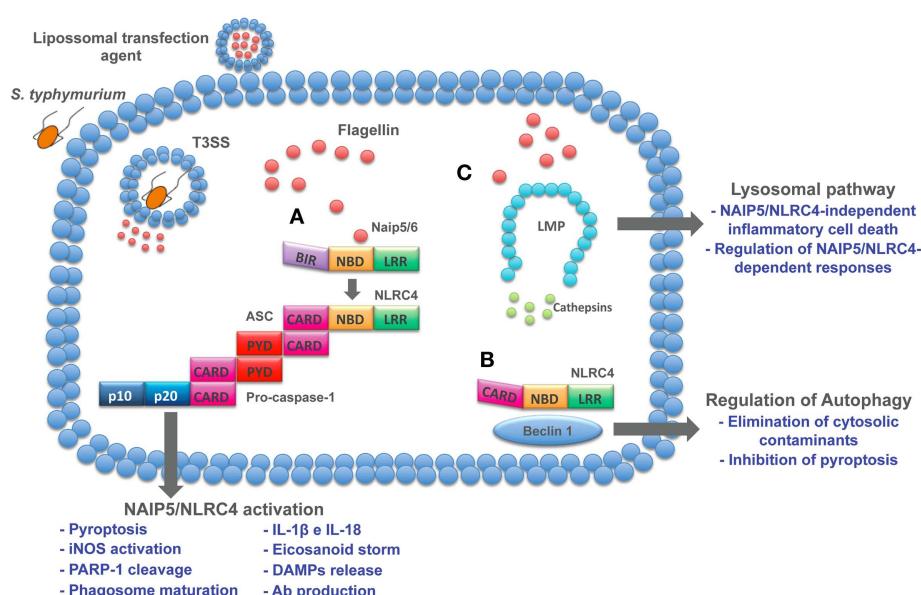


FIGURE 1 | Cytosolic pathways induced by flagellin. Flagellin delivered to cell cytosol through bacterial secretion systems or transfection agents activates different pathways. **(A)** NAIP5/6-NLRC4 activation induces a series of cellular and humoral responses involved in host control of infections. **(B)** In resting cells, NLRC4 is complexed with Beclin-1, thus inhibiting autophagy. When flagellin is detected by NAIP5/6, NLRC4 is recruited to assemble inflammasome complex and release Beclin-1 to initiate autophagy. As a host protection response,

autophagy is able to eliminate cytosolic cargo and inhibits pyroptosis, thus preventing cell loss and inflammation. Therefore, these emerging effector responses induced by flagellin open up new avenues to explore its immune potential as therapeutic targets. **(C)** Lysosomal destabilization leads to cathepsins release to cell cytosol, resulting in the induction of inflammasome-independent cell death that contributes to macrophage control of infection and regulation of NAIP5/NLRc4-dependent responses.

amplifying the potential of these proteins to detect bacterial infections (18, 19, 113, 114). Despite this important information, the molecular signatures of agonists recognized by NAIP/NLRc4 inflammasomes still require further study. Moreover, NLRc4 has been associated with host resistance against a mucosal *Candida albicans* infection (115) and in a colitis-associated colorectal cancer (CAC) model (116, 117). Interestingly, in both cases, NLRc4 seems to exert a protective role in non-hematopoietic compartments. However, the precise mechanism of NLRc4 activation in these models is unknown, raising the possibility that NLRc4 functions as an adaptor molecule for other NLR members in addition to NAIP and providing new insights into inflammasome signaling.

NAIP/NLRc4 are most likely the best-studied inflammasomes in the context of host resistance against infections. In addition to the extensively described IL-1 β and IL-18 secretion and pyroptosis, other important effector mechanisms mediated by these inflammasomes have recently emerged (Figure 1). Moreover, flagellin, the best studied NAIP/NLRc4 ligand, has been reported to activate distinct pathways, such as autophagy (95) and a lysosome pathway (27) (Figure 1). Although the precise mechanism involved in the lysosome disruption by flagellin is still under investigation, it culminates in an inflammatory process of cell death that is accompanied by IL-1 α secretion and contributes to the control of *S. typhimurium* by macrophages. This peculiar process of cell death occurs in the absence of inflammasome components. Additionally, the inhibition of cathepsin B disrupted IL-1 β secretion and pyroptosis in response to cytosolic flagellin, indicating a role for lysosomal proteases in the regulation of NAIP/NLRc4-dependent responses. Because human cells do not express NAIP5 or NAIP6 (18), the activation of the lysosomal pathway by flagellin might be an alternative pathway used when human cells interact with flagellated bacteria that reach cell cytosol. In the context of therapeutic strategies, this knowledge could be an important gain, as the immune properties of flagellin have been extensively exploited in different models. At least in the context of anti-tumor vaccination (118) and antibody production (90, 91), the protective and adjuvancy roles of flagellin require its cytosolic detection. Together, these reports open up new avenues to explore the immune potential of NAIP/NLRc4 agonists as therapeutic targets.

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REFERENCES

- Bortoluci KR, Medzhitov R. Control of infection by pyroptosis and autophagy: role of TLR and NLR. *Cell Mol Life Sci* (2010) **67**(10):1643–51. doi:10.1007/s00018-010-0335-5
- Broz P, von Moltke J, Jones JW, Vance RE, Monack DM. Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell Host Microbe* (2010) **8**(6):471–83. doi:10.1016/j.chom.2010.11.007
- Broz P, Monack DM. Noncanonical inflammasomes: caspase-11 activation and effector mechanisms. *PLoS Pathog* (2013) **9**(2):e1003144. doi:10.1371/journal.ppat.1003144
- Maslanik T, Mahaffey L, Tannura K, Beninson L, Greenwood BN, Fleshner M. The inflammasome and danger associated molecular patterns (DAMPs) are implicated in cytokine and chemokine responses following stressor exposure. *Brain Behav Immun* (2013) **28**:54–62. doi:10.1016/j.bbi.2012.10.014
- Ting JP, Lovering RC, Alnemri ES, Bertin J, Boss JM, Davis BK, et al. The NLR gene family: a standard nomenclature. *Immunity* (2008) **28**(3):285–7. doi:10.1016/j.immuni.2008.02.005
- Martinon F, Mayor A, Tschoop J. The inflammasomes: guardians of the body. *Annu Rev Immunol* (2009) **27**:229–65. doi:10.1146/annurev.immunol.021908.132715
- Schroder K, Tschoop J. The inflammasomes. *Cell* (2010) **140**(6):821–32. doi:10.1016/j.cell.2010.01.040
- Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, et al. Non-canonical inflammasome activation targets caspase-11. *Nature* (2011) **479**(7371):117–21. doi:10.1038/nature10558
- Martinon F, Burns K, Tschoop J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* (2002) **10**(2):417–26. doi:10.1016/S1097-2765(02)00599-3
- Molofsky AB, Byrne BG, Whitfield NN, Madigan CA, Fuse ET, Tateda K, et al. Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *J Exp Med* (2006) **203**(4):1093–104. doi:10.1084/jem.20051659
- Ren T, Zamboni DS, Roy CR, Dietrich WF, Vance RE. Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. *PLoS Pathog* (2006) **2**(3):e18. doi:10.1371/journal.ppat.0020018
- Franchi L, Amer A, Body-Malapel M, Kanneganti TD, Ozören N, Jagirdar R, et al. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in *Salmonella*-infected macrophages. *Nat Immunol* (2006) **7**(6):576–82. doi:10.1038/ni1346
- Miao EA, Alpuche-Aranda CM, Dors M, Clark AE, Bader MW, Miller SI, et al. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol* (2006) **7**(6):569–75. doi:10.1038/ni1344
- Geddes BJ, Wang L, Huang WJ, Lavelle M, Manji GA, Brown M, et al. Human CARD12 is a novel CED4/Apaf-1 family member that induces apoptosis. *Biochem Biophys Res Commun* (2001) **284**(1):77–82. doi:10.1006/bbrc.2001.4928
- Poyet JL, Srinivasula SM, Tnani M, Razmara M, Fernandes-Alnemri T, Alnemri ES. Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. *J Biol Chem* (2001) **276**(30):28309–13. doi:10.1074/jbc.C100250200
- Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by toll-like receptor 5. *Nature* (2001) **410**(6832):1099–103. doi:10.1038/35074106
- Lightfield KL, Persson J, Trinidad NJ, Brubaker SW, Kofoed EM, Sauer JD, et al. Differential requirements for NAIP5 in activation of the NLRc4 inflammasome. *Infect Immun* (2011) **79**(4):1606–14. doi:10.1128/IAI.01187-10
- Zhao Y, Yang J, Shi J, Gong YN, Lu Q, Xu H, et al. The NLRc4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* (2011) **477**(7366):596–600. doi:10.1038/nature10510
- Kofoed EM, Vance RE. Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* (2011) **477**(7366):592–5. doi:10.1038/nature10394
- Faustin B, Lartigue L, Bruey JM, Luciano F, Sergienko E, Bailly-Maitre B, et al. Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Mol Cell* (2007) **25**(5):713–24. doi:10.1016/j.molcel.2007.01.032
- Lightfield KL, Persson J, Brubaker SW, Witte CE, von Moltke J, Dunipace EA, et al. Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. *Nat Immunol* (2008) **9**(10):1171–8. doi:10.1038/ni.1646
- Halff EF, Diebold CA, Versteeg M, Schouten A, Brondijk TH, Huizinga EG. Formation and structure of a NAIP5-NLRc4 inflammasome induced by direct interactions with conserved N- and C-terminal regions of flagellin. *J Biol Chem* (2012) **287**(46):38460–72. doi:10.1074/jbc.M112.393512
- Kofoed EM, Vance RE. NAIPs: building an innate immune barrier against bacterial pathogens. NAIPs function as sensors that initiate innate immunity by detection of bacterial proteins in the host cell cytosol. *Bioessays* (2012) **34**(7):589–98. doi:10.1002/bies.201200013
- Diez E, Lee SH, Gauthier S, Yaraghi Z, Tremblay M, Vidal S, et al. Bircle is the gene within the Lgn1 locus associated with resistance to *Legionella pneumophila*. *Nat Genet* (2003) **33**(1):55–60. doi:10.1038/ng1065
- Zamboni DS, Kobayashi KS, Kohlsdorf T, Ogura Y, Long EM, Vance RE, et al. The Bircle cytosolic pattern-recognition receptor contributes to the

- detection and control of *Legionella pneumophila* infection. *Nat Immunol* (2006) **7**(3):318–25. doi:10.1038/ni1305
26. Tenthorey JL, Kofoed EM, Daugherty MD, Malik HS, Vance RE. Molecular basis for specific recognition of bacterial ligands by NAIP/NLRc4 inflammasomes. *Mol Cell* (2014) **54**(1):17–29. doi:10.1016/j.molcel.2014.02.018
27. Lage SL, Buzzo CL, Amaral EP, Matteucci KC, Massis LM, Icimoto MY, et al. Cytosolic flagellin-induced lysosomal pathway regulates inflammasome-dependent and -independent macrophage responses. *Proc Natl Acad Sci U S A* (2013) **110**(35):E3321–30. doi:10.1073/pnas.1305316110
28. Sutterwala FS, Ogura Y, Zamboni DS, Roy CR, Flavell RA. NALP3: a key player in caspase-1 activation. *J Endotoxin Res* (2006) **12**(4):251–6. doi:10.1179/096805106X118771
29. Hu Z, Yan C, Liu P, Huang Z, Ma R, Zhang C, et al. Crystal structure of NLRC4 reveals its autoinhibition mechanism. *Science* (2013) **341**(6142):172–5. doi:10.1126/science.1236381
30. Qu Y, Misaghi S, Izrael-Tomasevic A, Newton K, Gilmour LL, Lamkanfi M, et al. Phosphorylation of NLRC4 is critical for inflammasome activation. *Nature* (2012) **490**(7421):539–42. doi:10.1038/nature11429
31. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* (2004) **430**(6996):213–8. doi:10.1038/nature02664
32. Miao EA, Leaf IA, Treuting PM, Mao DP, Dors M, Sarkar A, et al. Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat Immunol* (2010) **11**(12):1136–42. doi:10.1038/ni.1960
33. Sutterwala FS, Mijares LA, Li L, Ogura Y, Kazmierczak BI, Flavell RA. Immune recognition of *Pseudomonas aeruginosa* mediated by the IPAF/NLRC4 inflammasome. *J Exp Med* (2007) **204**(13):3235–45. doi:10.1084/jem.20071239
34. Miao EA, Ernst RK, Dors M, Mao DP, Aderem A. *Pseudomonas aeruginosa* activates caspase 1 through Ipaf. *Proc Natl Acad Sci U S A* (2008) **105**(7):2562–7. doi:10.1073/pnas.0712183105
35. Brodsky IE, Palm NW, Sadanand S, Ryndak MB, Sutterwala FS, Flavell RA, et al. A Yersinia effector protein promotes virulence by preventing inflammasome recognition of the type III secretion system. *Cell Host Microbe* (2010) **7**(5):376–87. doi:10.1016/j.chom.2010.04.009
36. Suzuki T, Franchi L, Toma C, Ashida H, Ogawa M, Yoshikawa Y, et al. Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in *Shigella*-infected macrophages. *PLoS Pathog* (2007) **3**(8):e111. doi:10.1371/journal.ppat.0030111
37. McCoy AJ, Koizumi Y, Higa N, Suzuki T. Differential regulation of caspase-1 activation via NLRP3/NLRC4 inflammasomes mediated by aerolysin and type III secretion system during *Aeromonas veronii* infection. *J Immunol* (2010) **185**(11):7077–84. doi:10.4049/jimmunol.1002165
38. Franchi L, Munoz-Planillo R, Nunez G. Sensing and reacting to microbes through the inflammasomes. *Nat Immunol* (2012) **13**(4):325–32. doi:10.1038/ni.2231
39. Cookson BT, Brennan MA. Pro-inflammatory programmed cell death. *Trends Microbiol* (2001) **9**(3):113–4. doi:10.1016/S0966-842X(00)01936-3
40. Chen LM, Kaniga K, Galan JE. *Salmonella* spp. are cytotoxic for cultured macrophages. *Mol Microbiol* (1996) **21**(5):1101–15. doi:10.1046/j.1365-2958.1996.471410.x
41. Lundberg U, Vinatzer U, Berdnik D, von Gabain A, Baccarini M. Growth phase-regulated induction of *Salmonella*-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. *J Bacteriol* (1999) **181**(11):3433–7.
42. Brennan MA, Cookson BT. *Salmonella* induces macrophage death by caspase-1-dependent necrosis. *Mol Microbiol* (2000) **38**(1):31–40. doi:10.1046/j.1365-2958.2000.02103.x
43. Amer A, Franchi L, Kanneganti TD, Body-Malapel M, Ozoren N, Brady G, et al. Regulation of *Legionella* phagosome maturation and infection through flagellin and host Ipaf. *J Biol Chem* (2006) **281**(46):35217–23. doi:10.1074/jbc.M604933200
44. Franchi L, Stoolman J, Kanneganti TD, Verma A, Rampal R, Núñez G. Critical role for Ipaf in *Pseudomonas aeruginosa*-induced caspase-1 activation. *Eur J Immunol* (2007) **37**(11):3030–9. doi:10.1002/eji.200737532
45. Fink SL, Cookson BT. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cell Microbiol* (2006) **8**(11):1812–25. doi:10.1111/j.1462-5822.2006.00751.x
46. Silveira TN, Zamboni DS. Pore formation triggered by *Legionella* spp. is an Nlrp4 inflammasome-dependent host cell response that precedes pyroptosis. *Infect Immun* (2010) **78**(3):1403–13. doi:10.1128/IAI.00905-09
47. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* (2009) **7**(2):99–109. doi:10.1038/nrmicro2070
48. Lage SL, Amarante-Mendes GP, Bortoluci KR. Evaluation of pyroptosis in macrophages using cytosolic delivery of purified flagellin. *Methods* (2013) **61**(2):110–6. doi:10.1016/j.ymeth.2013.02.010
49. Bergsbaken T, Fink SL, den Hartigh AB, Loomis WP, Cookson BT. Coordinated host responses during pyroptosis: caspase-1-dependent lysosome exocytosis and inflammatory cytokine maturation. *J Immunol* (2011) **187**(5):2748–54. doi:10.4049/jimmunol.1100477
50. Van der Meer JW, Van de Gevel JS, Van Hinsbergh VW, Leijh PC. The influence of culture conditions and serum lipids on interleukin-1 production by human monocytes. *J Immunol Methods* (1988) **108**(1–2):19–26. doi:10.1016/0022-1759(88)90397-3
51. Delaleu N, Bickel M. Interleukin-1 beta and interleukin-18: regulation and activity in local inflammation. *Periodontol 2000* (2004) **35**:42–52. doi:10.1111/j.0906-6713.2004.003569.x
52. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* (2009) **27**:519–50. doi:10.1146/annurev.immunol.021908.132612
53. Dinarello CA. Anti-inflammatory agents: present and future. *Cell* (2010) **140**(6):935–50. doi:10.1016/j.cell.2010.02.043
54. Gabay C, Lamachia C, Palmer G. IL-1 pathways in inflammation and human diseases. *Nat Rev Rheumatol* (2010) **6**(4):232–41. doi:10.1038/nrrheum.2010.4
55. Sims JE, Smith DE. The IL-1 family: regulators of immunity. *Nat Rev Immunol* (2010) **10**(2):89–102. doi:10.1038/nri2691
56. Dinarello CA. Grand challenge in inflammation. *Front Immunol* (2012) **3**:12. doi:10.3389/fimmu.2012.00012
57. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev* (2001) **12**(1):53–72. doi:10.1016/S1359-6101(00)00015-0
58. Siegmund B, Fantuzzi G, Rieder F, Gamboni-Robertson F, Lehr HA, Hartmann G, et al. Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-gamma and TNF-alpha production. *Am J Physiol Regul Integr Comp Physiol* (2001) **281**(4):R1264–73.
59. Bastos KR, Barboza R, Sardinha L, Russo M, Alvarez JM, Lima MR. Role of endogenous IFN-gamma in macrophage programming induced by IL-12 and IL-18. *J Interferon Cytokine Res* (2007) **27**(5):399–410. doi:10.1089/jir.2007.0128
60. Mosley B, Urdal DL, Prickett KS, Larsen A, Cosman D, Conlon PJ, et al. The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. *J Biol Chem* (1987) **262**(7):2941–4.
61. Puren AJ, Fantuzzi G, Dinarello CA. Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in human blood mononuclear cells and mouse spleen cells. *Proc Natl Acad Sci U S A* (1999) **96**(5):2256–61. doi:10.1073/pnas.96.5.2256
62. Berda-Haddad Y, Robert S, Salers P, Zekraoui L, Farnarier C, Dinarello CA, et al. Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1alpha. *Proc Natl Acad Sci U S A* (2011) **108**(51):20684–9. doi:10.1073/pnas.1116848108
63. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* (2011) **117**(14):3720–32. doi:10.1182/blood-2010-07-273417
64. Carta S, Lavieri R, Rubartelli A. Different members of the IL-1 family come out in different ways: DAMPs vs. cytokines? *Front Immunol* (2013) **4**:123. doi:10.3389/fimmu.2013.00123
65. Dinarello CA. Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme. *Ann N Y Acad Sci* (1998) **856**:1–11. doi:10.1111/j.1749-6632.1998.tb08307.x
66. Bellora F, Castriconi R, Doni A, Cantoni C, Moretta L, Mantovani A, et al. M-CSF induces the expression of a membrane-bound form of IL-18 in a subset of human monocytes differentiating in vitro toward macrophages. *Eur J Immunol* (2012) **42**(6):1618–26. doi:10.1002/eji.201142173
67. von Moltke J, Ayres JS, Kofoed EM, Chavarría-Smith J, Vance RE. Recognition of bacteria by inflammasomes. *Annu Rev Immunol* (2013) **31**:73–106. doi:10.1146/annurev-immunol-032712-095944
68. Dinarello CA, Wolff SM. The role of interleukin-1 in disease. *N Engl J Med* (1993) **328**(2):106–13. doi:10.1056/NEJM199301143280207

69. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* (2009) **30**(4):576–87. doi:10.1016/j.jimmuni.2009.02.007
70. Lasigliè D, Traggiai E, Federici S, Alessio M, Buoncompagni A, Accogli A, et al. Role of IL-1 beta in the development of human T(H)17 cells: lesson from NLPR3 mutated patients. *PLoS One* (2011) **6**(5):e20014. doi:10.1371/journal.pone.0020014
71. Kupz A, Guarda G, Gebhardt T, Sander LE, Short KR, Diavatopoulos DA, et al. NLRc4 inflammasomes in dendritic cells regulate noncognate effector function by memory CD8(+) T cells. *Nat Immunol* (2012) **13**(2):162–9. doi:10.1038/ni.2195
72. Kolb R, Liu GH, Janowski AM, Sutterwala FS, Zhang W. Inflammasomes in cancer: a double-edged sword. *Protein Cell* (2014) **5**(1):12–20. doi:10.1007/s13238-013-0001-4
73. Janowski AM, Kolb R, Zhang W, Sutterwala FS. Beneficial and detrimental roles of NLRs in carcinogenesis. *Front Immunol* (2013) **4**:370. doi:10.3389/fimmu.2013.00370
74. von Moltke J, Trinidad NJ, Moayeri M, Kintzer AF, Wang SB, van Rooijen N, et al. Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. *Nature* (2012) **490**(7418):107–11. doi:10.1038/nature11351
75. Chen CJ, Kono H, Golenoock D, Reed G, Akira S, Rock KL. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat Med* (2007) **13**(7):851–6. doi:10.1038/nm1603
76. Howard AD, Kostura MJ, Thornberry N, Ding GJ, Limjoco G, Weidner J, et al. IL-1-converting enzyme requires aspartic acid residues for processing of the IL-1 beta precursor at two distinct sites and does not cleave 31-kDa IL-1 alpha. *J Immunol* (1991) **147**(9):2964–9.
77. Zheng Y, Humphrey M, Maguire JJ, Bennett MR, Clarke MC. Intracellular interleukin-1 receptor 2 binding prevents cleavage and activity of interleukin-1alpha, controlling necrosis-induced sterile inflammation. *Immunity* (2013) **38**(2):285–95. doi:10.1016/j.jimmuni.2013.01.008
78. Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS, et al. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* (1995) **267**(5206):2000–3. doi:10.1126/science.7535475
79. Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, et al. Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. *Cell* (1995) **80**(3):401–11. doi:10.1016/0092-8674(95)90490-5
80. Keller M, Rüegg A, Werner S, Beer HD. Active caspase-1 is a regulator of unconventional protein secretion. *Cell* (2008) **132**(5):818–31. doi:10.1016/j.cell.2007.12.040
81. Gross O, Yazdi AS, Thomas CJ, Masin M, Heinz LX, Guarda G, et al. Inflammasome activators induce interleukin-1alpha secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity* (2012) **36**(3):388–400. doi:10.1016/j.jimmuni.2012.01.018
82. Fettelschoss A, Kistowska M, LeibundGut-Landmann S, Beer HD, Johansen P, Senti G, et al. Inflammasome activation and IL-1beta target IL-1alpha for secretion as opposed to surface expression. *Proc Natl Acad Sci U S A* (2011) **108**(44):18055–60. doi:10.1073/pnas.1109176108
83. Barry KC, Fontana MF, Portman JL, Dugan AS, Vance RE. IL-1alpha signaling initiates the inflammatory response to virulent *Legionella pneumophila* in vivo. *J Immunol* (2013) **190**(12):6329–39. doi:10.4049/jimmunol.1300100
84. Muller S, Scaffidi P, Degryse B, Bonaldi T, Ronfani L, Agresti A, et al. New EMBO members' review: the double life of HMGB1 chromatin protein: architectural factor and extracellular signal. *EMBO J* (2001) **20**(16):4337–40. doi:10.1093/emboj/20.16.4337
85. Lamkanfi M, Sarkar A, Vande Walle L, Vitari AC, Amer AO, Wewers MD, et al. Inflammasome-dependent release of the alarmin HMGB1 in endotoxemia. *J Immunol* (2010) **185**(7):4385–92. doi:10.4049/jimmunol.1000803
86. Lu B, Nakamura T, Inouye K, Li J, Tang Y, Lundbäck P, et al. Novel role of PKR in inflammasome activation and HMGB1 release. *Nature* (2012) **488**(7413):670–4. doi:10.1038/nature11290
87. Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachì A, et al. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *EMBO J* (2003) **22**(20):5551–60. doi:10.1093/emboj/cdg516
88. Evankovich J, Cho SW, Zhang R, Cardinal J, Dhupar R, Zhang L, et al. High mobility group box 1 release from hepatocytes during ischemia and reperfusion injury is mediated by decreased histone deacetylase activity. *J Biol Chem* (2010) **285**(51):39888–97. doi:10.1074/jbc.M110.128348
89. Nyström S, Antoine DJ, Lundbäck P, Lock JG, Nita AF, Höglstrand K, et al. TLR activation regulates damage-associated molecular pattern isoforms released during pyroptosis. *EMBO J* (2013) **32**(1):86–99. doi:10.1038/embj.2012.328
90. Vijay-Kumar M, Carvalho FA, Aitken JD, Fifadara NH, Gewirtz AT. TLR5 or NLRc4 is necessary and sufficient for promotion of humoral immunity by flagellin. *Eur J Immunol* (2010) **40**(12):3528–34. doi:10.1002/eji.201040421
91. Lopez-Yglesias AH, Zhao X, Quarles EK, Lai MA, VandenBos T, Strong RK, et al. Flagellin induces antibody responses through a TLR5- and inflammasome-independent pathway. *J Immunol* (2014) **192**(4):1587–96. doi:10.4049/jimmunol.1301893
92. Sanders CJ, Franchi L, Yarovinsky F, Uematsu S, Akira S, Núñez G, et al. Induction of adaptive immunity by flagellin does not require robust activation of innate immunity. *Eur J Immunol* (2009) **39**(2):359–71. doi:10.1002/eji.200838804
93. Sanders CJ, Moore DA III, Williams IR, Gewirtz AT. Both radioresistant and hemopoietic cells promote innate and adaptive immune responses to flagellin. *J Immunol* (2008) **180**(11):7184–92. doi:10.4049/jimmunol.180.11.7184
94. Buzzo CL, Campopiano JC, Massis LM, Lage SL, Cassado AA, Leme-Souza R, et al. A novel pathway for inducible nitric-oxide synthase activation through inflammasomes. *J Biol Chem* (2010) **285**(42):32087–95. doi:10.1074/jbc.M110.124297
95. Byrne BG, Dubuisson JF, Joshi AD, Persson JJ, Swanson MS. Inflammasome components coordinate autophagy and pyroptosis as macrophage responses to infection. *MBio* (2013) **4**(1):e620–612. doi:10.1128/mBio.00620-12
96. Jouani N, Kobiyama K, Shiina M, Ogata K, Ishii KJ, Takeshita F. NLRP4 negatively regulates autophagic processes through an association with beclin1. *J Immunol* (2011) **186**(3):1646–55. doi:10.4049/jimmunol.1001654
97. Fortier A, de Chastellier C, Balor S, Gros P, Bircle Naip5 rapidly antagonizes modulation of phagosome maturation by *Legionella pneumophila*. *Cell Microbiol* (2007) **9**(4):910–23. doi:10.1111/j.1462-5822.2006.00839.x
98. Akhter A, Gavrilin MA, Frantz L, Washington S, Ditty C, Limoli D, et al. Caspase-7 activation by the Nlr4c/Ipfaf inflammasome restricts *Legionella pneumophila* infection. *PLoS Pathog* (2009) **5**(4):e1000361. doi:10.1371/journal.ppat.1000361
99. Lamkanfi M, Kanneganti TD, Van Damme P, Vanden Berghe T, Vanoverberghe I, Vandekerckhove J, et al. Targeted peptidecentric proteomics reveals caspase-7 as a substrate of the caspase-1 inflammasomes. *Mol Cell Proteomics* (2008) **7**(12):2350–63. doi:10.1074/mcp.M800132-MCP200
100. Man SM, Tourlomousis P, Hopkins L, Monie TP, Fitzgerald KA, Bryant CE. *Salmonella* infection induces recruitment of caspase-8 to the inflammasome to modulate IL-1beta production. *J Immunol* (2013) **191**(10):5239–46. doi:10.4049/jimmunol.1301581
101. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, et al. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* (1995) **376**(6535):37–43. doi:10.1038/376037a0
102. Casciola-Rosen L, Rosen A, Petri M, Schlissel M. Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. *Proc Natl Acad Sci U S A* (1996) **93**(4):1624–9. doi:10.1073/pnas.93.4.1624
103. Nosseri C, Coppola S, Ghibelli L. Possible involvement of poly(ADP-ribosyl) polymerase in triggering stress-induced apoptosis. *Exp Cell Res* (1994) **212**(2):367–73. doi:10.1006/excr.1994.1156
104. Malireddi RK, Ippagunta S, Lamkanfi M, Kanneganti TD. Cutting edge: proteolytic inactivation of poly(ADP-ribose) polymerase 1 by the Nlrp3 and Nlr4c inflammasomes. *J Immunol* (2010) **185**(6):3127–30. doi:10.4049/jimmunol.1001512
105. Kanneganti TD, Lamkanfi M, Kim YG, Chen G, Park JH, Franchi L, et al. Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* (2007) **26**(4):433–43. doi:10.1016/j.jimmuni.2007.03.008
106. Kim MY, Zhang T, Kraus WL. Poly(ADP-ribosylation) by PARP-1: “PARlaying” NAD⁺ into a nuclear signal. *Genes Dev* (2005) **19**(17):1951–67. doi:10.1101/gad.1331805
107. Hassa PO, Haenni SS, Elser M, Hottiger MO. Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? *Microbiol Mol Biol Rev* (2006) **70**(3):789–829. doi:10.1128/MMBR.00040-05
108. Kraus WL, Lis JT. PARP goes transcription. *Cell* (2003) **113**(6):677–83. doi:10.1016/S0092-8674(03)00433-1

109. Krishnakumar R, Kraus WL. PARP-1 regulates chromatin structure and transcription through a KDM5B-dependent pathway. *Mol Cell* (2010) **39**(5):736–49. doi:10.1016/j.molcel.2010.08.014
110. Erener S, Pétrilli V, Kassner I, Minotti R, Castillo R, Santoro R, et al. Inflammasome-activated caspase 7 cleaves PARP1 to enhance the expression of a subset of NF-κappaB target genes. *Mol Cell* (2012) **46**(2):200–11. doi:10.1016/j.molcel.2012.02.016
111. Bergsbaken T, Cookson BT. Macrophage activation redirects yersinia-infected host cell death from apoptosis to caspase-1-dependent pyroptosis. *PLoS Pathog* (2007) **3**(11):e161. doi:10.1371/journal.ppat.0030161
112. Aits S, Jaattela M. Lysosomal cell death at a glance. *J Cell Sci* (2013) **126**(Pt 9):1905–12. doi:10.1242/jcs.091181
113. Rayamajhi M, Zak DE, Chavarria-Smith J, Vance RE, Miao EA. Cutting edge: mouse NAIP1 detects the type III secretion system needle protein. *J Immunol* (2013) **191**(8):3986–9. doi:10.4049/jimmunol.1301549
114. Yang J, Zhao Y, Shi J, Shao F. Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation. *Proc Natl Acad Sci U S A* (2013) **110**(35):14408–13. doi:10.1073/pnas.1306376110
115. Tomalka J, Ganesan S, Azodi E, Patel K, Majmudar P, Hall BA, et al. A novel role for the NLRc4 inflammasome in mucosal defenses against the fungal pathogen *Candida albicans*. *PLoS Pathog* (2011) **7**(12):e1002379. doi:10.1371/journal.ppat.1002379
116. Hu B, Elinav E, Huber S, Booth CJ, Strowig T, Jin C, et al. Inflammation-induced tumorigenesis in the colon is regulated by caspase-1 and NLRc4. *Proc Natl Acad Sci U S A* (2010) **107**(50):21635–40. doi:10.1073/pnas.1016814108
117. Hu B, Elinav E, Flavell RA. Inflammasome-mediated suppression of inflammation-induced colorectal cancer progression is mediated by direct regulation of epithelial cell proliferation. *Cell Cycle* (2011) **10**(12):1936–9. doi:10.4161/cc.10.12.16008
118. Garaude J, Kent A, van Rooijen N, Blander JM. Simultaneous targeting of toll- and nod-like receptors induces effective tumor-specific immune responses. *Sci Transl Med* (2012) **4**(120):120ra16. doi:10.1126/scitranslmed.3002868

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Targeting C-type lectin receptors for cancer immunity

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C-type lectin receptors (CLRs) are a large family of soluble and trans-membrane pattern recognition receptors that are widely and primarily expressed on myeloid cells. CLRs are important for cell-cell communication and host defense against pathogens through the recognition of specific carbohydrate structures. Similar to a family of Toll-like receptors, CLRs signaling are involved in the various steps for initiation of innate immune responses and promote secretion of soluble factors such as cytokines and interferons. Moreover, CLRs contribute to endocytosis and antigen presentation, thereby fine-tune adaptive immune responses. In addition, there may also be a direct activation of acquired immunity. On the other hand, glycans, such as mannose structures, Lewis-type antigens, or GalNAc are components of tumor antigens and ligate CLRs, leading to immunoregulation. Therefore, agonists or antagonists of CLRs signaling are potential therapeutic reagents for cancer immunotherapy. We aim to overview the current knowledge of CLRs signaling and the application of their ligands on tumor-associating immune response.

Keywords: C-type lectin receptors, innate immunity, cancer immunity, immunoregulation

Introduction

Interaction between tumors and the immune system is a complex and dynamic process. The immune system consists of innate and adaptive immunity whose cooperative interactions are required for eliminating pathogens efficiently. Similar protective mechanisms are effective against cancer cells; the endogenous non-self which potentially grow into harmful cell mass. To prevent and suppress such tumor progression, the immune system utilize host defense mechanisms (1, 2).

Protecting self from harmful pathogens, and facilitating the symbiosis with harmless environmental microorganisms are the original mission of immune system. Above all, the innate immune system provides the first line of host defense against invading pathogens, with use of soluble factors, anti-microbial peptides, compliments, and natural antibodies. Initial activation of innate immune cells are mediated via pattern recognition receptors (PRRs) by recognizing characteristic structures of microorganisms (3, 4). Known PRRs are categorized into Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), and cyclic GMP-AMP synthase (cGAS) that has been recently identified.

Toll-like receptors and CLRs are involved in antigen capture, presentation, and activation of immune responses by enhancing cytokine/chemokine production and up-regulation of MHC class II molecules (5–7). NLRs predominantly recognize microbial products and endogenous danger signals, and enhance caspase activity to produce activated IL-1 β (8). RLRs and cGAS are involved in cytosolic recognition of nucleic acids and other microbial components, i.e., RLRs are sensors of

cytosolic dsRNA and cGAS are sensors of DNA, respectively, and both induce type I IFN production (9, 10).

C-type lectin receptors are a large family of receptors that encompass upwards of 1000 members with diverse functions including cell adhesion, complement activation, tissue remodeling, platelet activation, endocytosis, phagocytosis, and activation of innate immunity (11, 12). CLRs contain one or more C-type lectin-like domains, which are important for the recognition of specific carbohydrate structures of pathogens and self-antigens (13). Because of their specificity for glycans, such as mannose structures, Lewis-type antigens, or GalNAc (14, 15), CLRs may also mediate specific interactions with tumor antigens and facilitate tumor rejection. On the other hand, tumor cells devise multiple strategies to inhibit effector anti-tumor immune responses through modulating CLRs signaling (16, 17). It is therefore important to identify CLRs signaling toward immune evasion and regulate them in a specific way, while making the best application of beneficial side of CLRs signaling to mount anti-tumor immunity (Figure 1).

The Immune Regulation by CLRs and Signaling Pathways

C-type lectin receptors are widely expressed on myeloid cells, such as macrophages, neutrophils, and dendritic cells (DCs). They

contain one or more C-type lectin-like domains, which are important for recognition and internalization of glycosylated antigens. Ligand activation of CLRs initiates intracellular signaling pathways that regulate the immune response. Mounting evidence has been shown that CLRs play roles in shaping innate immune response. Many CLRs such as dectin-1, dectin-2, dectin-3, Mincle, and DEC-205 have been demonstrated to trigger cellular immune responses, including DC maturation, chemotaxis, reactive oxygen species production, and inflammasome activation (18, 19). The innate immune cells stimulated through CLRs acquire the capacity to secrete pro-inflammatory and anti-inflammatory cytokines such as TNF- α , IL-12, IL-6, IL-1 β , and IL-10 (20–22). On the other hand, ligand engagement of some CLRs, such as MICL and DCIR, has inhibitory effects on host immunity through controlling DC maturation, activation, and proliferation (23–25).

The ability of CLRs to exhibit activation or inhibition of immune response is regulated by the specific motifs in their cytoplasmic tails. Intracellular signaling through CLRs with immune-receptor tyrosine-based activation motif (ITAM) domains result in cell activation, whereas CLRs which possess immune-receptor tyrosine-based inhibition motif (ITIM) domains usually mediate inhibitory functions (18, 26). The tyrosine residues are phosphorylated by Src family kinases and a tri-molecular complex composed of CARD9, Bcl10, and MALT1 is involved in the subsequent activation of NF- κ B and expression of inflammatory

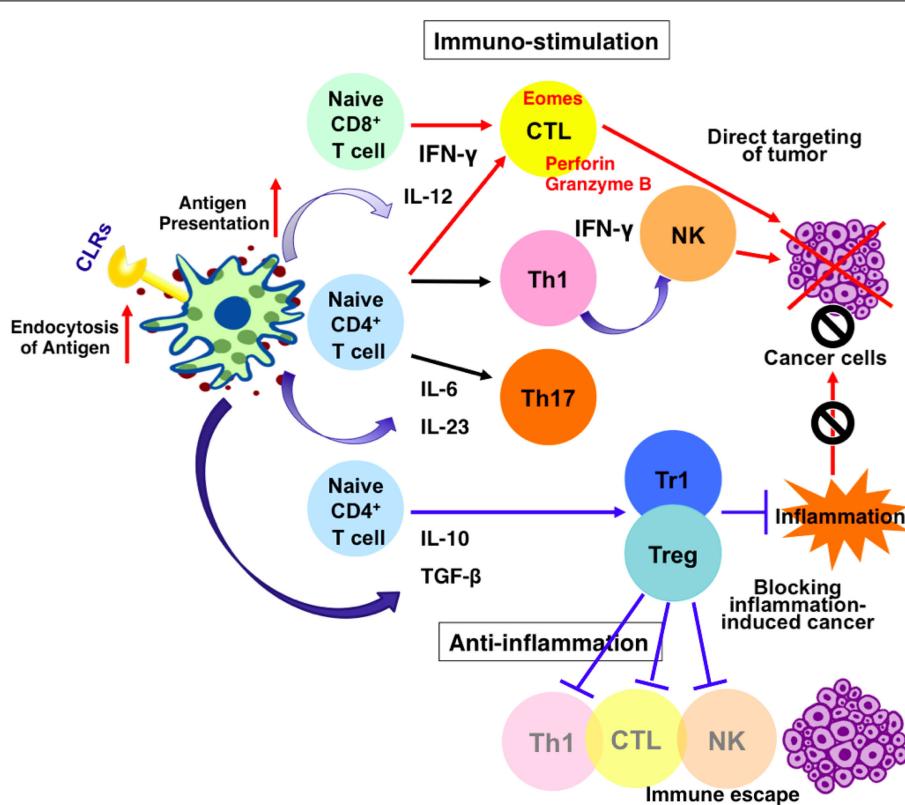
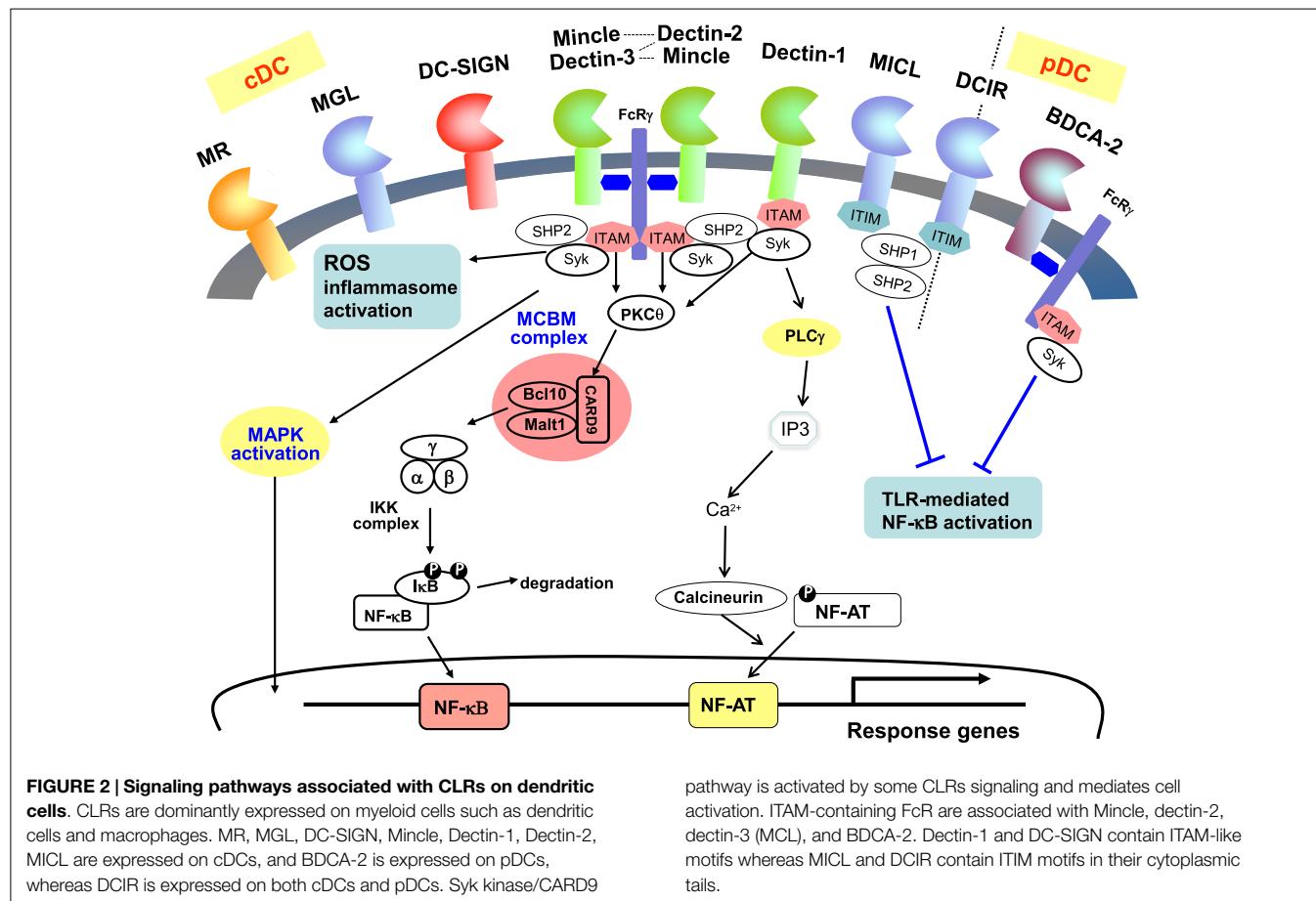


FIGURE 1 | Effects of CLRs signaling on dendritic cells and anti-cancer immune response. Stimulation of CLRs enhances endocytosis of antigens and up-regulate antigen presentation. It also increases the production of mediators such as cytokines and interferons. Thus, CLRs-ligands possibly contribute to enhance anti-tumor immunity via two independent mechanisms.

One mechanism leads to enhancement of tumoricidal activity of NK cells and cytotoxic T lymphocytes (CTL) via induction of IFN- γ and target cancer cells directly. The other mechanism support maturation of anti-inflammatory cells and lower the level of local inflammation, blocking inflammation-induced cancer.



cytokines (6, 27, 28). Syk/CARD9 pathway is utilized by dectin-1, dectin-2, dectin-3, or Mincle and plays important roles in bridging the innate immunity and adaptive immunity. Dectin-1 directly signals through Syk using cytoplasmic ITAM and activates NF- κ B, whereas dectin-2, dectin-2/dectin-3 heterodimer, and Mincle couple to Syk via the FcR γ and mediate NF- κ B activation (29–32) (summarized and depicted in Figure 2). Signaling through Syk/IRF5 is crucial for the production of dectin-1-mediated IFN- β (33). Furthermore, it is reported that dectin-1 activates inflammasomes and caspase-1, leading to production of IL-1 β (34).

Stimulation of these CLRs has been shown to drive the development of Th1, Th17, and CD8 $^{+}$ cytotoxic T lymphocytes (CTLs) cells immune responses through triggering the production of multiple cytokines (26, 35–37). In particular, dectin-1 has been found to activate NFAT also and enhance IL-2 and IL-10 production in DCs (38). A further study found that Src-homology phosphatase (SHP)-2 is an essential component, which facilitates the recruitment of Syk to the dectin-1 or the ITAM-containing adaptor FcR γ of dectin-2/3 and Mincle, and mediates the induction of Th17 responses (39). Given that T-cell immunity is essential for anti-tumor immunity, activation of ITAM-based CLRs signaling should support the development of protective immunity.

Recently, the important role of CLRs in inducing immunological tolerance has also been demonstrated. In the case of inhibitory CLRs containing ITIMs, such as DCIR (on dendritic cells) or MICL (on granulocytes and monocytes), SHP is an essential

element. Ligation of these CLRs results in phosphorylation of ITIM domain, leading to SHP-1 and SHP-2 activation and inhibits cellular activation (25). Ligation of DCIR increases the number and function of Foxp3 $^{+}$ Treg cells, thus attenuates airway hyper responsiveness and inflammation (40). BDCA-2 and DC-SIGN do not contain a cytoplasmic ITIM motif but signaling through these CLRs has been shown to modulate TLR signaling through alternative pathways (41) and be critical for the maintenance of Foxp3 $^{+}$ Treg cells (42, 43). Moreover, several CLRs such as DC-ASGPR, SIGNR1, and dectin-1 are shown to play an important role in triggering IL-10-producing suppressive CD4 $^{+}$ T cells (44–47). Recently, it is highlighted that inflammation-induced cancers are prevented by anti-inflammatory mechanisms including Tregs (48). Therefore, the anti-inflammatory pathway lead by CLRs activation may also become a therapeutic strategy for reducing the risk of such diseases (Figure 1).

Recognition of Tumor-Associated Antigen by CLRs

Tumors are recognized by the immune system through tumor antigens, including membrane proteins and altered carbohydrate molecules of glycoproteins or glycolipids on the cell surface (49). Tumor-associated carbohydrate antigens (TACAs) can be specifically recognized by CLRs. It has been shown that DC-SIGN recognizes carcinoembryonic antigen (CEA), a well-known

tumor-associated antigen overexpressed on almost all human colorectal, gastric, and pancreatic adenocarcinomas, 70% of non-small cell lung carcinomas, and 50% of breast carcinomas A (50). DC-SIGN also exhibits high affinity for Mac-2-binding protein (Mac-2BP), which increases in patients with pancreatic, breast, and lung cancers (51).

Macrophage galactose type C-type lectin (MGL) is involved in the recognition and binding of tumor-associated Neu5Ac-Tn and Neu5Gc-Tn antigens (52). It has also been demonstrated that DCs are able to recognize cancer-specific glycosylation changes of the mucin 1 (MUC1), in particular, the carbohydrate sialyl Lewis X, and the sialyl TN epitope through MGL and DC-SIGN (53, 54). In addition, MUC1, CA-125, and TAG-72 show strong binding activity to mannose receptor (MR) and induce its internalization (55–57). Further, mannose-binding lectin (MBL) has been shown to recognize glycoproteins from a human colorectal carcinoma cell line in a fucose-dependent manner (58–60).

A critical role of dectin-1, a receptor for β -glucans (61, 62), has recently been shown in recognition of N-glycan structures on tumor cells. N-glycosidase treatment markedly reduced the binding of dectin-1 to tumor cells. Importantly, tumoricidal activity of splenocytes was reduced when tumor cells were pretreated with N-glycosidase (63).

Plasmacytoid dendritic cells (pDCs) are responsible for production of type I interferons (IFN- α and β), type III IFNs (IFN- λ /IL-28/29), and pro-inflammatory cytokines. Antigen presentation by CpG-activated pDC influenced anti-tumor immune responses by promoting efficient Th17 differentiation (64). A study showed that BDCA-2 exclusively expressed on pDCs binds tumor cells via asialo-oligosaccharides containing terminal residues of galactose (65) and potently suppresses the ability of pDCs to produce type I IFNs. Such direct regulation and/or cross-regulation of TLRs signaling by BDCA-2, an inhibitory CLR,

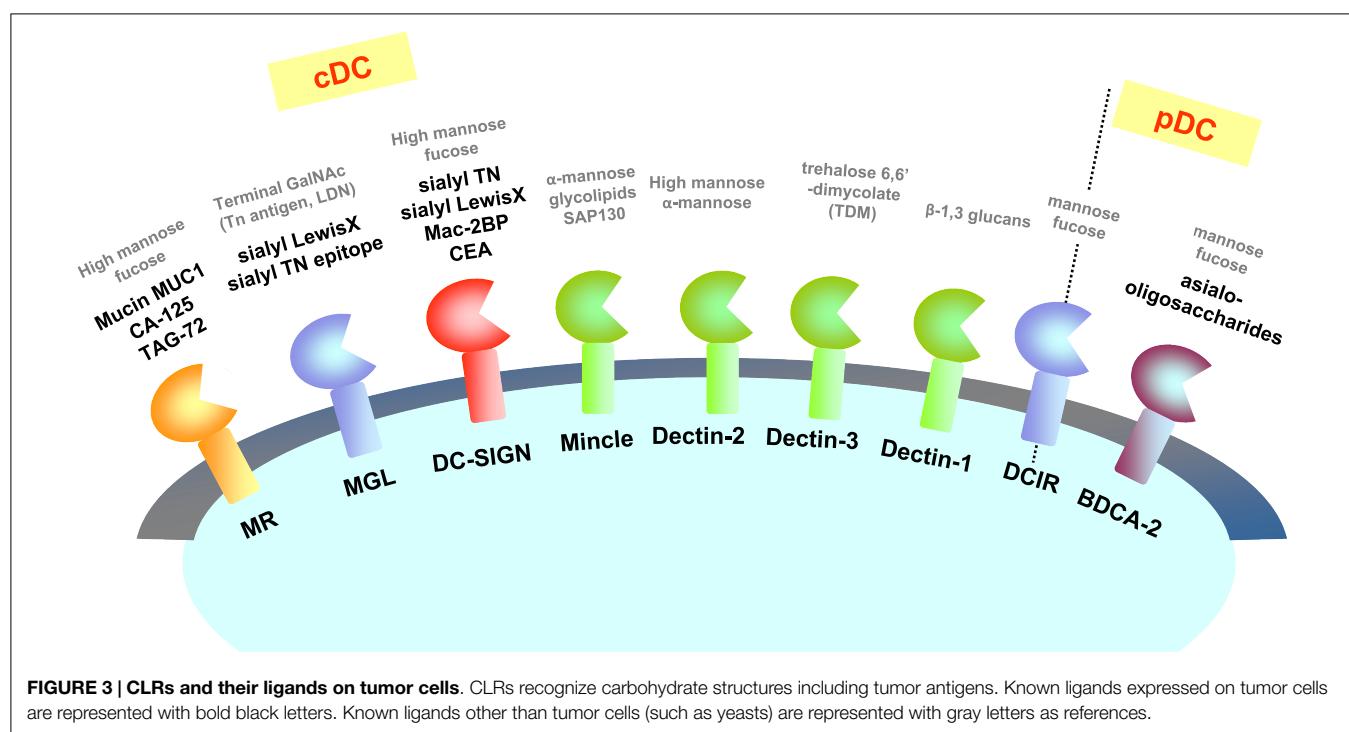
may also suppress beneficial adaptive immune response *in vivo* (Figure 3).

CLRs in Induction of Anti-Tumor Immune Response

Effective immunological eradication of tumors requires NK cells and tumor-specific CD8 $^{+}$ and CD4 $^{+}$ T cells. The potential role of CLRs improving anti-tumor activity of immune cells has been investigated. A study showed that MGL interacts with tumor-associated Tn antigens and efficiently internalized with antigens for presentation to CD4 $^{+}$ T cells (5). Furthermore, engagement of MGL using α -N-acetylgalactosamine-carrying tumor-associated antigens promotes the up-regulation of maturation markers of DCs, decrease phagocytosis, enhance motility, and most importantly increase antigen-specific CD8 $^{+}$ T-cell activation (54).

DC-SIGN is another important CLR in inducing anti-tumor immune responses. It is reported that Lewis X oligosaccharides-heparanase complex activate and enhance the maturation of DCs, leading to enhancement of antigen-specific IFN- γ production and cytotoxic T-cell response. Furthermore, the modified DCs also significantly suppress the established tumor growth and prolong the life span of tumor-bearing mice (66). In addition, glycan-modified liposomes lead to efficient antigen presentation of DCs in the presence of LPS and augment CD4 $^{+}$ and CD8 $^{+}$ effector T-cell activation via DC-SIGN-dependent pathway (67). The potency of MR to improve anti-tumor immune responses has also been conducted. Cross-presentation of antigen and strong antigen-specific immune response were induced by conjugation of glycan ligands to MR (68), which resulted in an efficient anti-tumor response and tumor clearance (69).

Dectin-1 is one of the most important CLRs and its contribution to anti-tumor immunity has been intensively studied.



Dectin-1 engagement is apparent to up-regulate costimulatory molecules such as CD80, produce TNF- α , IL-6, IL-2, IL-10, IL-12, and IL-23, and elicit potent CTL responses that protect mice from tumor challenge (35). Targeting of dectin-1 with its ligands β -glucan has been shown to increase the infiltration of activated T cells into the tumor. On the other hand, the number of tumor-caused immunosuppressive regulatory T cells and myeloid-derived suppressor cells are decreased (70, 71). More recently, the critical role of dectin-1 on enhancement of NK-mediated killing of tumor cells has been demonstrated. Dectin-1 recognize N-glycan structures on the surface of some tumor cells, and cause the activation of IRF5 transcription factor and downstream gene induction, for the full-blown tumorcidal activity of NK cells (63).

As described above, MR and DC-SIGN are major players for both immune evasion and eradication of tumor cells. Further information is necessary to clarify how these CLRs signaling affect the direction of the immunological outcome. Whether cell types or expression level is important, or ligands and microenvironment is the key, or maybe both are closely related. It is known the nature of ligands (i.e., size, form, or chemical side chains of ligands) directly modulate CLRs signaling (62). Further investigation on such regulation of CLRs signaling should lead to make the best application of beneficial side of CLRs signaling to mount anti-tumor immunity.

CLRs and Tumor Immune Evasion

C-type lectin receptors mediate beneficial effect on anti-tumor immunity via enhancement of type I and type II interferon production. On the other hand, CLRs signaling also play roles on induction of anti-inflammatory factors and molecules (23), and suppress TLRs-mediated protective immunity, thereby tolerating cancer cells escape from immune surveillance. Some examples of such process are induction of specific tolerance to tumor antigens, TGF- β and/or IL-10 production, down-regulation of MHC molecules, or up-regulation of FasL expression (72). Several studies have shown the involvement of CLRs on dysfunction of anti-tumor immune responses. The interaction between DC-SIGN and tumor-associated Le glycans results in enhanced IL-10 production, and impairs production of pro-inflammatory cytokines in tumor-associated macrophages (TAMs) from breast adenocarcinoma and melanoma patients, which leads to decrease capacity to elicit anti-tumor T-cell responses (73). Ligation of DC-SIGN and tumor-associated Le glycans also strongly enhance LPS-induced anti-inflammatory cytokine secretions of IL-6 and IL-10 by monocyte-derived DCs (50). Therefore, ligation of DC-SIGN might cause tumor progression by contributing to the maintenance of an immunosuppressive environment.

Other CLR associated with tumor immune evasion is MR. The research study showed that tumor-activated liver sinusoidal endothelial cells (LSECs) affect liver sinusoidal lymphocytes (LSLs) anti-tumor cytotoxicity and IFN- γ /IL-10 secretion through MR-dependent mechanisms. Further, immunosuppressive effects of tumor-activated LSECs on LSLs were abrogated by way of anti-mouse MR antibodies or MR $^{-/-}$ mice (74).

Recently, the important role of CLRs on modulating the function of tumor-associated cells in tumor microenvironment has

been demonstrated. TAMs are a major component of the tumor stroma, which contribute to the evasion of tumors from immune control by producing immune-suppressive cytokines such as IL-10 and TGF- β (75). It has been found that TAMs from human ovarian carcinoma abundantly express MR and dectin-1, MDL-1, MGL, DCIR. MR engagement by tumoral mucins and an agonist anti-MR antibody modulates cytokine production by TAMs toward an immune-suppressive profile: increase of IL-10, absence of IL-12, and decrease of the Th1-attracting chemokine CCL3, indicating that tumoral mucin-mediated activation of the MR on TAMs is important for their immune-suppressive phenotype (57).

In addition to expressing in immune cells, some CLRs have been shown to express on tumor cells, and involved in suppressing human immune system function. LSECtin, a cell-surface member of the C-type lectin DC-SIGN, has been found to express in B16 melanoma cells and inhibit tumor-specific T-cell responses (76). It is therefore important to identify such self-recognition toward immune evasion and regulate them in a specific way.

Genetic Variation of CLRs and Cancers

Host genetic background is one of important factors influencing susceptibility to cancer. Recently, study on single nucleotide polymorphisms (SNP) has been widely used to explore genetic susceptibility. SNPs in CLRs loci have been investigated to clarify its relationship to inflammatory responses. Because chronic inflammation is highly associated with the onset and progression of a multiplicity of human cancer, it is possible SNPs in CLRs associate with cancer susceptibility. Lu et al. (77) evaluated the correlation between colorectal cancer (CRC) risk and SNPs in three C-type lectin genes, i.e., DC-SIGN, MBL, and REG4. They found that polymorphisms in DC-SIGN gene promoter were associated with increased risk in CRC patients, while a SNP in REG4 might be a useful marker for CRC progression. The association of polymorphisms of genes encoding DC-SIGN with nasopharyngeal carcinoma risk has also been investigated. Three SNPs in the GG genotype of the rs2287886, AA genotype of the -939 promoter polymorphism, and the G allele of the rs735239 are connected with increased risk of nasopharyngeal carcinoma (78).

Mannose-binding lectin, soluble CLRs, is a plasma col lectin and one of the key molecules involved in modulating innate immune system. Low level of serum MBL is associated with increased risk of colon cancer. Polymorphisms in the 3'-untranslated region of MBL2 at rs10082466, rs2120132, rs2099902, and rs10450310 reduce MBL plasma levels and activity (79). Odds ratio for homozygous variants versus wild-type ranged from 3.17 to 4.51, whereas the 3'-UTR region haplotype consisting of these four variants had an OR of 2.10.

Ligand Treatment or Blockade of CLRs and Cancer

Based on the immune-regulatory effects of CLRs on cellular immunity, application of their ligands to cancer therapy is a scheme of promising scope. Several CLR agonists or antagonists are candidates for anti-cancer drugs. β -glucan as dectin-1 agonists has been extensively investigated for their

anti-tumor activity. In murine lung carcinoma models, orally administered particulate β -glucans significantly inhibited tumor growth (71, 80). Both oral and intraperitoneal injection of highly purified soluble β -glucan derived from *Grifola frondosa* were reported to exert anti-tumor effects in experimental murine mammary and colon adenocarcinoma tumor models (70, 81). In addition to their direct effects on specific immunity, β -glucans significantly augment the therapeutic efficacy mediated by anti-tumor monoclonal antibodies (mAbs) in murine breast, liver metastasis, lung, and lymphoma tumor models as well as in human neuroblastoma, lymphoma, and melanoma xenograft models (82). In human, the combination therapy of β -glucan and conventional chemotherapy was reported to improve the long-term survival of patients with ovarian cancer (83). A meta-analysis shows that the addition of lentinan (a purified β -glucans isolated from shiitake mushroom) to chemotherapy prolonged the survival of patients with advanced gastric cancer as compared to chemotherapy alone (84).

Some mechanisms have been proposed to explain the therapeutic response of β -glucan on anti-tumor activity. First, β -glucans are capable of eliciting anti-tumor innate and adaptive immune response via dectin-1-dependent pathway. As discussed above, β -glucans play an essential role in activating DCs and macrophages both *in vitro* and *in vivo*, leading to enhanced antigen-specific CD4 $^{+}$ and CD8 $^{+}$ T-cell responses. Moreover, β -glucans modulate the suppressive tumor microenvironment and facilitate anti-tumoral cellular immunity.

The other important role of CLRs is to serve as sensors that transduce tumor antigen into DCs. Some CLRs, including MGL, MR, DNGR-1, and DEC-205, have been found to deliver exogenous antigens on MHC-I for inducing efficient CTL immune response and MHC-II for stimulation of CD4 $^{+}$ T cells (68, 85, 86). Moreover, targeted delivery of tumor antigens via DC-SIGN, DNGR-1, and DEC-205 with an appropriate adjuvant is capable to prevent development or mediate eradication of tumor in grafted mouse models (87–90).

References

1. Kalb ML, Glaser A, Stary G, Koszik F, Stingl G. TRAIL(+) human plasmacytoid dendritic cells kill tumor cells *in vitro*: mechanisms of imiquimod- and IFN- α -mediated antitumor reactivity. *J Immunol* (2012) **188**:1583–91. doi:10.4049/jimmunol.1102437
2. Baginska J, Viry E, Paggetti J, Medves S, Berchem G, Moussay E, et al. The critical role of the tumor microenvironment in shaping natural killer cell-mediated anti-tumor immunity. *Front Immunol* (2013) **4**:490. doi:10.3389/fimmu.2013.00490
3. Huang B, Zhao J, Unkeless JC, Feng ZH, Xiong H. TLR signaling by tumor and immune cells: a double-edged sword. *Oncogene* (2008) **27**:218–24. doi:10.1038/sj.onc.1210904
4. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. *Nat Immunol* (2010) **11**:373–84. doi:10.1038/ni.1863
5. van Vliet SJ, Aarnoudse CA, Broks-van den Berg VC, Boks M, Geijtenbeek TB, van Kooyk Y. MGL-mediated internalization and antigen presentation by dendritic cells: a role for tyrosine-5. *Eur J Immunol* (2007) **37**:2075–81. doi:10.1002/eji.200636838
6. Hardison SE, Brown GD. C-type lectin receptors orchestrate antifungal immunity. *Nat Immunol* (2012) **13**:817–22. doi:10.1038/ni.2369
7. Miyake Y, Toyonaga K, Mori D, Kakuta S, Hoshino Y, Oyamada A, et al. C-type lectin MCL is an FcR γ -coupled receptor that mediates the adjuvanticity of mycobacterial cord factor. *Immunity* (2013) **38**:1050–62. doi:10.1016/j.immuni.2013.03.010
8. Koizumi Y, Toma C, Higa N, Nohara T, Nakasone N, Suzuki T. Inflammasome activation via intracellular NLRs triggered by bacterial infection. *Cell Microbiol* (2012) **14**(2):149–54. doi:10.1111/j.1462-5822.2011.01707.x
9. Chan YK, Gack MU. RIG-I-like receptor regulation in virus infection and immunity. *Curr Opin Virol* (2015) **12C**:7–14. doi:10.1016/j.coviro.2015.01.004
10. Li XD, Wu J, Gao D, Wang H, Sun L, Chen ZJ. Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. *Science* (2013) **341**(6152):1390–4. doi:10.1126/science.1244040
11. Weis WI, Taylor ME, Drickamer K. The C-type lectin superfamily in the immune system. *Immunol Rev* (1998) **163**:19–34. doi:10.1111/j.1600-065X.1998.tb01185.x
12. Zelensky AN, Gready JE. The C-type lectin-like domain superfamily. *FEBS J* (2005) **272**:6179–217. doi:10.1111/j.1742-4658.2005.05031.x
13. Osorio F, Reis e Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity* (2011) **34**:651–64. doi:10.1016/j.immuni.2011.05.001
14. Denda-Nagai K, Aida S, Saba K, Suzuki K, Moriyama S, Oo-Puthinan S, et al. Distribution and function of macrophage galactose-type C-type lectin 2 (MGL2/CD301b): efficient uptake and presentation of glycosylated antigens by dendritic cells. *J Biol Chem* (2010) **285**:19193–204. doi:10.1074/jbc.M110.113613

Along with the rapid and thorough innate immune systems, targeting CLRs has emerged as a translational approach to treat a wide variety of cancers. However, there still are some problems yet resolved and further research is required for improving the anti-tumor strategies via CLRs. Some CLRs signaling results in immunosuppressive responses, for instance, and lead to tumor immune escape. Drugs targeting immune checkpoint molecules such as PD-1, PD-L1, and CTLA-4 have recently been demonstrated beneficial and safe (91, 92). The combination of strategy targeting CLRs and immune checkpoints may improve anti-tumor effectiveness.

Concluding Remarks

C-type lectin receptors are multifunctional receptors that have a key role in the recognition of pathogens and regulating innate and adaptive immune responses. In fact, abundant evidence supports that CLRs, especially on DCs, contribute to the recognition of TACA. CLRs also play important roles in inducing anti-tumor immune response and regulate tumor-promoting inflammation. On the other hand, the function of CLRs in tumor remains unknown, therefore CLRs may act as double-edged swords in tumor-associated immune response. Specific regulation of CLRs signaling by modulating tumor microenvironment such as glycoligands and immune cells should lead to the best application of CLRs biology.

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15. Geijtenbeek TB, Van Vliet SJ, Koppelaar EA, Sanchez-Hernandez M, Vandebroucke-Grauls CM, Appelmelk B, et al. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med* (2003) **197**:7–17. doi:10.1084/jem.20021229
16. Kerkar SP, Restifo NP. Cellular constituents of immune escape within the tumor microenvironment. *Cancer Res* (2012) **72**:3125–30. doi:10.1158/0008-5472.CAN-11-4094
17. Wang W, Guo H, Geng J, Zheng X, Wei H, Sun R, et al. Tumor-released Galectin-3, a soluble inhibitory ligand of human NKp30, plays an important role in tumor escaping from NK cell attack. *J Biol Chem* (2014) **289**:33311–9. doi:10.1074/jbc.M114.603464
18. Sancho D, Reis e Sousa C. Signaling by myeloid C-type lectin receptors in immunity and homeostasis. *Annu Rev Immunol* (2012) **30**:491–529. doi:10.1146/annurev-immunol-031210-101352
19. Zhu LL, Zhao XQ, Jiang C, You Y, Chen XP, Jiang YY, et al. C-type lectin receptors Dectin-3 and Dectin-2 form a heterodimeric pattern-recognition receptor for host defense against fungal infection. *Immunity* (2013) **39**(2):324–34. doi:10.1016/j.jimmuni.2013.05.017
20. Ifrim DC, Joosten LA, Kullberg BJ, Jacobs L, Jansen T, Williams DL, et al. *Candida albicans* primes TLR cytokine responses through a Dectin-1/Raf-1-mediated pathway. *J Immunol* (2013) **190**:4129–35. doi:10.4049/jimmunol.1202611
21. Yonekawa A, Saito S, Hoshino Y, Miyake Y, Ishikawa E, Suzukawa M, et al. Dectin-2 is a direct receptor for mannose-capped lipoarabinomannan of mycobacteria. *Immunity* (2014) **41**:402–13. doi:10.1016/j.jimmuni.2014.08.005
22. Behler F, Steinwede K, Balboa L, Ueberberg B, Maus R, Kirchhoff G, et al. Role of Mincle in alveolar macrophage-dependent innate immunity against mycobacterial infections in mice. *J Immunol* (2012) **189**:3121–9. doi:10.4049/jimmunol.1201399
23. Yan H, Ohno N, Tsuji NM. The role of C-type lectin receptors in immune homeostasis. *Int Immunopharmacol* (2013) **16**(3):353–7. doi:10.1016/j.intimp.2013.04.013
24. Marshall AS, Willment JA, Pyz E, Dennehy KM, Reid DM, Dri P, et al. Human MICL (CLEC12A) is differentially glycosylated and is down-regulated following cellular activation. *Eur J Immunol* (2006) **36**:2159–69. doi:10.1002/eji.200535628
25. Lambert AA, Barabé F, Gilbert C, Tremblay MJ. DCIR-mediated enhancement of HIV-1 infection requires the ITIM-associated signal transduction pathway. *Blood* (2011) **117**:6589–99. doi:10.1182/blood-2011-01-331363
26. Geijtenbeek TB, Gringhuis SI. Signaling through C-type lectin receptors: shaping immune responses. *Nat Rev Immunol* (2009) **9**:465–79. doi:10.1038/nri2569
27. Drummond RA, Brown GD. Signalling C-type lectins in antimicrobial immunity. *PLoS Pathog* (2013) **9**:e1003417. doi:10.1371/journal.ppat.1003417
28. Hoving JC, Wilson GJ, Brown GD. Signalling C-type lectin receptors, microbial recognition and immunity. *Cell Microbiol* (2014) **16**:185–94. doi:10.1111/cmi.12249
29. Rogers NC, Slack EC, Edwards AD, Nolte MA, Schulz O, Schweighoffer E, et al. Syk-dependent cytokine induction by dectin-1 reveals a novel pattern recognition pathway for C-type lectins. *Immunity* (2005) **22**:507–17. doi:10.1016/j.jimmuni.2005.06.005
30. Hara H, Saito T. CARD9 versus CARMA1 in innate and adaptive immunity. *Trends Immunol* (2009) **30**(5):234–42. doi:10.1016/j.it.2009.03.002
31. Sato K, Yang XL, Yudate T, Chung JS, Wu J, Luby-Phelps K, et al. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. *J Biol Chem* (2006) **281**:38854–66. doi:10.1074/jbc.M606542200
32. Yamasaki S, Ishikawa E, Sakuma M, Ogata K, Saito T. Mincle is an ITAM-couples activating receptor that senses damaged cells. *Nat Immunol* (2008) **9**:1179–88. doi:10.1038/ni.1651
33. del Fresno C, Soulard D, Roth S, Blazek K, Udalova I, Sancho D, et al. Interferon- β production via Dectin-1-Syk-IRF5 signaling in dendritic cells is crucial for immunity to *C. albicans*. *Immunity* (2013) **38**(6):1176–86. doi:10.1016/j.jimmuni.2013.05.010
34. Gringhuis SI, Kaptein TM, Wevers BA, Theelen B, van der Vlist M, Boekhout T, et al. Dectin-1 is an extracellular pathogen sensor for the induction and processing of IL-1 β via a noncanonical caspase-8 inflammasome. *Nat Immunol* (2012) **13**:246–54. doi:10.1038/ni.2222
35. Leibundgut-Landmann S, Osorio F, Brown GD, Reis e Sousa C. Stimulation of dendritic cells via the dectin-1/Syk pathway allows priming of cytotoxic T-cell responses. *Blood* (2008) **112**:4971–80. doi:10.1182/blood-2008-05-158469
36. Saito S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity* (2010) **32**:681–91. doi:10.1016/j.jimmuni.2010.05.001
37. Desel C, Werninghaus K, Ritter M, Jozefowski K, Wenzel J, Russkamp N, et al. The Mincle-activating adjuvant TDB induces MyD88-dependent Th1 and Th17 responses through IL-1R signaling. *PLoS One* (2013) **8**:e53531. doi:10.1371/journal.pone.0053531
38. Xu S, Huo J, Lee KG, Kurosaki T, Lam KP. Phospholipase Cgamma2 is critical for Dectin-1-mediated Ca $^{2+}$ flux and cytokine production in dendritic cells. *J Biol Chem* (2009) **284**:7038–46. doi:10.1074/jbc.M806650200
39. Deng Z, Ma S, Zhou H, Zang A, Fang Y, Li T, et al. Tyrosine phosphatase SHP-2 mediates C-type lectin receptor-induced activation of the kinase Syk and anti-fungal TH17 responses. *Nat Immunol* (2015) **16**(6):642–52. doi:10.1038/ni.3155
40. Massoud AH, Yona M, Xue D, Chouiali F, Alturaihi H, Ablona A, et al. Dendritic cell immunoreceptor: a novel receptor for intravenous immunoglobulin mediates induction of regulatory T cells. *J Allergy Clin Immunol* (2014) **133**:853–63.e5. doi:10.1016/j.jaci.2013.09.02
41. Jahn PS, Zanker KS, Schmitz J, Dzionek A. BDCA-2 signaling inhibits TLR-9-agonist-induced plasmacytoid dendritic cell activation and antigen presentation. *Cell Immunol* (2010) **265**(1):15–22. doi:10.1016/j.cellimm.2010.06.005
42. Chappell CP, Giltay NV, Draves KE, Chen C, Hayden-Ledbetter MS, Shlomchik MJ, et al. Targeting antigens through blood dendritic cell antigen 2 on plasmacytoid dendritic cells promotes immunologic tolerance. *J Immunol* (2014) **192**:5789–801. doi:10.4049/jimmunol.1303259
43. Cai M, Wu J, Mao C, Ren J, Li P, Li X, et al. A lectin-EGF antibody promotes regulatory T cells and attenuates nephrotoxic nephritis via DC-SIGN on dendritic cells. *J Transl Med* (2013) **11**:103. doi:10.1186/1479-5876-11-103
44. Zhou Y, Kawasaki H, Hsu SC, Lee RT, Yao X, Plunkett B. Oral tolerance to food-induced systemic anaphylaxis mediated by the C-type lectin SIGNR1. *Nat Med* (2010) **16**:1128–33. doi:10.1038/nm.2201
45. Li D, Romain G, Flamar AL, Duluc D, Dullaerts M, Li XH, et al. Targeting self- and foreign antigens to dendritic cells via DC-ASGPR generates IL-10-producing suppressive CD4+ T cells. *J Exp Med* (2012) **209**:109–21. doi:10.1016/j.jem.20110399
46. Kawashima S, Hirose K, Iwata A, Takahashi K, Ohkubo A, Tamachi T, et al. β -glucan curdlan induces IL-10-producing CD4+ T cells and inhibits allergic airway inflammation. *J Immunol* (2012) **189**(12):5713–21. doi:10.4049/jimmunol.1201521
47. Karumuthil-Melothil S, Gudi R, Johnson BM, Perez N, Vasu C. Fungal β -glucan, a Dectin-1 ligand, promotes protection from type 1 diabetes by inducing regulatory innate immune response. *J Immunol* (2014) **193**:3308–21. doi:10.4049/jimmunol.1400186
48. Diakos CI, Charles KA, McMillan DC, Clarke SJ. Cancer-related inflammation and treatment effectiveness. *Lancet Oncol* (2014) **15**(11):e493–503. doi:10.1016/S1470-2045(14)70263-3
49. Novellino L, Castelli C, Parmiani G. A listing of human tumor antigens recognized by T cells: March 2004 update. *Cancer Immunol Immunother* (2005) **54**:187–207. doi:10.1007/s00262-004-0560-6
50. Nonaka M, Ma BY, Murai R, Nakamura N, Baba M, Kawasaki N, et al. Glycosylation-dependent interactions of C-type lectin DC-SIGN with colorectal tumor-associated Lewis glycans impair the function and differentiation of monocyte-derived dendritic cells. *J Immunol* (2008) **180**:3347–56. doi:10.4049/jimmunol.180.5.3347
51. Nonaka M, Ma BY, Imaeda H, Kawabe K, Kawasaki N, Hodohara K, et al. Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) recognizes a novel ligand, Mac-2-binding protein, characteristically expressed on human colorectal carcinomas. *J Biol Chem* (2011) **286**:22403–13. doi:10.1074/jbc.M110.215301
52. Mortezaei N, Behnken HN, Kurze AK, Ludewig P, Buck F, Meyer B, et al. Tumor-associated Neu5Ac-Tn and Neu5Gc-Tn antigens bind to C-type lectin CLEC10A (CD301, MGL). *Glycobiology* (2013) **23**:844–52. doi:10.1093/glycob/cwt021
53. Aarnoudse CA, Vallejo JJG, Saeland E, Van Kooyk Y. Recognition of tumor glycans by antigen-presenting cells. *Curr Opin Immunol* (2006) **18**:105–11. doi:10.1016/j.coi.2005.11.001
54. Napoletano C, Zizzari IG, Rughetti A, Rahimi H, Irimura T, Clausen H, et al. Targeting of macrophage galactose-type C-type lectin (MGL) induces DC signaling and activation. *Eur J Immunol* (2012) **42**(4):936–45. doi:10.1002/eji.201142086

55. Chieppa M, Bianchi G, Doni A, Del Prete A, Sironi M, Laskarin G, et al. Cross-linking of the mannose receptor on monocyte-derived dendritic cells activates an anti-inflammatory immunosuppressive program. *J Immunol* (2003) **171**:4552–60. doi:10.4049/jimmunol.171.9.4552
56. Hiltbold EM, Vlad AM, Ciborowski P, Watkins SC, Finn OJ. The mechanism of unresponsiveness to circulating tumor antigen MUC1 is a block in intracellular sorting and processing by dendritic cells. *J Immunol* (2000) **165**:3730–41. doi:10.4049/jimmunol.165.7.3730
57. Allavena P, Chieppa M, Bianchi G, Solinas G, Fabbri M, Laskarin G, et al. Engagement of the mannose receptor by tumoral mucins activates an immune suppressive phenotype in human tumor-associated macrophages. *Clin Dev Immunol* (2010) **2010**:547179. doi:10.1155/2010/547179
58. Terada M, Khoo KH, Inoue R, Chen CI, Yamada K, Sakaguchi H, et al. Characterization of oligosaccharide ligands expressed on SW1116 cells recognized by mannan-binding protein. A highly fucosylated polygalactosamine type N-glycan. *J Biol Chem* (2005) **280**:10897–913. doi:10.1074/jbc.M413092200
59. Kawasaki N, Lin CW, Inoue R, Khoo KH, Kawasaki N, Ma BY, et al. Highly fucosylated N-glycan ligands for mannan-binding protein expressed specifically on CD26 (DPPVI) isolated from a human colorectal carcinoma cell line, SW1116. *Glycobiology* (2009) **19**:437–50. doi:10.1093/glycob/cwn158
60. Nonaka M, Imaeda H, Matsumoto S, Yong Ma B, Kawasaki N, Mekata E, et al. Mannan-binding protein, a C-type serum lectin, recognizes primary colorectal carcinomas through tumor-associated Lewis glycans. *J Immunol* (2014) **192**:1294–301. doi:10.4049/jimmunol.1203023
61. Brown GD. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat Rev Immunol* (2006) **6**:33–43. doi:10.1038/nri1745
62. Goodridge HS, Reyes CN, Becker CA, Katsumoto TR, Ma J, Wolf AJ, et al. Activation of the innate immune receptor Dectin-1 upon formation of a ‘phagocytic synapse’. *Nature* (2011) **472**(7344):471–5. doi:10.1038/nature10071
63. Chiba S, Ikushima H, Ueki H, Yanai H, Kimura Y, Hangai S, et al. Recognition of tumor cells by Dectin-1 orchestrates innate immune cells for anti-tumor responses. *Elife* (2014) **3**:e04177. doi:10.7554/elife.04177
64. Guery L, Dubrot J, Lippens C, Brighouse D, Malinge P, Irla M, et al. Ag-presenting CpG-activated pDCs prime Th17 cells that induce tumor regression. *Cancer Res* (2014) **74**:6430–40. doi:10.1158/0008-5472.CAN-14-1149
65. Riboldi E, Daniele R, Parola C, Inforzato A, Arnold PL, Bosio D, et al. Human C-type lectin domain family 4, member C (CLEC4C/BDCA-2/CD303) is a receptor for asialo-galactosyl-oligosaccharides. *J Biol Chem* (2011) **286**:35329–33. doi:10.1074/jbc.C111.290494
66. Chen H, Yuan B, Zheng Z, Liu Z, Wang S, Lewis X. Oligosaccharides-heparanase complex targeting to DCs enhance antitumor response in mice. *Cell Immunol* (2011) **269**(2):144–8. doi:10.1016/j.cellimm.2011.03.021
67. Unger WW, van Beelen AJ, Bruijns SC, Joshi M, Fehres CM, van Bloois L, et al. Glycan-modified liposomes boost CD4+ and CD8+ T-cell responses by targeting DC-SIGN on dendritic cells. *J Control Release* (2012) **160**(1):88–95. doi:10.1016/j.jconrel.2012.02.007
68. Singh SK, Streng-Ouwendijk I, Litjens M, Kalay H, Burgdorf S, Saeland E, et al. Design of neo-glycoconjugates that target the mannose receptor and enhance TLR-independent cross-presentation and Th1 polarization. *Eur J Immunol* (2011) **41**(4):916–25. doi:10.1002/eji.201040762
69. He LZ, Crocker A, Lee J, Mendoza-Ramirez J, Wang XT, Vitale LA, et al. Antigenic targeting of the human mannose receptor induces tumor immunity. *J Immunol* (2007) **178**(10):6259–67. doi:10.4049/jimmunol.178.10.6259
70. Masuda Y, Inoue M, Miyata A, Mizuno S, Nanba H. Maitake beta-glucan enhances therapeutic effect and reduces myelosuppression and nephrotoxicity of cisplatin in mice. *Int Immunopharmacol* (2009) **9**:620–6. doi:10.1016/j.intimp.2009.02.005
71. Tian J, Ma J, Ma K, Guo H, Baidoo SE, Zhang Y, et al. β -Glucan enhances antitumor immune responses by regulating differentiation and function of monocytic myeloid-derived suppressor cells. *Eur J Immunol* (2013) **43**(5):1220–30. doi:10.1002/eji.201242841
72. Chouaib S, Asselin-Paturel C, Mami-Chouaib F, Caignard A, Blay JY. The host-tumor immune conflict: from immunosuppression to resistance and destruction. *Immunol Today* (1997) **18**(10):493–7. doi:10.1016/S0167-5699(97)01115-8
73. Dominguez-Soto A, Sierra-Filardi E, Puig-Kröger A, Pérez-Maceda B, Gómez-Aguado F, Corcuer MT, et al. Dendritic cell-specific ICAM-3-grabbing nonintegrin expression on M2-polarized and tumor-associated macrophages is macrophage-CSF dependent and enhanced by tumor-derived IL-6 and IL-10. *J Immunol* (2011) **186**(4):2192–200. doi:10.4049/jimmunol.1000475
74. Arteta B, Lasuen N, Lopategi A, Sveinbjörnsson B, Smedsrød B, Vidal-Vanaclocha F. Colon carcinoma cell interaction with liver sinusoidal endothelium inhibits organ-specific antitumor immunity through interleukin-1-induced mannose receptor in mice. *Hepatology* (2010) **51**(6):2172–82. doi:10.1002/hep.23590
75. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. *Immunity* (2014) **41**(1):49–61. doi:10.1016/j.immuni.2014.06.010
76. Xu F, Liu J, Liu D, Liu B, Wang M, Hu Z, et al. LSECtin expressed on melanoma cells promotes tumor progression by inhibiting antitumor T-cell responses. *Cancer Res* (2014) **74**(13):3418–28. doi:10.1158/0008-5472.CAN-13-2690
77. Lu S, Bevier M, Huhn S, Sainz J, Lascorz J, Pardini B, et al. Genetic variants in C-type lectin genes are associated with colorectal cancer susceptibility and clinical outcome. *Int J Cancer* (2013) **133**(10):2325–33. doi:10.1002/ijc.28251
78. Xu YF, Liu WL, Dong JQ, Liu WS, Feng QS, Chen LZ, et al. Sequencing of DC-SIGN promoter indicates an association between promoter variation and risk of nasopharyngeal carcinoma in cantonese. *BMC Med Genet* (2010) **11**:161. doi:10.1186/1471-2350-11-161
79. Zanetti KA, Haznadar M, Welsh JA, Robles AI, Ryan BM, McClary AC, et al. 3'-UTR and functional secretor haplotypes in mannose-binding lectin 2 are associated with increased colon cancer risk in African Americans. *Cancer Res* (2012) **72**(6):1467–77. doi:10.1158/0008-5472.CAN-11-3073
80. Li B, Cai Y, Qi C, Hansen R, Ding C, Mitchell TC, et al. Orally administered particulate beta-glucan modulates tumor-capturing dendritic cells and improves antitumor T-cell responses in cancer. *Clin Cancer Res* (2010) **16**(21):5153–64. doi:10.1158/1078-0432.CCR-10-0820
81. Masuda Y, Inoue H, Ohta H, Miyake A, Konishi M, Nanba H. Oral administration of soluble β -glucans extracted from *Grifola frondosa* induces systemic antitumor immune response and decreases immunosuppression in tumor-bearing mice. *Int J Cancer* (2013) **133**(1):108–19. doi:10.1002/ijc.27999
82. Modak S, Koehne G, Vickers A, O'Reilly RJ, Cheung NK. Rituximab therapy of lymphoma is enhanced by orally administered (1 – >3),(1 – >4)-D-beta-glucan. *Leuk Res* (2005) **29**:679–83. doi:10.1016/j.leukres.2004.10.008
83. Inoue M, Tanaka Y, Sugita N, Yamasaki M, Yamanaka T, Minagawa J, et al. Improvement of long-term prognosis in patients with ovarian cancers by adjuvant sizofiran immunotherapy: a prospective randomized controlled study. *Biotherapy* (1993) **6**(1):13–8. doi:10.1007/BF01877381
84. Oba K, Kobayashi M, Matsui T, Kodera Y, Sakamoto J. Individual patient based meta-analysis of lentinan for unresectable/recurrent gastric cancer. *Anticancer Res* (2009) **29**(7):2739–45.
85. Napoletano C, Rughetti A, Agervig Tarp MP, Coleman J, Bennett EP, Picco G, et al. Tumor-associated Tn-MUC1 glycoform is internalized through the macrophage galactose-type C-type lectin and delivered to the HLA class I and II compartments in dendritic cells. *Cancer Res* (2007) **67**(17):8358–67. doi:10.1158/0008-5472.CAN-07-1035
86. Singh SK, Streng-Ouwendijk I, Litjens M, Saeland E, van Kooyk Y. Tumour-associated glycan modifications of antigen enhance MGL2 dependent uptake and MHC class I restricted CD8 T cell responses. *Int J Cancer* (2011) **128**(6):1371–83. doi:10.1002/ijc.25458
87. Mahnke K, Qian Y, Fondel S, Brueck J, Becker C, Enk AH. Targeting of antigens to activated dendritic cells in vivo cures metastatic melanoma in mice. *Cancer Res* (2005) **65**(15):7007–12. doi:10.1158/0008-5472.CAN-05-0938
88. Kretz-Rommel A, Qin F, Dakappagari N, Torensma R, Faas S, Wu D, et al. In vivo targeting of antigens to human dendritic cells through DC-SIGN elicits stimulatory immune responses and inhibits tumor growth in grafted mouse models. *J Immunother* (2007) **30**(7):715–26. doi:10.1097/CJI.0b013e318135472c
89. Sancho D, Mourao-Sa D, Joffre OP, Schulz O, Rogers NC, Pennington DJ, et al. Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin. *J Clin Invest* (2008) **118**(6):2098–110. doi:10.1172/JCI34584
90. Macho-Fernandez E, Cruz LJ, Ghinnagow R, Fontaine J, Bialecki E, Frisch B, et al. Targeted delivery of α -galactosylceramide to CD8 α + dendritic cells optimizes type I NKT cell-based antitumor responses. *J Immunol* (2014) **193**(2):961–9. doi:10.4049/jimmunol.1303029
91. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. *N Engl J Med* (2015) **372**(21):2018–28. doi:10.1056/NEJMoa1501824

92. Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, et al. Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. *N Engl J Med* (2015) 372(21):2006–17. doi:10.1056/NEJMoa1414428

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Pattern recognition and signaling mechanisms of RIG-I and MDA5

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Most organisms rely on innate immune receptors to recognize conserved molecular structures from invading microbes. Two essential innate immune receptors, RIG-I and MDA5, detect viral double-stranded RNA in the cytoplasm. The inflammatory response triggered by these RIG-I-like receptors (RLRs) is one of the first and most important lines of defense against infection. RIG-I recognizes short RNA ligands with 5'-triphosphate caps. MDA5 recognizes long kilobase-scale genomic RNA and replication intermediates. Ligand binding induces conformational changes and oligomerization of RLRs that activate the signaling partner MAVS on the mitochondrial and peroxisomal membranes. This signaling process is under tight regulation, dependent on post-translational modifications of RIG-I and MDA5, and on regulatory proteins including unanchored ubiquitin chains and a third RLR, LGP2. Here, we review recent advances that have shifted the paradigm of RLR signaling away from the conventional linear signaling cascade. In the emerging RLR signaling model, large multimeric signaling platforms generate a highly cooperative, self-propagating, and context-dependent signal, which varies with the subcellular localization of the signaling platform.

Keywords: pathogen-associated molecular pattern, nucleic-acid sensor, RecA-like DEAD-box (DExD/H-box) RNA helicase, caspase recruitment domain, signal transduction, signalosome, prion-like switch, amyloid-like aggregation

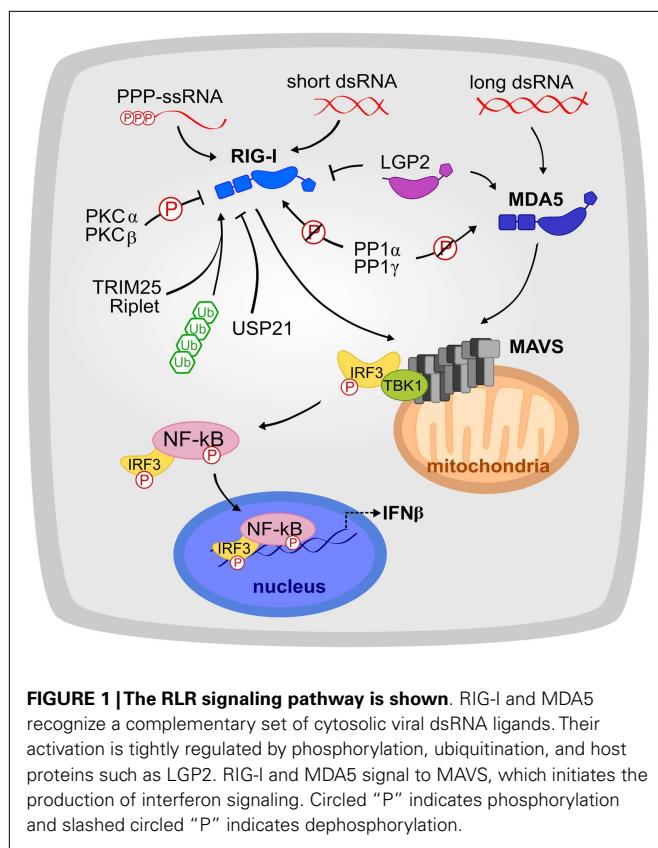
INTRODUCTION

Eukaryotic organisms rely on their innate immune system to detect viruses and other microbes. Innate immune receptors detect chemical patterns or structures that are broadly conserved in microbes, including bacterial cell wall components, microbial nucleic acids, and certain highly conserved proteins. These pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors that fall into several families, including Toll-like receptors (TLRs), NOD like receptors (NLRs), C-type lectin receptors (CLRs), and RIG-I-like receptors (RLRs). At the cell surface and in endocytic compartments, TLRs are the most important family of molecular sentries for the innate immune recognition of a wide range of microbial patterns outside the cytosol (1). CLRs, such as Dectin1, are localized on the cell surface and principally recognize fungal pathogens (2). In the cytosol, NODs and other NLRs recognize cell wall fragments and other bacterial components (3). This review will focus on the RLRs, which are found in the cytosol and recognize viral double-stranded RNA (dsRNA). Innate immune receptors from all families have in common that they nucleate the assembly of large multimeric protein complexes with their signaling adaptors, which include most notably MyD88, MAVS, ASC, and RIP2 (4). These oligomeric assemblies rapidly activate and amplify potent inflammatory antimicrobial responses, principally through the activation of NF-κB, type I interferons, or caspase 1.

Nucleic acids are the largest, and arguably the most important class of ligands for innate immune receptors. To avoid signaling

in response to endogenous nucleic acids, which are ubiquitous in the cytoplasm and nucleus, innate immune sensors must recognize specific patterns in specific subcellular locations. (1) A subfamily of TLRs (TLRs 3, 7, 8, and 9) recognizes microbial DNA and RNA ligands exclusively in endolysosomal compartments (5–9). In the cytosol, two essential immune sensors, RIG-I and MDA5, detect viral dsRNA (10–12). Several different sensors recognize double-stranded DNA (dsDNA) in the cytoplasm, including proteins from the AIM2 family, the DDX family, RNA polymerase III, and cyclic GMP–AMP synthase (13, 14). Ligand binding by each of these sensors induces a conformational change that directs the cooperative assembly of large oligomeric signaling platforms, leading to the recruitment and activation of signaling adaptors (4). The rapidly ensuing inflammatory response culminates in activation of the NF-κB and type I interferon signaling pathways (Figure 1). This response is one of the first and most important lines of defense against infection and is responsible for the activation of the adaptive immune system (1). Innate immune receptors therefore play pivotal roles as master-regulators of inflammation.

Many viruses deliver an RNA genome into the cytoplasm or rely on a replication or transcription step that generates viral RNA in the cytoplasm. Infection by these viruses is primarily detected by RIG-I and MDA5, also referred to as the RLRs. RIG-I and MDA5 sense complementary sets of viral RNA ligands (10–12, 15). RIG-I recognizes 5'-phosphorylated blunt ends of viral genomic dsRNA, whereas MDA5 binds internally to long dsRNA with no end specificity (10–12). RIG-I and MDA5 both



have tandem N-terminal caspase recruitment domains (CARDs) with death domain folds, a DExD/H-box helicase (consisting of two RecA-like helicase domains, Hel1 and Hel2, and an insert domain, Hel2i), and a C-terminal domain (CTD) (Figure 2A). In the absence of dsRNA, RIG-I has a closed inactive conformation (16). RNA binding through the helicase and CTD domains (17, 18) releases the CARDs, which then recruit and activate the signaling adaptor MAVS (IPS-1) (19). In contrast, MDA5 does not sequester its CARDs (20) and cooperatively assembles into ATP-sensitive filaments on dsRNA (20–22). Moreover, the MDA5 CTD is required for cooperative filament assembly but not for RNA binding (20, 23, 24). The MDA5 CARDs have been proposed to nucleate the assembly of MAVS into its active polymeric form (20, 25) in a process that can be promoted by K63-linked polyubiquitin chains (26). The self-propagating amyloid-like properties of MAVS polymers amplify signaling (25). RLR signaling is regulated by numerous host and viral factors through various mechanisms, including ubiquitin-dependent proteolytic degradation and cleavage of MAVS by virally encoded proteases (27–29). A third RLR, LGP2, lacks CARDs and exerts co-stimulatory and inhibitory functions on MDA5 and RIG-I, respectively (30–33).

Recent biochemical, biophysical, and cellular studies have greatly advanced our understanding at the molecular level of the mechanisms of pattern recognition and signaling by RIG-I and MDA5. Here, we review these studies and their implications on the current models of microbe-induced inflammation, auto-inflammation, and inflammation-induced cancer.

RECOGNITION OF dsRNA IN THE CYTOSOL BY RIG-I AND MDA5

THE MOLECULAR DETERMINANTS OF LIGAND RECOGNITION BY RLRs

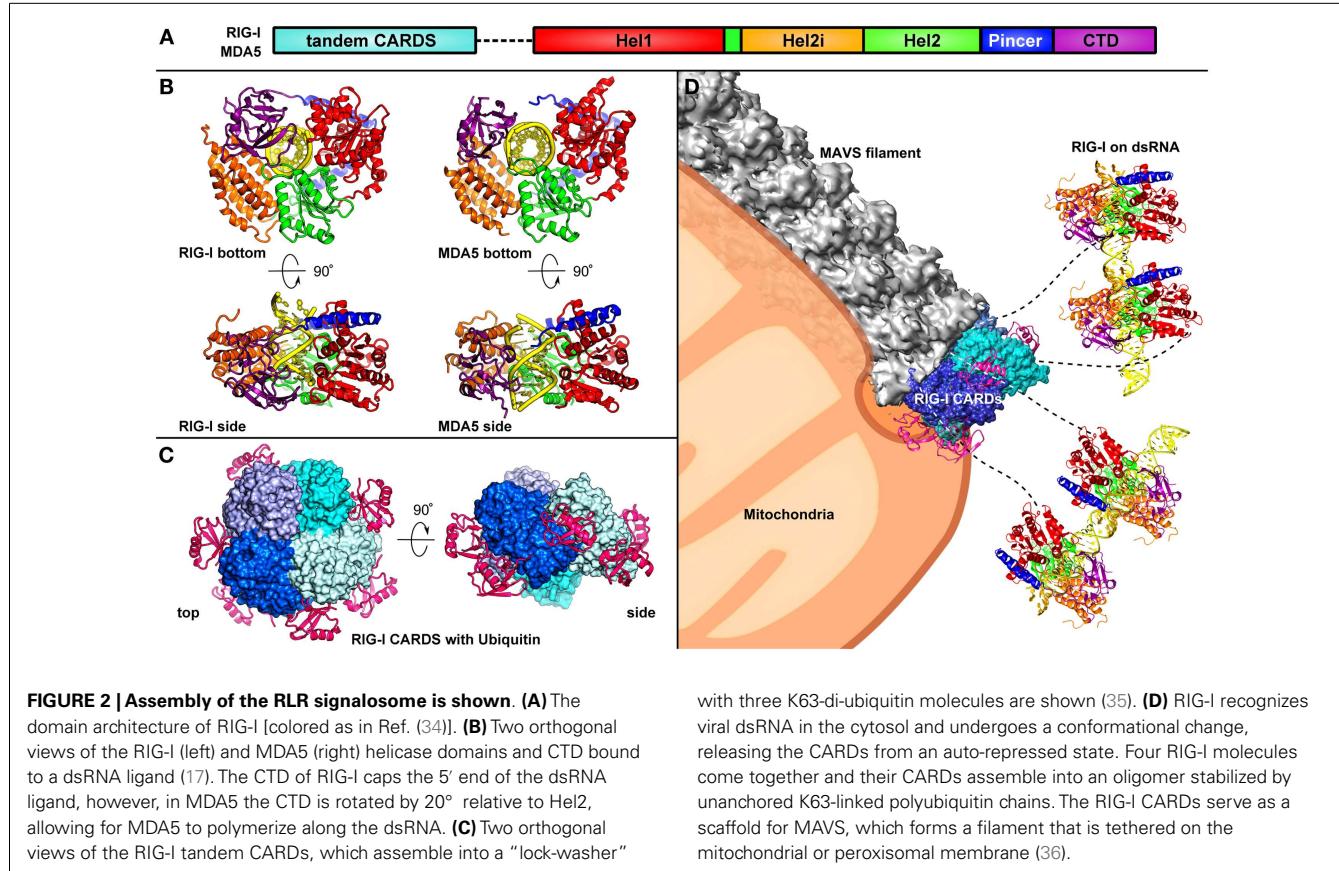
RIG-I preferentially binds to short (<300 bp) dsRNAs that have blunt ends and a 5' triphosphate (5'-ppp) moiety, facilitating discrimination between host and viral dsRNA (10–12). Crystal structures of RIG-I bound to a 12-bp dsRNA ligand and of unliganded RIG-I have provided detailed insights into the mechanism of activation of this receptor. In the absence of dsRNA ligand, RIG-I is in an auto-repressed state: the domains in the helicase domain are in an open conformation and the tandem CARDs form contacts with the Hel2i domain. This conformation sterically prevents the CARDs from binding to polyubiquitin or to CARDs from other binding partners, thereby preventing signaling to MAVS (16).

Upon the presentation of a viral dsRNA, RIG-I undergoes significant conformational rearrangement. The CTD binds tightly to the 5'-ppp and the helicase domains wrap around dsRNA, adopting a more compact configuration (16–18) (Figure 2B). RIG-I recognizes RNA primarily through non-specific interactions with the phosphate sugar backbone, predominantly by the Hel2i domain. This conformational change allows ATP to bind RIG-I, a necessary step for the activation of RIG-I (16–18). Although the CARDs were absent from the RNA-bound RIG-I crystal structures, biochemical studies and small angle X-ray scattering data indicate that the tandem CARDs are released from the Hel2i domain in the active form of RIG-I (17, 18).

In contrast to RIG-I, MDA5 preferentially binds internally to long dsRNA (>1,000 bp) with no end specificity (10–12) and cooperatively assembles into a filament on the dsRNA (20, 21). Unlike RIG-I, the CARDs of MDA5 are not sequestered in the absence of ligand (20). The forced proximity of the CARDs upon MDA5 filament formation induces oligomerization of MDA5 CARDs, forming a scaffold for binding and oligomerization of MAVS CARD (see Activation of MAVS and Downstream Signaling). Notably, the atomic structures of the MDA5 CARDs have not yet been determined.

A crystal structure of the MDA5 helicase domains and CTD bound to dsRNA revealed how MDA5, despite having a similar domain architecture as RIG-I, recognizes dsRNA in a different manner (Figure 2B). The helicase domains of MDA5 wrap around dsRNA similarly to the helicase domains of RIG-I (34, 37). However, consistent with the observation that MDA5 is not preferentially activated by 5'-ppp dsRNA (10–12), the MDA5 CTD is rotated by 20°, bringing it closer to the dsRNA, as compared to the RIG-I structure. The CTD also forms contact with Hel1 in MDA5, such that MDA5 forms a closed ring around the dsRNA (37). This orientation of the CTD promotes cooperative filament formation along dsRNA, initiated from internal sites in the dsRNA rather than from one of the ends (20, 21, 34).

The RLRs are part of the DExD/H-box helicase family based on their domain architecture (33), but they do not appear to have dsRNA helicase activity. Instead, ATP binding and hydrolysis have been implicated in filament formation. ATP binding strengthens the interaction between MDA5 and the dsRNA (34). ATP hydrolysis, however, causes MDA5 to dissociate from the dsRNA (20, 38). At the ends of the MDA5-RNA filaments, ATP hydrolysis causes



depolymerization, providing a mechanism for shutting down the signal and for recycling of MDA5. MDA5 filament assembly and disassembly dynamics provide the specificity for long dsRNA (20, 38). RIG-I was also shown recently to form ATP-dependent filaments, although the RIG-I filaments are shorter and less stable than MDA5 filaments (34, 39).

LGP2, the third RLR, has similar helicase and CTD domains as RIG-I and MDA5, but it lacks the tandem CARDs (33). LGP2 recognizes the termini of dsRNA through similar types of protein-RNA contacts as RIG-I and MDA5 (23, 33, 40, 41). ATP hydrolysis enhances RNA recognition by LGP2 (42). Because it does not have CARDs, LGP2 does not recruit MAVS or induce MAVS signaling. LGP2 affects signaling in response to viral stimuli, however, by modulating the RIG-I and MDA5 signals (see Regulation of RLR Signaling) (30–33).

ROLE OF UNANCHORED LYSINE 63-LINKED UBIQUITIN CHAINS IN RLR ACTIVATION

The oligomerization of the RNA sensors RIG-I and MDA5 that activates the antiviral innate immune response depends on unanchored lysine 63-linked polyubiquitin chains (19, 26). In 2010, Chen and colleagues reconstituted the RIG-I pathway *in vitro* and demonstrated that unanchored K63-linked polyubiquitin chains are required for a full signaling response as measured by IRF3 dimerization (19). Polyubiquitin chains containing as few as four ubiquitin molecules bind non-covalently to the RIG-I CARDs and can be covalently attached to RIG-I by the E3 ligase TRIM25 (19,

43). Furthermore, RIG-I interacted with K63-linked polyubiquitin chains from HEK293T cells in co-immunoprecipitation experiments (19). Similar studies generalized these findings to MDA5 and showed that K63-ubiquitin chains promoted oligomerization of the MDA5 CARDs (26).

A recent crystal structure of the tandem CARDs of RIG-I bound to K63-diubiquitin revealed the molecular basis of the CARD-ubiquitin interaction (Figure 2C) (35). K63-ubiquitin chains promote the assembly of RIG-I CARDs into a tetrameric "lock-washer" structure by stabilizing intermolecular CARD-CARD interactions. This RIG-I tetramer recruits and activates MAVS (see next section) (35). Monoubiquitin is not sufficient to promote RIG-I signaling because a single ubiquitin domain does not make enough contacts to significantly stabilize RIG-I oligomerization through CARD-CARD interactions (19, 35).

Although ubiquitin chains promote RIG-I tetramerization, RIG-I and MDA5 can both assemble into oligomeric filaments and induce MAVS filament formation and signaling in the absence of polyubiquitin chains. Indeed, under certain experimental conditions, namely in the absence of polyubiquitin and as a result of ATP hydrolysis, RIG-I has been observed to form filaments along dsRNA (34, 39, 44). Similarly, MDA5 signaling is thought to be triggered by the formation of MDA5 filaments along dsRNA, which is a ubiquitin-independent process (20, 21). The forced juxtaposition of RLR CARDs upon RLR filament formation is thought to be sufficient to activate MAVS signaling (34). Both RIG-I CARDs and MDA5 CARDs have, however, been shown to

bind K63 polyubiquitin chains (26). Hence the question arises of whether K63-linked ubiquitin chains always participate in RLR signaling, or whether they are only required under specific physiological conditions that do not favor RLR filament formation. Because RIG-I has much higher affinity for the 5'-ppp end of viral ligands than it does for the phosphate backbone alone, it has been proposed that RIG-I is more likely to bind to the 5'-ppp end of the dsRNA (34). If sufficient polyubiquitin is available, RIG-I does not form a filament and instead remains at the end of the dsRNA, and the tetrameric CARD lock-washer scaffold is formed (34, 35). K63-linked polyubiquitin chains stabilize the CARDs oligomer through non-covalent interactions. Covalent linkage of the ubiquitin chains to RIG-I by TRIM25 can provide further stabilization of the RIG-I oligomer, thereby increasing interferon signaling capacity (19, 35, 43). If the local concentration of polyubiquitin is insufficient to induce RIG-I CARDs tetramer formation, ATP hydrolysis may enable RIG-I to translocate along dsRNA and assemble into filaments (39), bringing the CARDs together by cooperative stacking of the helicase domains and leading to ubiquitin-independent signal activation. Unlike RIG-I, MDA5 has no known RNA end-preference and MDA5 has a higher propensity to form filaments than RIG-I (26, 34). Hence, the physiological role of unanchored polyubiquitin chains in MDA5 signaling remains less well understood than in RIG-I.

ACTIVATION OF MAVS AND DOWNSTREAM SIGNALING

In the textbook view of RLR signaling, the signal is propagated sequentially from the ligand-bound RLR to MAVS to the cytosolic protein kinases IKK and TBK1, which in turn activate the transcription factors NF- κ B and IRF3, respectively (45). Activated NF- κ B and IRF3 are translocated into the nucleus, where they induce expression of type I interferons and other inflammatory antimicrobial molecules. The discovery that ligand binding induces RIG-I and MDA5 to assemble into large oligomeric platforms with MAVS on the mitochondrial and peroxisomal membranes has, however, shifted the paradigm for RLR signaling away from the model of a linear signaling cascade. As reviewed in the previous section, both RIG-I and MDA5 form filaments along dsRNA ligands. For RIG-I the forced juxtaposition of its CARDs, along with binding of K63-linked polyubiquitin chains, promotes the formation of a tetrameric lock-washer structure (Figure 2C), which serves as a platform to recruit MAVS (35). Structural and biochemical data suggest that the minimal signaling unit for MDA5 is much larger than for RIG-I and contains at least 11 MDA5 molecules (34). These oligomeric RLR CARD assemblies have been proposed to nucleate the formation of MAVS polymers (Figure 2D) (20, 25). Notably, the polymeric form of MAVS, but not its monomeric form, activates downstream RLR signaling (25). Moreover, once MAVS polymers have been nucleated they are self-propagating, drawing soluble-form MAVS monomers into the polymer. The MAVS CARD, even when isolated from the C-terminal and transmembrane domains, recapitulates this behavior *in vitro* (25). MAVS CARD polymers were recently found to consist of helical filaments (36), similar to those formed by the death domains of MyD88 (4, 46). The switch from a soluble form to a self-propagating helical fiber is reminiscent of amyloids and prions,

and indeed MAVS CARD functions like a *bona fide* prion in yeast (47). Thus, MAVS has a prion-like mechanism of signal activation and amplification. ASC, the adaptor of the NLRP3 inflammasome, was recently shown to have a similar prion-like mechanism of signal transduction (47).

A transmembrane domain tethers MAVS to the mitochondrial or peroxisomal membrane. MAVS polymerization may therefore cause some remodeling of the membrane in these organelles (Figure 2D) (36). In support of this notion, MAVS facilitates cell death by disrupting the mitochondrial membrane potential and by activating caspases (48). Notably, the signaling output from MAVS is different depending on whether it occurs at the peroxisomal or mitochondrial membrane. Peroxisomal MAVS induces the rapid interferon-independent expression of defense factors, which precedes the activation of the principal interferon-dependent pathway by mitochondrial MAVS that amplifies and stabilizes the antiviral response (49). Thus, MAVS signaling is dependent on cellular localization, and peroxisomes are an important site of antiviral signal transduction (49).

REGULATION OF RLR SIGNALING

The inflammatory response resulting from RLR signaling unavoidably occurs at a cost to normal tissue function. Multiple regulatory mechanisms have evolved to allow rapid activation, amplification, and inactivation of RLR signaling, and to achieve the optimal trade-off between the cost and benefit of the inflammatory response (50). Polyubiquitination has been one of the most extensively studied modifications of RIG-I and MDA5, so it is not surprising that E3 ligases and deubiquitinases have been implicated in modulating the RLR response. TRIM25, the most exhaustively studied E3 ligase, covalently attaches K63-linked polyubiquitin to RIG-I CARDs to initiate or promote signaling (26, 43). The E3 ligase Riplet has recently been identified as a necessary component of RIG-I signaling (51). USP21 negatively regulates RIG-I signaling by deubiquitinating RIG-I (52).

In addition to ubiquitination, phosphorylation is slowly emerging as an important regulatory mechanism for RLR signaling. Phosphorylation of Ser8 and Thr170 in the CARDs of RIG-I antagonizes RIG-I signaling (53, 54). Based on the crystal structure of RIG-I in complex with K63-linked diubiquitin (35), we expect phosphorylation of Ser8 but not Thr170 to interfere with ubiquitin binding. Phosphorylation of RIG-I CARD has also been proposed to inhibit recruitment of TRIM25 and MAVS (53, 54). The RIG-I phosphorylation sites are not conserved in MDA5, but MDA5 does have a suppressing phosphorylation site in its first CARD, at Ser88 (55). Conventional protein kinases α and β (PKC α/β) have been identified to be responsible for RIG-I phosphorylation (56). RIG-I and MDA5 are thought to be constitutively phosphorylated until presentation of viral RNA, at which time the RLRs must be dephosphorylated by phosphoprotein phosphatase 1 α and γ (PP1 α/γ) (55).

Besides post-translational modification of the RLRs, RLR signaling is also modulated by several different proteins, derived both from the host and from pathogens. One such protein is the third RLR, LGP2. Because it lacks CARDs, LGP2 cannot activate MAVS; however, its ability to recognize dsRNA allows it to modulate

the signaling capacities of RIG-I and MDA5. LGP2 downregulates signaling by RIG-I (32, 33). This activity was attributed to LGP2 competitively recognizing the same viral ligand as RIG-I. In contrast, LGP2 enhances MDA5 signaling (30, 33, 42). The molecular mechanism of this enhancement remains unclear, but LGP2 appears to facilitate recognition of viral RNA by MDA5 through interactions between the LGP2 CTD and RNA (41). Indeed, a recent study identified a specific picornaviral RNA ligand (in the antisense L region) to which LGP2 binds tightly, thereby stimulating MDA5 signaling (31).

The seemingly contradictory roles of LGP2 in RLR signaling remain an open question. The experimental approaches used to study LGP2 in relation to MDA5 and RIG-I have been different, potentially explaining some of the differences. As evidence accumulates for the opposing roles of LGP2 on RLR signaling, however, the emerging perspective is that LGP2 can control the balance between RIG-I and MDA5 responses during viral infection.

Pathogen evasion tactics against RLR-mediated immune response are extensive and occur at every level of signaling [reviewed in Ref. (57)]. A complete description of these tactics is beyond the scope of this review, so we highlight below a few representative examples of different modes of RLR evasion. MAVS is the primary target of viral factors for inhibiting RLR signaling. MAVS is cleaved by hepatitis C virus NS3/4A protease (28, 29), enterovirus 71 protease 2Apro (58), GB virus B NS3/4A (59), and coxsackie virus B 3C protease, which also cleaves TRIF (60). In a distinct mechanism of RLR signal inhibition, paramyxovirus V proteins disrupt the fold of MDA5 (61). Another major mechanism for evasion of the RLR innate immune response is masking or hiding of viral RNA ligands by viral proteins, such as VP35 from Ebola and Marburg viruses, which coat the ends and backbone of dsRNA to prevent RLR recognition (62–64). Similarly, nucleoproteins from arenaviruses bind to the ends of viral dsRNA and digest the RNA in a 3'-5' direction, thereby making the RNA a weaker ligand for RLRs (65–68). Interestingly, MAVS was recently also shown to be under cellular control. A truncated variant of MAVS resulting from alternative translation initiation interferes with interferon production induced by full-length MAVS (69).

CONCLUSION

RIG-I and MDA5 are the principal sensors of viral dsRNA in the cytoplasm. The interferon-dependent inflammatory response triggered by RLR ligand binding is one of the first and most important lines of defense against infection. RIG-I and MDA5 recognize distinct and complementary sets of viral dsRNA ligands. The molecular signaling mechanisms of RIG-I and MDA5 differ in some respects but also share certain key features. Differences include the sequestration of CARDs by RIG-I but not by MDA5 in the absence of ligand, the much greater propensity of MDA5 to form filaments along dsRNA, and the different contribution of K63-linked ubiquitin chains, which remains poorly defined for MDA5. Common features in RLR signaling include proximity-induced assembly of CARD oligomers, which serve as platforms to nucleate MAVS CARD polymerization, and signal amplification through the amyloid-like properties of the MAVS CARD. Together, the recent advances reviewed here shift

the paradigm of RLR signaling away from the prototypical linear signaling cascade to a model in which signaling is activated by the cooperative assembly of an oligomeric signaling platform. The signal output depends on the cellular localization of MAVS (mitochondria or peroxisome), and signaling is finely regulated by a multitude of cellular and pathogen-derived factors. Key outstanding questions include when, where, and how ubiquitin chains potentiate RIG-I and MDA5 signaling, exactly how RLRs interact with MAVS, and how LGP2 and other factors modulate RLR signaling.

OUTSTANDING QUESTIONS

- Do K63-linked ubiquitin chains always participate in RLR signaling, or are they only required under specific physiological conditions that do not favor RLR filament formation?
 - Is the mechanism of action of K63-linked ubiquitin chains the same for RIG-I and MDA5?
- What are the molecular and structural bases of MAVS activation by RLR oligomers?
 - How do RIG-I CARD tetramers, stabilized by K63-linked ubiquitin, nucleate MAVS filament assembly?
 - How do MDA5 CARDs nucleate MAVS filament assembly? Does this process require K63-linked ubiquitin chains?
- What are the underlying molecular mechanisms for the opposite activities of LGP2 on RIG-I and MDA5 signaling?

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REFERENCES

1. Janeway CA Jr, Medzhitov R. Innate immune recognition. *Annu Rev Immunol* (2002) **20**:197–216. doi:10.1146/annurev.immunol.20.083001.084359
2. Vautier S, MacCallum DM, Brown GD. C-type lectin receptors and cytokines in fungal immunity. *Cytokine* (2012) **58**(1):89–99. doi:10.1016/j.cyto.2011.08.031
3. Choi JS, Ryter SW. Inflammasomes: molecular regulation and implications for metabolic and cognitive diseases. *Mol Cells* (2014) **37**:441–8. doi:10.1434/molcells.2014.0104
4. Ferrao R, Li J, Bergamin E, Wu H. Structural insights into the assembly of large oligomeric signalosomes in the Toll-like receptor-interleukin-1 receptor superfamily. *Sci Signal* (2012) **5**(226):re3. doi:10.1126/scisignal.2003124
5. Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur J Immunol* (2002) **32**(7):1958–68. doi:10.1002/1521-4141(200207)32:7<1958::AID-IMMU1958>3.0.CO;2-U
6. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, et al. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J Immunol* (2003) **171**(6):3154–62. doi:10.4049/jimmunol.171.9.4934-b
7. Nishiya T, Kajita E, Miwa S, DeFranco AL. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. *J Biol Chem* (2005) **280**(44):37107–17. doi:10.1074/jbc.M504951200
8. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* (2004) **5**(2):190–8. doi:10.1038/ni1028
9. Rigby RE, Webb LM, Mackenzie KJ, Li Y, Leitch A, Reijns MA, et al. RNA:DNA hybrids are a novel molecular pattern sensed by TLR9. *EMBO J* (2014) **33**(6):542–58. doi:10.1002/embj.201386117
10. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* (2006) **441**(7089):101–5. doi:10.1038/nature04734

11. Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, et al. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* (2006) **314**:994–7. doi:10.1126/science.1132505
12. Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, et al. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5' phosphates. *Science* (2006) **314**:997–1001. doi:10.1126/science.1132998
13. Holm CK, Paludan SR, Fitzgerald KA. DNA recognition in immunity and disease. *Curr Opin Immunol* (2013) **25**(1):13–8. doi:10.1016/j.co.2012.12.006
14. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* (2013) **339**(6121):786–91. doi:10.1126/science.1232458
15. Feng Z, Hensley L, McKnight KL, Hu F, Madden V, Ping L, et al. A pathogenic picornavirus acquires an envelope by hijacking cellular membranes. *Nature* (2013) **496**(7445):367–71. doi:10.1038/nature12029
16. Kowalinski E, Lunardi T, McCarthy AA, Louber J, Brunel J, Grigorov B, et al. Structural basis for the activation of innate immune pattern-recognition receptor RIG-I by viral RNA. *Cell* (2011) **147**(2):423–35. doi:10.1016/j.cell.2011.09.039
17. Jiang F, Ramanathan A, Miller MT, Tang GQ, Gale M, Patel SS, et al. Structural basis of RNA recognition and activation by innate immune receptor RIG-I. *Nature* (2011) **479**(7373):423–7. doi:10.1038/nature10537
18. Luo D, Ding SC, Vela A, Kohlway A, Lindenbach BD, Pyle AM. Structural insights into RNA recognition by RIG-I. *Cell* (2011) **147**(2):409–22. doi:10.1016/j.cell.2011.09.023
19. Zeng W, Sun L, Jiang X, Chen X, Hou F, Adhikari A, et al. Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. *Cell* (2010) **141**(2):315–30. doi:10.1016/j.cell.2010.03.029
20. Berke IC, Modis Y. MDA5 cooperatively forms dimers and ATP-sensitive filaments upon binding double-stranded RNA. *EMBO J* (2012) **31**(7):1714–26. doi:10.1038/emboj.2012.19
21. Peisley A, Lin C, Wu B, Orme-Johnson M, Liu M, Walz T, et al. Cooperative assembly and dynamic disassembly of MDA5 filaments for viral dsRNA recognition. *Proc Natl Acad Sci U S A* (2011) **108**(52):21010–5. doi:10.1073/pnas.1113651108
22. Berke IC, Yu X, Modis Y, Egelman EH. MDA5 assembles into a polar helical filament on double-stranded RNA. *Proc Natl Acad Sci U S A* (2012) **109**:18437–41. doi:10.1073/pnas.1212186109
23. Takahasi K, Kumeta H, Tsuduki N, Narita R, Shigemoto T, Hirai R, et al. Solution structures of cytosolic RNA sensor MDA5 and LGP2 C-terminal domains: identification of the RNA recognition loop in RIG-I-like receptors. *J Biol Chem* (2009) **284**(26):17465–74. doi:10.1074/jbc.M109.007179
24. Li X, Lu C, Stewart M, Xu H, Strong RK, Igumenova T, et al. Structural basis of double-stranded RNA recognition by the RIG-I-like receptor MDA5. *Arch Biochem Biophys* (2009) **488**(1):23–33. doi:10.1016/j.abb.2009.06.008
25. Hou F, Sun L, Zheng H, Skaug B, Jiang QX, Chen ZJ. MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell* (2011) **146**(3):448–61. doi:10.1016/j.cell.2011.06.041
26. Jiang X, Kinch LN, Brautigam CA, Chen X, Du F, Grishin NV, et al. Ubiquitin-induced oligomerization of the RNA sensors RIG-I and MDA5 activates antiviral innate immune response. *Immunity* (2012) **36**:959–73. doi:10.1016/j.jimmuni.2012.03.022
27. Arimoto K, Takahashi H, Hishiki T, Konishi H, Fujita T, Shimotohno K. Negative regulation of the RIG-I signaling by the ubiquitin ligase RNF125. *Proc Natl Acad Sci U S A* (2007) **104**(18):7500–5. doi:10.1073/pnas.0611551104
28. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* (2005) **437**(7062):1167–72. doi:10.1038/nature04193
29. Li XD, Sun L, Seth RB, Pineda G, Chen ZJ. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc Natl Acad Sci U S A* (2005) **102**(49):17717–22. doi:10.1073/pnas.0508531102
30. Childs KS, Randall RE, Goodbourn S. LGP2 plays a critical role in sensitizing mda-5 to activation by double-stranded RNA. *PLoS One* (2013) **8**(5):e64202. doi:10.1371/journal.pone.0064202
31. Deddouche S, Gobau D, Rehwinkel J, Chakravarty P, Begum S, Maillard PV, et al. Identification of an LGP2-associated MDA5 agonist in picornavirus-infected cells. *Elife* (2014) **3**:e01535. doi:10.7554/elife.01535
32. Rothenfusser S, Goutagny N, DiPerna G, Gong M, Monks BG, Schoenemeyer A, et al. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J Immunol* (2005) **175**(8):5260–8. doi:10.4049/jimmunol.175.8.5260
33. Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, et al. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* (2005) **175**(5):2851–8. doi:10.4049/jimmunol.175.5.2851
34. Peisley A, Wu B, Yao H, Walz T, Hur S. RIG-I forms signaling-competent filaments in an ATP-dependent, ubiquitin-independent manner. *Mol Cell* (2013) **51**(5):573–83. doi:10.1016/j.molcel.2013.07.024
35. Peisley A, Wu B, Xu H, Chen ZJ, Hur S. Structural basis for ubiquitin-mediated antiviral signal activation by RIG-I. *Nature* (2014) **509**(7498):110–4. doi:10.1038/nature13140
36. Xu H, He X, Zheng H, Huang LJ, Hou F, Yu Z, et al. Structural basis for the prion-like MAVS filaments in antiviral innate immunity. *Elife* (2014) **3**:e01489. doi:10.7554/elife.01489
37. Wu B, Peisley A, Richards C, Yao H, Zeng X, Lin C, et al. Structural basis for dsRNA recognition, filament formation, and antiviral signal activation by MDA5. *Cell* (2013) **152**(1–2):276–89. doi:10.1016/j.cell.2012.11.048
38. Peisley A, Jo MH, Lin C, Wu B, Orme-Johnson M, Walz T, et al. Kinetic mechanism for viral dsRNA length discrimination by MDA5 filaments. *Proc Natl Acad Sci U S A* (2012) **109**(49):E3340–9. doi:10.1073/pnas.1208618109
39. Patel JR, Jain A, Chou YY, Baum A, Ha T, Garcia-Sastre A. ATPase-driven oligomerization of RIG-I on RNA allows optimal activation of type-I interferon. *EMBO Rep* (2013) **14**(9):780–7. doi:10.1038/embor.2013.102
40. Murali A, Li X, Ranjith-Kumar CT, Bhardwaj K, Holzenburg A, Li P, et al. Structure and function of LGP2, a DEX(D/H) helicase that regulates the innate immunity response. *J Biol Chem* (2008) **283**(23):15825–33. doi:10.1074/jbc.M800542200
41. Li X, Ranjith-Kumar CT, Brooks MT, Dharmia S, Herr AB, Kao C, et al. The RIG-I-like receptor LGP2 recognizes the termini of double-stranded RNA. *J Biol Chem* (2009) **284**(20):13881–91. doi:10.1074/jbc.M900818200
42. Bruns AM, Pollpeter D, Hadizadeh N, Myong S, Marko JE, Horvath CM. ATP hydrolysis enhances RNA recognition and antiviral signal transduction by the innate immune sensor, laboratory of genetics and physiology 2 (LGP2). *J Biol Chem* (2013) **288**(2):938–46. doi:10.1074/jbc.M112.424416
43. Gack MU, Shin YC, Joo CH, Urano T, Liang C, Sun L, et al. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* (2007) **446**(7138):916–20. doi:10.1038/nature05732
44. Binder M, Eberle F, Seitz S, Mucke N, Huber CM, Kiani N, et al. Molecular mechanism of signal perception and integration by the innate immune sensor retinoic acid-inducible gene-I (RIG-I). *J Biol Chem* (2011) **289**(31):27278–87. doi:10.1074/jbc.M111.256974
45. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* (2004) **5**(7):730–7. doi:10.1038/ni1087
46. Lin SC, Lo YC, Wu H. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature* (2010) **465**(7300):885–90. doi:10.1038/nature09212
47. Cai X, Chen J, Xu H, Liu S, Jiang QX, Halfmann R, et al. Prion-like polymerization underlies signal transduction in antiviral immune defense and inflammasome activation. *Cell* (2014) **156**(6):1207–22. doi:10.1016/j.cell.2014.01.063
48. Yu CY, Chiang RL, Chang TH, Liao CL, Lin YL. The interferon stimulator mitochondrial antiviral signaling protein facilitates cell death by disrupting the mitochondrial membrane potential and by activating caspases. *J Virol* (2010) **84**(5):2421–31. doi:10.1128/JVI.02174-09
49. Dixit E, Boulant S, Zhang Y, Lee AS, Odendall C, Shum B, et al. Peroxisomes are signaling platforms for antiviral innate immunity. *Cell* (2010) **141**(4):668–81. doi:10.1016/j.cell.2010.04.018
50. Okin D, Medzhitov R. Evolution of inflammatory diseases. *Curr Biol* (2012) **22**(17):R733–40. doi:10.1016/j.cub.2012.07.029
51. Oshiumi H, Miyashita M, Matsumoto M, Seya T. A distinct role of Riplet-mediated K63-linked polyubiquitination of the RIG-I repressor domain in human antiviral innate immune responses. *PLoS Pathog* (2013) **9**(8):e1003533. doi:10.1371/journal.ppat.1003533

52. Fan Y, Mao R, Yu Y, Liu S, Shi Z, Cheng J, et al. USP21 negatively regulates antiviral response by acting as a RIG-I deubiquitinase. *J Exp Med* (2014) **211**(2):313–28. doi:10.1084/jem.20122844
53. Gack MU, Nistal-Villan E, Inn KS, Garcia-Sastre A, Jung JU. Phosphorylation-mediated negative regulation of RIG-I antiviral activity. *J Virol* (2010) **84**(7):3220–9. doi:10.1128/JVI.02241-09
54. Nistal-Villan E, Gack MU, Martinez-Delgado G, Maharaj NP, Inn KS, Yang H, et al. Negative role of RIG-I serine 8 phosphorylation in the regulation of interferon-beta production. *J Biol Chem* (2010) **285**(26):20252–61. doi:10.1074/jbc.M109.089912
55. Wies E, Wang MK, Maharaj NP, Chen K, Zhou S, Finberg RW, et al. Dephosphorylation of the RNA sensors RIG-I and MDA5 by the phosphatase PP1 is essential for innate immune signaling. *Immunity* (2013) **38**(3):437–49. doi:10.1016/j.immuni.2012.11.018
56. Maharaj NP, Wies E, Stoll A, Gack MU. Conventional protein kinase C-alpha (PKC-alpha) and PKC-beta negatively regulate RIG-I antiviral signal transduction. *J Virol* (2012) **86**(3):1358–71. doi:10.1128/JVI.06543-11
57. Zinzula L, Tramontano E. Strategies of highly pathogenic RNA viruses to block dsRNA detection by RIG-I-like receptors: hide, mask, hit. *Antiviral Res* (2013) **100**(3):615–35. doi:10.1016/j.antiviral.2013.10.002
58. Wang B, Xi X, Lei X, Zhang X, Cui S, Wang J, et al. Enterovirus 71 protease 2Apro targets MAVS to inhibit anti-viral type I interferon responses. *PLoS Pathog* (2013) **9**(3):e1003231. doi:10.1371/journal.ppat.1003231
59. Chen Z, Benureau Y, Rijnbrand R, Yi J, Wang T, Warter L, et al. GB virus B disrupts RIG-I signaling by NS3/4A-mediated cleavage of the adaptor protein MAVS. *J Virol* (2007) **81**(2):964–76. doi:10.1128/JVI.02076-06
60. Mukherjee A, Morosky SA, Delorme-Axford E, Dybdahl-Sissoko N, Oberste MS, Wang T, et al. The coxsackievirus B 3C protease cleaves MAVS and TRIF to attenuate host type I interferon and apoptotic signaling. *PLoS Pathog* (2011) **7**(3):e1001311. doi:10.1371/journal.ppat.1001311
61. Motz C, Schuhmann KM, Kirchhofer A, Moldt M, Witte G, Conzelmann KK, et al. Paramyxovirus V proteins disrupt the fold of the RNA sensor MDA5 to inhibit antiviral signaling. *Science* (2013) **339**(6120):690–3. doi:10.1126/science.1230949
62. Ramachandran S, Kota P, Ding F, Dokholyan NV. Automated minimization of steric clashes in protein structures. *Proteins* (2011) **79**(1):261–70. doi:10.1002/prot.22879
63. Ramanan P, Edwards MR, Shabman RS, Leung DW, Endlich-Frazier AC, Borek DM, et al. Structural basis for Marburg virus VP35-mediated immune evasion mechanisms. *Proc Natl Acad Sci U S A* (2012) **109**(50):20661–6. doi:10.1073/pnas.1213559109
64. Leung DW, Prins KC, Borek DM, Farahbakhsh M, Tufariello JM, Ramanan P, et al. Structural basis for dsRNA recognition and interferon antagonism by Ebola VP35. *Nat Struct Mol Biol* (2010) **17**(2):165–72. doi:10.1038/nsmb.1765
65. Hastie KM, Kimberlin CR, Zandonatti MA, MacRae IJ, Saphire EO. Structure of the Lassa virus nucleoprotein reveals a dsRNA-specific 3' to 5' exonuclease activity essential for immune suppression. *Proc Natl Acad Sci U S A* (2011) **108**(6):2396–401. doi:10.1073/pnas.1016404108
66. Hastie KM, Liu T, Li S, King LB, Ngo N, Zandonatti MA, et al. Crystal structure of the Lassa virus nucleoprotein-RNA complex reveals a gating mechanism for RNA binding. *Proc Natl Acad Sci U S A* (2011) **108**(48):19365–70. doi:10.1073/pnas.1108515108
67. Qi X, Lan S, Wang W, Schelde LM, Dong H, Wallat GD, et al. Cap binding and immune evasion revealed by Lassa nucleoprotein structure. *Nature* (2010) **468**(7325):779–83. doi:10.1038/nature09605
68. Jiang X, Huang Q, Wang W, Dong H, Ly H, Liang Y, et al. Structures of arenaviral nucleoproteins with triphosphate dsRNA reveal a unique mechanism of immune suppression. *J Biol Chem* (2013) **288**(23):16949–59. doi:10.1074/jbc.M112.420521
69. Brubaker SW, Gauthier AE, Mills EW, Ingolia NT, Kagan JC. A bicistronic MAVS transcript highlights a class of truncated variants in antiviral immunity. *Cell* (2014) **156**(4):800–11. doi:10.1016/j.cell.2014.01.021

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Recognition of human oncogenic viruses by host pattern-recognition receptors

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Human oncogenic viruses include Epstein–Barr virus, hepatitis B virus, hepatitis C virus, human papilloma virus, human T-cell lymphotropic virus, Kaposi's associated sarcoma virus, and Merkel cell polyomavirus. It would be expected that during virus–host interaction, the immune system would recognize these pathogens and eliminate them. However, through evolution, these viruses have developed a number of strategies to avoid such an outcome and successfully establish chronic infections. The persistent nature of the infection caused by these viruses is associated with their oncogenic potential. In this article, we will review the latest information on the interaction between oncogenic viruses and the innate immune system of the host. In particular, we will summarize the available knowledge on the recognition by host pattern-recognition receptors of pathogen-associated molecular patterns present in the incoming viral particle or generated during the virus' life cycle. We will also review the data on the recognition of cell-derived danger associated molecular patterns generated during the virus infection that may impact the outcome of the host–pathogen interaction and the development cancer.

Keywords: PRRs, oncogenic viruses, cancer, innate immunity, innate sensors

INTRODUCTION

Seven human viruses have been found so far to cause approximately 10–20% of human cancers worldwide (1). They include the herpesviruses, Epstein–Barr virus (EBV) and Kaposi's associated sarcoma virus (KSHV), the hepatitis B (HBV) and hepatitis C (HCV) viruses, high-risk human papillomaviruses (HPV) (the most clinically important ones being types 16 and 18, but most probably a few others will be found to be relevant to cancer development as well in the future), the human T-cell lymphotropic virus-1 (HTLV-1), and the recently discovered Merkel cell polyomavirus (MCPyV) (1). The mechanisms by which these viruses cause cancer are diverse. They have prolonged latency periods, during which viral factors combine with other environmental factors in the setting of the genetic background of each particular host (2). However, it could be proposed that these viruses have no intention of generating disease in their hosts, as evidenced by the overall rate of disease/infected humans worldwide for each virus (Table 1). Although exact numbers are not available for every region in the world, the number of humans that suffer a disease associated with each oncogenic virus, as compared to the number of people infected with each virus is evidently low. It appears that during evolution these viruses have found a balance of “live and let live” with their host. Until very recently in history, humans were not living long enough to considerably suffer from the diseases attributed to these viruses (3). Today, however, human longevity is greatly extended, and although the burden of diseases associated with oncogenic viruses is still low in comparison with the number of infected people, the goal of medicine is, of course, to eradicate diseases. Understanding the interactions of these viruses with the host will certainly help to achieve this goal. Of particular importance is their

interaction with the innate immune system, which functions to recognize non-self like microorganisms, and also plays a critical role in recognition of modified self that indicates damage or danger (4).

Germline-encoded pattern-recognition receptors (PRRs) recognize chemically distinct moieties in microorganisms or “pathogen-associated molecular patterns” (PAMPs) (12). PRRs can also recognize endogenous host molecules that in different ways signal danger (“damage” or “danger”-associated molecular patterns’ or “DAMPs”) (13, 14). It is noteworthy that the “D” in DAMPs is used interchangeably for “danger” or “damage.” However, “danger” would seem to be more appropriate, as there could be danger without damage, and it would be more in line with the original “danger” theory proposed by Matzinger several years ago (15).

There are two families of transmembrane PRRs, namely toll-like receptors (TLRs) (16) and C-type lectin receptors (CLRs) (17). They are positioned to scan the extracellular and endosomal spaces. The families of cytoplasmic PRRs include the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (18) and the nucleotide-binding, oligomerization domain (NOD)-like receptors (NLRs) (19, 20), as well as a large number of DNA sensors that converge in the adaptor for cytosolic DNA sensing stimulator of interferon genes (STING). An excellent very comprehensive review on nucleic acid sensing was recently published (21). The double-stranded (ds)RNA-dependent protein kinase R (PKR) and the 2',5'-oligoadenylate synthetases (OAS) are considered part of the cytoplasmic PRRs as well (22). Recently, a nuclear DNA sensor was identified, IFI-16, a PYHIN protein that, together with the cytoplasmic AIM-2 DNA sensor, was proposed to form a new family of innate DNA sensors (“AIM2-like receptors” or “ALRs”)

Table 1 | Oncoviruses induce cancer in only a fraction of infected humans.

Virus	Family	Infected worldwide (estimated)	Percentage developing disease*	Reference
HBV	Hepadnaviridae	400 million	HCC: 340,000/year (1% approximately)	Busca and Kumar (5)
HCV	Flaviviridae	210 million; 80% persistent infection	HCC: 195,000/year (1% approximately)	Eksioglu et al. (6)
EBV	Herpesviridae	90% Human population (approximately 6.3 billion?)	Most people do not develop disease	Zauner and Nadal (7)
KSHV	Herpesviridae	Not ubiquitous, perhaps between 5 and 50% of the population	Varies	Areste and Blackbourn (8)
HPV	Papillomaviridae	50–80% Of sexually active adults; one or more HPV types during lifetime	Invasive cervical cancer: 500,000 cases/year	Sunthamala et al. (9)
HTLV-1	Retroviridae	10–20 million	2–3% ATL; 0.25–4% HAM/TSP	Oliere et al. (10)
MCPyV	Polyomaviridae	Reports vary, between 20 and 80% of population tested	MCC: 1600 cases year in USA	Bhatia et al. (11)

*Numbers are approximate, and may vary in different geographical regions.

HCC, hepatocellular carcinoma; MCC, Merkel cell carcinoma. See other abbreviations in text.

(23). Importantly, some of the members of the NLR and ALR families form a molecular complex termed “inflammasomes,” molecular platforms that control the secretion of the pro-inflammatory cytokines interleukin-1b and -18 (14). Some of the members of the already mentioned families of receptors recognize DNA. However, there is a growing list of DNA sensors not belonging to these families, recognizing both pathogen’s DNA as well as modified or displaced self DNA (24). Finally, although it is not be in the scope of this review, it is relevant to mention here that there is a particular set of immune proteins called “intrinsic antiviral factors.” Unlike PRRs that function against viruses by triggering a cascade of antiviral signaling events, intrinsic antiviral factors directly block viruses at different points of their life cycle (25). Each of these families of proteins does work in concert in order to eradicate viruses. However, viruses have evolved a myriad of mechanisms to evade and subvert these host antiviral defenses in order to ensure their evolutionary survival (26).

There is abundant information on the mechanisms by which the seven oncogenic viruses block the molecular pathways of the innate immune system at the level of intracellular adaptors, and the reader is referred to the several extensive published reviews in the specific sections below. However, much less is known on the recognition of these viruses by the sensors that physically interact with viral PAMPs. Here, we will focus on the latest findings on the growing list of innate immune sensors that have been implicated in sensing each known human oncogenic virus (Figure 1). We believe that by combining this information in one single review, parallelisms and differences between these very distinct viruses, which trigger the same human disease, i.e., cancer, may be revealed.

HEPATITIS C VIRUS

Hepatitis C virus is a single-stranded RNA virus, with an enveloped nucleocapsid of about 50 nm. It is transmitted via parenteral route, and there are millions of people infected with HCV worldwide, for which there is no available vaccine (27). During its evolution with the host, it has developed a number of mechanisms to avoid being eliminated by the innate immune system, establishing chronic infection of the liver. This chronic infection triggers injury

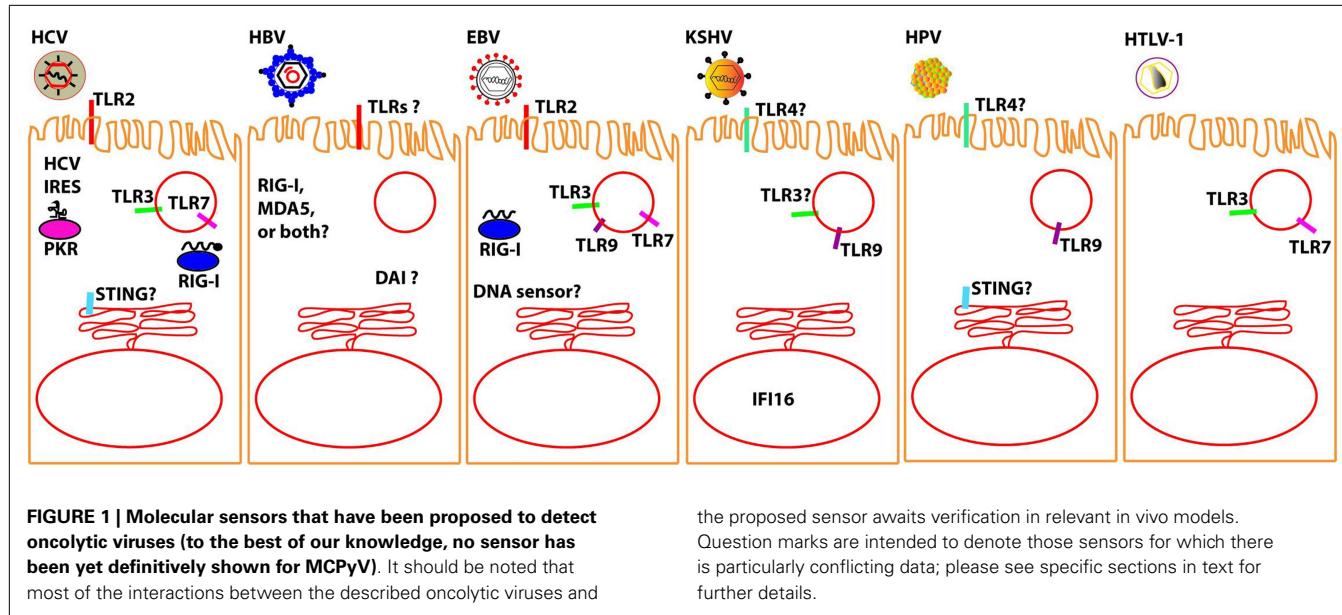
to the liver, which is believed to be the basis for the development of liver cancer. HCV and its interaction with the adaptive and innate immune systems is a very active field of research, and many recent review articles have exhaustively discussed these topics (6, 27–32). However, the sensing of the virus and the innate pathways activated during the first days of infection in humans remain largely unknown (33). Understanding of these steps is critical, as they are likely to set the stage for the ultimate outcome of the infection.

CELLULAR MEMBRANE AND ENDOSOME SENSING

TLR2 has been proposed to sense HCV proteins at the cell surface (30). TLR3 has been shown to be relevant for the activation of the transcription factors IRF-3 and NF-κB in response to HCV-RNA (28). TLR7 was also shown to be relevant in HCV sensing (34), and the proposed mechanism suggested the existence of a cell–cell RNA transfer process where HCV-infected cells activated plasmacytoid dendritic cells (pDCs) in *trans*. This was shown to be the case as well by Dreux et al., who reported the transfer of HCV-RNA containing exosomes from infected cells to pDCs (35).

INTRACELLULAR SENSING

Hepatitis C virus recognition in the cytosol is mediated by the host RNA-dependent PKR, which identifies an internal ribosome entry site (IRES) in HCV genome. In contrast, the virus’ 3’ poly-U/UC sequence, short dsRNA regions, and 5’ triphosphate of the uncapped HCV-RNA are recognized by RIG-I [reviewed in detail by Horner (28); Horner and Gale (29)]. A detailed analysis of the HCV-RNA that activates RIG-I was described by Schnell et al. (36), who reported a 34-nt poly-uridine “core” of the 5’-ppp poly-U/UC sequence as a critical structure for RIG-I activation. Recently, a new mechanism by which HCV controls interferon (IFN) induction was described, where RIG-I is ubiquitinated through the di-ubiquitin-like protein ISG15, one of the early interferon responsive genes (ISGs) (37). Other investigators, however, propose a different mechanism of RIG-I activation, where Riplet-mediated K63-linked polyubiquitination releases RIG-I RD autorepression, allowing the access of downstream signaling factors to the RIG-I



protein (38). These differences in the proposed models of RIG-I activation may be due to the use of different cell types and experimental conditions. More recent data also suggest that the STING may be relevant for HCV recognition (39, 40). The mechanism these investigators propose implicates direct interaction of HCV NS4B with STING, blocking IFN beta production downstream of both STING and RIG-I. Finally, although human biopsies provide limited opportunities for mechanistic studies, they are critical since they allow a snapshot view of the tissue that is infected in the actual host. Consistent with this concept, Mozer-Lisewska et al. reported that in liver from patients with chronic hepatitis C infection, the expression of TLR1, 2, 4, NALP, and RIG-I helicase was markedly increased, suggesting that these PRRs may be important for the pathogenesis of chronic viral hepatitis by HCV in humans (41).

HEPATITIS B VIRUS

Hepatitis B virus genome consists of partial dsDNA, its nucleocapsid is enveloped, and is transmitted via the parenteral route; although there is a vaccine available, millions of people are infected (27). The major challenge for mechanistic analysis of HBV interaction with the innate immune system is the lack of a suitable animal model. Woodchuck infected with the woodchuck hepatitis virus (WHV) (42) is an accepted study model, but available immunological tools are limited. Researchers use transfected cells or mice hydrodynamically injected with HBV replicative plasmids, but they cannot faithfully recapitulate the *in vivo* infection process. Even with these caveats in mind, the field is advancing toward an understanding of the interaction between HBV and the human innate immune system. Until recently, it was believed that the virus was just a stealth pathogen that could not be detected by PRRs (43–45). However, it is becoming clear that HBV just have a number of very efficient strategies to block innate immunity, and they were recently reviewed in Ref. (5, 46). Indirect data seem to support the fact that PRR sensing of HBV is important for HBV

pathogenesis. For example, Guo et al. showed that transfection in cells with the plasmids expressing adaptors for PRRs signaling pathways (myeloid differentiation primary response gene 88, or MyD88), TIR-domain-containing adaptor-inducing beta interferon (TRIF), or the RIG-I/MDA5 adaptor, interferon promoter stimulator 1 (IPS-1), reduced HBV DNA and RNA levels (47). However, it is difficult to conclude that the data obtained in this *in vitro* system correlates with the behavior of the virus in naturally infected hosts.

CELLULAR MEMBRANE AND ENDOSOME SENSING

Using the HBV/WHV model, Zhang et al. described that addition of TLR2 ligands activate NF- κ B, PI3K/Akt, and different arms of the MAPK signaling pathways to induce pro-inflammatory cytokines, leading to the reduction of WHV replication and gene expression in HepG2.2.15 cells and primary woodchuck hepatocytes (48). However, in a previous study using an HBV transgenic mice model, a single intravenous injection of exogenous ligands specific for TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9 showed that all of the ligands except for TLR2 inhibited HBV replication in the liver non-cytopathically in an alpha/beta IFN-dependent manner (49). Differences in these results could easily be attributed to the different model systems used, and warrant further investigation. In a more relevant study model, i.e., the chimpanzee, Lanford et al. showed that the small molecule GS-9620, which activates TLR7 signaling in immune cells, provided long-term suppression of serum and liver HBV DNA (50). Based on these and other results, TLR ligands are being developed as drugs for the treatment of chronic viral infections, including HBV (51).

INTRACELLULAR SENSING

RIG-I and MDA5 are important PRRs responsible for recognition of viral RNAs produced during viral infection, and represent targets for immunosuppression during HBV infection. Lu and Liao demonstrated that in human Huh7 cells transfected and in

the livers of mice hydrodynamically injected with HBV replicative plasmids, the expression of MDA5, but not RIG-I, was increased, and it was the critical protein for HBV detection (52). It is interesting that mice heterozygous for MDA5 also had an increase in HBV replication, indicating the existence of a possible threshold in MDA5 expression level necessary for its function as a HBV sensor. In another study, Zhao et al. proposed that RIG-I, and not MDA5, is the protein involved in HBV sensing (53). Although it is not clear as yet which specific sensor is involved, viral RNA sensing in the cytoplasm is clearly occurring during HBV infection. Studies using hepatocytes (54), 293 cells (55), or the cytoplasmic fraction of HBx transgenic mouse livers (56) showed that hepatitis B virus X (HBX) protein interacts with MAVS (also called IPS-1, a critical molecule in RNA signaling pathways) (57), and prevents the induction of IFN genes. DNA sensing mechanisms are also likely to be relevant, since in the cell line Huh7, Chen et al. showed that DAI can inhibit HBV replication, where the inhibitory effect was associated with activation of NF- κ B, and was independent of IRF-3 or cytokines (58).

In summary, it is clear that many more studies identifying new mechanisms of HBV detection by the innate immune system are likely to follow. The true challenge will be to reconcile those *in vitro* identified pathways with the mechanisms of HBV control in more relevant infectious models, i.e., the chimpanzee, and translate this knowledge into human settings.

HERPESVIRUSES: EPSTEIN–BARR VIRUS AND KAPOSI'S ASSOCIATED SARCOMA VIRUS

There is a significant body of data demonstrating that herpesviruses can be sensed by the innate immune system at the cellular membrane, in the endosomes, and in the cytosol. Furthermore, recent studies showed that herpesviruses can also be sensed in the nuclei. A recent comprehensive review on herpesviridae was published by Paludan and Bowie (24). EBV and KSHV are the two members of this virus family that have been identified as having growth transforming potential, and therefore, we focus on these here.

EPSTEIN–BARR VIRUS

Epstein–Barr virus was discovered approximately 50 years ago. It is an enveloped virus with a dsDNA genome, for which there is extensive knowledge about its biology (59). The innate immune recognition of EBV was also reviewed in detail (60, 61).

CELLULAR MEMBRANE AND ENDOSOME SENSING

Epstein–Barr virus can be sensed by TLR2 in certain cells; however, the exact virion component being sensed is still unclear (62). Ariza et al. proposed that deoxyuridine triphosphate nucleotidohydrolase (dUTPase), a non-structural protein encoded by EBV, is sensed by TLR2 and initiates a MyD-88 dependent response (63). This group further extended their results to demonstrate that the protein was secreted in exosomes inducing NF- κ B activation and cytokine secretion in primary DCs and peripheral blood mononuclear cells (PBMCs) (64). However, these results should be interpreted with caution given that the studies were done using an *in vitro* experimental system. EBV produces non-coding RNAs or “Epstein–Barr virus-encoded small RNA” (“EBER”). TLR3 is a

sensor of viral dsRNA. Very interestingly, it was discovered that a substantial amount of EBER was released from EBV-infected cells in exosomes that stimulated DCs to produce type-I IFN. Most importantly, they found EBER in sera from patients with EBV-related diseases, suggesting that EBER could be responsible for immune activation by EBV, inducing type I IFN and proinflammatory cytokines (65). These results were further discussed by the same group (66). TLR7 has not been proposed as a direct sensor for EBV. However, Valente et al. reported that the aberrant activation of TLR7 in EBV-infected cells might induce the expression of the EBV-protein LMP1 (67). As LMP1 is known to prime cells to express IFN, and both TLR7 and IFNs are believed to be involved in the development of systemic lupus erythematosus (SLE, or simply “lupus”), the association of EBV infection and autoimmunity clearly warrants further investigation.

Interestingly, Severa et al. showed that EBV can activate pDCs through TLR9 and TLR7, in combination with functional autophagic machinery (68). However, these pDCs were not able to mature and induced an inefficient T-cell response, suggesting a new virus escape mechanism potentially related to EBV induced diseases. Another important finding reported by van Gent et al. showed that EBV encoded deubiquitinase, BPLF1, interferes with NF- κ B activation mediated by TLR signaling (69). TLR9 can initiate a response by detecting EBV DNA in the endosomes. However, Fathallah et al. showed that EBV infection of human primary B cells results in the strong inhibition of TLR9 transcription by the EBV oncprotein latent membrane protein 1 (LMP1) (70). The role of TLR9 in EBV infection has been exhaustively reviewed in Ref. (7).

INTRACELLULAR SENSING

In the cytosol, EBV EBERs are recognized by RIG-I (62). Moreover, RIG-I has been proposed to indirectly sense EBV DNA by recognizing the 5'-triphosphate transcribed by the host RNA polymerase III (71). However, there are conflicting results that need to be resolved by further experimentation to clarify the role of RNAPol-III in EBV sensing mechanism (62). There are numerous DNA sensors in the cytosol, and although some of them have been shown to recognize other herpesviruses (62), the relevance of cytosolic DNA sensors to EBV remains unclear.

KAPOSI'S ASSOCIATED SARCOMA VIRUS

This virus, formally classified as human herpesvirus 8 (HHV-8), is associated with Kaposi's sarcoma (KS), among other pathologies (72). It is a big enveloped virus with a dsDNA genome (73). Employing many proteins and micro-RNAs, KSHV modulates the innate and adaptive immune system of the host at multiple levels. A number of excellent reviews on these topics have been recently published (8, 73–75).

CELLULAR MEMBRANE AND ENDOSOME SENSING

Only recently, researchers have started investigating the role of TLR-mediated sensing of KSHV. Although a direct interaction of KSHV with a TLR has not been reported, the virus downregulates the expression of TLR4 soon after infection in endothelial cells (76). West and Damania, however, showed that in monocytes TLR3 expression is upregulated after KSHV infection (77).

Gregory et al. showed that agonists specific for TLR7/8 reactivated latent KSHV and induced viral lytic gene transcription and replication (78). Moreover, the same was accomplished by secondary infection with vesicular stomatitis virus (VSV), which also activates those same TLRs. More recently, pDCs were shown to respond to KSHV in TLR9-dependent manner (79). Finally, it has been shown that stimulation of the TLR3–TRIF axis increases the expression of the KSHV protein RTA (replication and transcription activator), only for RTA to degrade TRIF in order to block the innate immune response (80, 81). Collectively, although these results do not demonstrate a direct interaction between KSHV and TLRs, they clearly indicate that there is a physiologically relevant interplay between them.

INTRACELLULAR SENSING

The field of intracellular sensing of KSHV has recently seen a number of very exciting discoveries. Gregory et al. reported that KSHV Orf63 blocks NLRP1-dependent innate immune responses, including caspase-1 activation and processing of interleukin-1 beta (IL-1beta) and IL-18, and significantly reduces NLRP1-dependent cell death (82). Moreover, the inhibition of Orf63 expression resulted in increased expression of IL-1beta during the KSHV infection that could have an effect on KSHV induced pathologies. In a new development in the field of innate immune sensing, Unterholzner et al. reported that IFI-16 acts as a nuclear sensor for HSV-1 (23). Based on their findings, they proposed the existence of a new family of “AIM-2 like receptors” or ALRs. In the same line of research, Kerur et al. found that the same protein is responsible for KSHV sensing through an IFI-16/ASC inflammasome assembled in the nuclei (83). They reported that caspase-1 activation is IFI-16/ASC inflammasome dependent, and it leads to IL-1b secretion. Moreover, the same group proposed that latent KSHV genome is continuously sensed in the nuclei through IFI-16 sensing mechanism (84). Further studies will be needed to shed light on the biological significance of these very exciting findings. Finally, West et al. suggested a role for MAVS and RIG-I dependent signaling mechanisms during KSHV infection (85). Therefore, all of the families of cytosolic sensors have been implicated in the recognition of KSHV. These results clearly indicate that KSHV has a complex interaction with host innate immunity by activating several PRRs. It is conceivable that activation of this network of innate immune receptors is a necessary step in the virus pathogenesis to establish lifelong persistence of the virus infection.

HUMAN PAPILLOMAVIRUSES

The HPV family encompasses a large number of stable dsDNA viruses (86). Infections with high-risk HPVs are causally associated with the development of anogenital cancers (87). It has been proposed that HPVs evade the innate immune response of the host cells by deregulating immunomodulatory factors such as cytokines and chemokines, thereby creating a microenvironment that favors malignancy (88). The combination of knowledge from the fields of basic HPV virology and vaccinology was the driving force for the successful development of clinically effective vaccines against HPV (89). However, the developed vaccines are prophylactic, not therapeutic, and cover only a subset of HPV types. It is certainly clear that improving our understanding of the interaction

of HPV with the innate immune system will improve the probability of success in developing better treatments. Similar to all other viruses described in this review, experimental systems that would be informative about HPV pathogenesis in humans are very limited. The vast majority of studies were performed using virus like particles (VLPs). This approach, and the differences between laboratories in their techniques for virus particles preparation, is partially responsible for the incomplete understanding of HPV biology. For example, the exact mechanism of virus entry into the cell remains incompletely defined (90). Along the same lines, the full spectrum of PRRs relevant to HPV recognition by the cell is yet to be determined.

CELLULAR MEMBRANE AND ENDOSOME SENSING

The current understanding of the interaction between HPV and PRRs is mostly based on studies aimed to potentiate immunological responses to HPV vaccines by modulating innate immunity. Therefore, research in the field has focused primarily on the role of TLRs. To date there are no publications on the involvement of cytosolic or nuclear sensors in HPV recognition. There is currently no evidence that any cellular PRRs interact with HPV directly [reviewed in Ref. (88)]. A comprehensive review on the role of TLRs in HPV infection has been recently published by Zhou et al. (91). Although TLR4 was suggested to bind HPV L1 directly, these studies were performed using VLPs, and although TLR9 may recognize HPV DNA in the endosomes, it is not clear whether the HPV DNA is exposed in the endosome during natural viral infections (91). More recently, it was described that an HPV16 transcriptional repressor complex associates with the TLR9 promoter, suggesting that blocking this TLR-mediated sensing pathway may be of significance for the virus pathogenesis (92). Collectively, these data indicate that although direct interaction between HPV and PRRs is yet to be shown, the virus does interfere with innate pathogen recognition machinery. In this regard, several recent publications describing how HPV may control cellular responses initiated by PRRs pathways should be mentioned. IL-1beta is a critical cytokine that mediates inflammation and is important for both innate and adaptive immunity. Using immortalized keratinocytes, it was shown that the high-risk HPV16 E6 oncoprotein can abrogate IL-1beta processing and secretion independently of the NALP3 inflammasome (93). The authors further demonstrated that pro-IL-1beta is degraded by a novel proteasome-dependent mechanism via the ubiquitin ligase E6-AP and p53. Moreover, in a panel of HPV-positive tissue samples, the authors found correlation between reduced amounts of IL-1beta and the stage of cellular progression toward cervical cancer (93). HPV was also shown to interfere with innate immune signaling pathways through virus-dependent upregulation of an intrinsic ubiquitin ligase, ubiquitin carboxyl-terminal hydrolase L1 (UCHL1). Upregulation of UCHL1 inhibited TRAF-3 dependent phosphorylation of interferon regulatory factor-3 (IRF-3), and the activation of NF- κ B (94). However, the role of this ubiquitin ligase *in vivo* remains unclear as these studies were performed in HPV infected keratinocytes. Using an *in vitro* approach, Sunthamala et al. found that HPV E2 protein interferes with innate immune signaling pathways by downregulating STING and IFN- κ (9). Importantly, they also demonstrated in clinical specimens that

STING and IFN- κ are downregulated in HPV low grade lesions when compared to normal tissues. Conceptually and mechanistically interesting findings were made by Kumar et al., who showed that Langerhans cells from cervical tumors lack TLR9 expression and are functionally anergic to TLR7, TLR8, and TLR9 ligands (95). These data suggest that apart from directly interacting with cellular PRRs, HPV may interfere with innate signaling pathways in neighboring cells in an indirect paracrine manner leading to PRRs signaling inhibition.

HUMAN T-CELL LYMPHOTROPIC VIRUS

Human T-cell lymphotropic virus-1 belongs to the retroviridae family and is an enveloped, round shaped particle with a single-stranded RNA genome (96). The diseases that induce are diverse and this diversity in clinical manifestations in response to HTLV-1 is likely associated with genetic heterogeneity of the host. The pathologies induced by this virus include the aggressive, fatal T-cell malignancy adult T-cell leukemia (ATL) and a chronic, progressive neurologic disorder called HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), among others. Unfortunately, the molecular mechanisms underlying the diversity in host responses to HTLV-1 remain unclear (96). The studies of host defense against HTLV-1 have largely focused on understanding the very strong CTL response against the virus. It is puzzling how the virus can establish a persistent infection in the face of such a response. One of the potential mechanisms to escape from the CTL response is the capacity of the virus to downregulate the expression of all but one viral protein (HBZ), thus directly reducing the immunogenicity of the infected cells (97). This capacity of the virus also makes its detection by the innate immune system very challenging. Several reviews have recently summarized the advances in the field of HTLV-1 interactions with the innate immune system (10, 97–99).

The fact that there is not an adequate animal model to study the virus interaction with innate immunity makes advancing in the field very challenging. Rabbits and monkeys models can be used; however, the available immunological tools are scarce. For HTLV-1, mice represent a very poor animal model. Finally, in contrast to the availability of human cervix samples for studies of HPV pathogenesis, access to central nervous system tissue of HTLV-1 infected individuals is not available (100, 101). Therefore, it is not surprising that as of yet there is no evidence of direct recognition of HTLV-1 by PRRs. Furthermore, the role of innate immunity in HTLV-1-associated diseases is not clear (99). Only recently, the induction of an innate immune response to HTLV-1 (102) was reported for the first time. The authors found that cell-free HTLV-1 stimulates pDCs to produce massive amounts of type-I IFN. The proposed mechanism of type-1 IFN induction was the degradation of the viral particles in the endosomal compartments, and consequent exposure of the ssRNA to TLR7. This model was supported by the indirect observations that an endosomal acidification inhibitor and a TLR7 specific blocker drastically inhibited pDC response to HTLV-1 measured by type-1 IFN production. Progress in understanding the innate immune responses to HTLV-1 may come from the use of humanized mouse models (100). For example, reconstitution of mice with WT or TLR7 deficient human cells may reveal the contribution

of the TLR7 innate immune signaling pathway to recognition of HTLV-1.

MERKEL CELL POLYOMAVIRUS

Merkel cell carcinoma (MCC) is a highly aggressive non-melanoma skin cancer arising from epidermal mechanoreceptor Merkel cells. In 2008, a novel human polyomavirus, MCPyV, was identified and is now implicated in MCC pathogenesis. Polyomaviruses are small, non-enveloped dsDNA viruses [for a detailed review on polyomaviruses and MCPyV in particular see Ref. (11, 46, 103, 104)]. Although little is known about this newly identified virus, it is plausible that, as with other oncogenic viruses, MCPyV has an array of mechanisms to block the innate immune responses. There is limited information on the innate immune recognition of this virus, as the field is in its infancy. It was reported that MCPyV large T antigen (LT) expression downregulates TLR9 expression in epithelial and MCC-derived cells (105), but nothing is known regarding the direct recognition of the virus by PRRs. More data are clearly needed on the interaction of this virus with the innate immune system.

CONCLUSION

Over evolutionary times, the battle between the oncogenic viruses and their hosts has arrived at a balance that ensures the survival of both organisms. However, with the current advances in vaccinology and drug development, it is plausible to imagine that we are potentially getting closer to limiting the impact these seven viruses have on the population of the world. Although a complete understanding of all of the complexity of interactions with the native host for all of the oncogenic viruses discussed in this review is still lacking, it is clear that the innate immune system is able to recognize their presence through a network of sensors. Undoubtedly, the understanding of virus interactions with the innate immune system will aid in the development of effective treatments against these pathogens. More research is clearly warranted to devise effective approaches to harness the tools of the innate immune system for elimination of these viral pathogens without negatively affecting their hosts.

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REFERENCES

1. Moore PS, Chang Y. Why do viruses cause cancer? Highlights of the first century of human tumour virology. *Nat Rev Cancer* (2010) **10**:878–89. doi:10.1038/nrc2961
2. Martin D, Gutkind JS. Human tumor-associated viruses and new insights into the molecular mechanisms of cancer. *Oncogene* (2008) **27**(Suppl 2):S31–42. doi:10.1038/onc.2009.351
3. Finch CE. Evolution in health and medicine Sackler colloquium: evolution of the human lifespan and diseases of aging: roles of infection, inflammation, and nutrition. *Proc Natl Acad Sci U S A* (2010) **107**(Suppl 1):1718–24. doi:10.1073/pnas.0909606106

4. Medzhitov R. Recognition of microorganisms and activation of the immune response. *Nature* (2007) **449**:819–26. doi:10.1038/nature06246
5. Busca A, Kumar A. Innate immune responses in hepatitis B virus (HBV) infection. *Virol J* (2014) **11**:22. doi:10.1186/1743-422X-11-22
6. Eksioglu EA, Zhu H, Bayouth L, Bess J, Liu HY, Nelson DR, et al. Characterization of HCV interactions with toll-like receptors and RIG-I in liver cells. *PLoS One* (2011) **6**:e21186. doi:10.1371/journal.pone.0021186
7. Zauner L, Nadal D. Understanding TLR9 action in Epstein-Barr virus infection. *Front Biosci* (2012) **17**:1219–31. doi:10.2741/3982
8. Areste C, Blackbourn DJ. Modulation of the immune system by Kaposi's sarcoma-associated herpesvirus. *Trends Microbiol* (2009) **17**:119–29. doi:10.1016/j.tim.2008.12.001
9. Sunthamala N, Thierry F, Teissier S, Pientong C, Kongyinyo B, Tangsiriwatthana T, et al. E2 proteins of high risk human papillomaviruses down-modulate STING and IFN- κ transcription in keratinocytes. *PLoS One* (2014) **9**:e91473. doi:10.1371/journal.pone.0091473
10. Oliere S, Douville R, Sze A, Belgaouai SM, Hiscott J. Modulation of innate immune responses during human T-cell leukemia virus (HTLV-1) pathogenesis. *Cytokine Growth Factor Rev* (2011) **22**:197–210. doi:10.1016/j.cytofr.2011.08.002
11. Bhatia S, Afanasiev O, Nghiem P. Immunobiology of Merkel cell carcinoma: implications for immunotherapy of a polyomavirus-associated cancer. *Curr Oncol Rep* (2011) **13**:488–97. doi:10.1007/s11912-011-0197-5
12. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* (2010) **140**:805–20. doi:10.1016/j.cell.2010.01.022
13. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol* (2010) **10**:826–37. doi:10.1038/nri2873
14. Schroder K, Tschopp J. The inflammasomes. *Cell* (2010) **140**:821–32. doi:10.1016/j.cell.2010.01.040
15. Matzinger P. Tolerance, danger, and the extended family. *Annu Rev Immunol* (1994) **12**:991–1045. doi:10.1146/annurev.immunol.12.1.991
16. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* (2011) **34**:637–50. doi:10.1016/j.immuni.2011.05.006
17. Osorio F, Reis e Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity* (2011) **34**:651–64. doi:10.1016/j.immuni.2011.05.001
18. Loo YM, Gale M Jr. Immune signaling by RIG-I-like receptors. *Immunity* (2011) **34**:680–92. doi:10.1016/j.immuni.2011.05.003
19. Wang H, Ryn WS. Hepatitis B virus polymerase blocks pattern recognition receptor signaling via interaction with DDX3: implications for immune evasion. *PLoS Pathog* (2010) **6**:e1000986. doi:10.1371/journal.ppat.1000986
20. Wen H, Miao EA, Ting JP. Mechanisms of NOD-like receptor-associated inflammasome activation. *Immunity* (2013) **39**:432–41. doi:10.1016/j.immuni.2013.08.037
21. Wu J, Chen ZJ. Innate immune sensing and signaling of cytosolic nucleic acids. *Annu Rev Immunol* (2014) **32**:461–88. doi:10.1146/annurev-immunol-032713-120156
22. Dauber B, Wolff T. Activation of the antiviral kinase PKR and viral countermeasures. *Viruses* (2009) **1**:523–44. doi:10.3390/v1030523
23. Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, et al. IFI16 is an innate immune sensor for intracellular DNA. *Nat Immunol* (2010) **11**:997–1004. doi:10.1038/ni.1932
24. Paludan SR, Bowie AG. Immune sensing of DNA. *Immunity* (2013) **38**:870–80. doi:10.1016/j.immuni.2013.05.004
25. Yan N, Chen ZJ. Intrinsic antiviral immunity. *Nat Immunol* (2012) **13**:214–22. doi:10.1038/ni.2229
26. Bowie AG, Unterholzner L. Viral evasion and subversion of pattern-recognition receptor signalling. *Nat Rev Immunol* (2008) **8**:911–22. doi:10.1038/nri2436
27. Park SH, Rehermann B. Immune responses to HCV and other hepatitis viruses. *Immunity* (2014) **40**:13–24. doi:10.1016/j.immuni.2013.12.010
28. Horner SM. Activation and evasion of antiviral innate immunity by hepatitis C virus. *J Mol Biol* (2014) **426**:1198–209. doi:10.1016/j.jmb.2013.10.032
29. Horner SM, Gale M Jr. Regulation of hepatic innate immunity by hepatitis C virus. *Nat Med* (2013) **19**:879–88. doi:10.1038/nm.3253
30. Howell J, Angus P, Gow P, Visvanathan K. Toll-like receptors in hepatitis C infection: implications for pathogenesis and treatment. *J Gastroenterol Hepatol* (2013) **28**:766–76. doi:10.1111/jgh.12170
31. Imran M, Waheed Y, Manzoor S, Bilal M, Ashraf W, Ali M, et al. Interaction of Hepatitis C virus proteins with pattern recognition receptors. *Virol J* (2012) **9**:126. doi:10.1186/1743-422X-9-126
32. Rosen HR. Emerging concepts in immunity to hepatitis C virus infection. *J Clin Invest* (2013) **123**:4121–30. doi:10.1172/JCI67714
33. Heim MH. Innate immunity and HCV. *J Hepatol* (2013) **58**:564–74. doi:10.1016/j.jhep.2012.10.005
34. Takahashi K, Asabe S, Wieland S, Garaigorta U, Gastaminza P, Isogawa M, et al. Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection. *Proc Natl Acad Sci U S A* (2010) **107**:7431–6. doi:10.1073/pnas.1002301107
35. Dreux M, Garaigorta U, Boyd B, Decembre E, Chung J, Whitten-Bauer C, et al. Short-range exosomal transfer of viral RNA from infected cells to plasmacytoid dendritic cells triggers innate immunity. *Cell Host Microbe* (2012) **12**:558–70. doi:10.1016/j.chom.2012.08.010
36. Schnell G, Loo YM, Marcotrigiano J, Gale M Jr. Uridine composition of the poly-U/UC tract of HCV RNA defines non-self recognition by RIG-I. *PLoS Pathog* (2012) **8**:e1002839. doi:10.1371/journal.ppat.1002839
37. Arnaud N, Dabo S, Akazawa D, Fukasawa M, Shinkai-Ouchi F, Hugon J, et al. Hepatitis C virus reveals a novel early control in acute immune response. *PLoS Pathog* (2011) **7**:e1002289. doi:10.1371/journal.ppat.1002289
38. Oshiumi H, Miyashita M, Matsumoto M, Seya T, distinct A. role of Riplet-mediated K63-linked polyubiquitination of the RIG-I repressor domain in human antiviral innate immune responses. *PLoS Pathog* (2013) **9**:e1003533. doi:10.1371/journal.ppat.1003533
39. Ding Q, Cao X, Lu J, Huang B, Liu YJ, Kato N, et al. Hepatitis C virus NS4B blocks the interaction of STING and TBK1 to evade host innate immunity. *J Hepatol* (2013) **59**:52–8. doi:10.1016/j.jhep.2013.03.019
40. Nitta S, Sakamoto N, Nakagawa M, Kakinuma S, Mishima K, Kusano-Kitazume A, et al. Hepatitis C virus NS4B protein targets STING and abrogates RIG-I-mediated type I interferon-dependent innate immunity. *Hepatology* (2013) **57**:46–58. doi:10.1002/hep.26017
41. Mozer-Lisewska I, Kowala-Piaskowska A, Mania A, Jenek R, Samara H, Kaczmarek E, et al. Expression of pattern recognition receptors in liver biopsy specimens of children chronically infected with HBV and HCV. *Folia Histochem Cytobiol* (2011) **49**:410–6. doi:10.5603/FHC.2011.0058
42. Fan H, Zhu Z, Wang Y, Zhang X, Lu Y, Tao Y, et al. Molecular characterization of the type I IFN receptor in two woodchuck species and detection of its expression in liver samples from woodchucks infected with woodchuck hepatitis virus (WHV). *Cytokine* (2012) **60**:179–85. doi:10.1016/j.cyto.2012.05.013
43. Dunn C, Peppa D, Khanna P, Nebbia G, Jones M, Brendish N, et al. Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. *Gastroenterology* (2009) **137**:1289–300. doi:10.1053/j.gastro.2009.06.054
44. Fisicaro P, Valdatta C, Boni C, Massari M, Mori C, Zerbini A, et al. Early kinetics of innate and adaptive immune responses during hepatitis B virus infection. *Gut* (2009) **58**:974–82. doi:10.1136/gut.2008.163600
45. Wieland S, Thimme R, Purcell RH, Chisari FV. Genomic analysis of the host response to hepatitis B virus infection. *Proc Natl Acad Sci U S A* (2004) **101**:6669–74. doi:10.1073/pnas.0401771101
46. Chang Y, Moore PS. Merkel cell carcinoma: a virus-induced human cancer. *Annu Rev Pathol* (2012) **7**:123–44. doi:10.1146/annurev-pathol-011110-130227
47. Guo H, Jiang D, Ma D, Chang J, Dougherty AM, Cuconati A, et al. Activation of pattern recognition receptor-mediated innate immunity inhibits the replication of hepatitis B virus in human hepatocyte-derived cells. *J Virol* (2009) **83**:847–58. doi:10.1128/JVI.02008-08
48. Zhang X, Ma Z, Liu H, Liu J, Meng Z, Broering R, et al. Role of toll-like receptor 2 in the immune response against hepadnaviral infection. *J Hepatol* (2012) **57**:522–8. doi:10.1016/j.jhep.2012.05.004
49. Isogawa M, Robek MD, Furuichi Y, Chisari FV. Toll-like receptor signaling inhibits hepatitis B virus replication in vivo. *J Virol* (2005) **79**:7269–72. doi:10.1128/JVI.79.11.7269-7272.2005
50. Lanford RE, Guerra B, Chavez D, Giavedoni L, Hodara VL, Brasky KM, et al. GS-9620, an oral agonist of toll-like receptor-7, induces prolonged suppression of hepatitis B virus in chronically infected chimpanzees. *Gastroenterology* (2013) **144**:1508–17, 1517.e1–10. doi:10.1053/j.gastro.2013.02.003

51. Zhang X, Kraft A, Broering R, Schlaak JF, Dittmer U, Lu M. Preclinical development of TLR ligands as drugs for the treatment of chronic viral infections. *Expert Opin Drug Discov* (2012) **7**:597–611. doi:10.1517/17460441.2012.689281
52. Lu HL, Liao F. Melanoma differentiation-associated gene 5 senses hepatitis B virus and activates innate immune signaling to suppress virus replication. *J Immunol* (2013) **191**:3264–76. doi:10.4049/jimmunol.1300512
53. Zhao G, An B, Zhou H, Wang H, Xu Y, Xiang X, et al. Impairment of the retinoic acid-inducible gene-I-IFN-beta signaling pathway in chronic hepatitis B virus infection. *Int J Mol Med* (2012) **30**:1498–504. doi:10.3892/ijmm.2012.1131
54. Wei C, Ni C, Song T, Liu Y, Yang X, Zheng Z, et al. The hepatitis B virus X protein disrupts innate immunity by downregulating mitochondrial antiviral signaling protein. *J Immunol* (2010) **185**:1158–68. doi:10.4049/jimmunol.0903874
55. Wang X, Li Y, Mao A, Li C, Li Y, Tien P. Hepatitis B virus X protein suppresses virus-triggered IRF3 activation and IFN-beta induction by disrupting the VISA-associated complex. *Cell Mol Immunol* (2010) **7**:341–8. doi:10.1038/cmi.2010.36
56. Kumar M, Jung SY, Hodgson AJ, Madden CR, Qin J, Slagle BL. Hepatitis B virus regulatory HBx protein binds to adaptor protein IPS-1 and inhibits the activation of beta interferon. *J Virol* (2011) **85**:987–95. doi:10.1128/JVI.01825-10
57. Goubaud D, Deddouche S, Reis ESC. Cytosolic sensing of viruses. *Immunity* (2013) **38**:855–69. doi:10.1016/j.immuni.2013.05.007
58. Chen QY, Liu YH, Li JH, Wang ZK, Liu JX, Yuan ZH, et al. Activator of interferon-regulatory factors inhibits hepatitis B virus replication. *World J Gastroenterol* (2012) **18**:2850–8. doi:10.3748/wjg.v18.i22.2850
59. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. *Nat Rev Cancer* (2004) **4**:757–68. doi:10.1038/nrc1452
60. Chijioka O, Azzi T, Nadal D, Munz C. Innate immune responses against Epstein Barr virus infection. *J Leukoc Biol* (2013) **94**:1185–90. doi:10.1189/jlb.0313173
61. Ning S. Innate immune modulation in EBV infection. *Herpesviridae* (2011) **2**:1. doi:10.1186/2042-4280-2-1
62. Paludan SR, Bowie AG, Horan KA, Fitzgerald KA. Recognition of herpesviruses by the innate immune system. *Nat Rev Immunol* (2011) **11**:143–54. doi:10.1038/nri2937
63. Ariza ME, Glaser R, Kaumaya PT, Jones C, Williams MV. The EBV-encoded dUTPase activates NF-kappa B through the TLR2 and MyD88-dependent signaling pathway. *J Immunol* (2009) **182**:851–9. doi:10.4049/jimmunol.182.2.851
64. Ariza ME, Rivaiola P, Glaser R, Chen M, Williams MV. Epstein-Barr virus encoded dUTPase containing exosomes modulate innate and adaptive immune responses in human dendritic cells and peripheral blood mononuclear cells. *PLoS One* (2013) **8**:e69827. doi:10.1371/journal.pone.0069827
65. Iwakiri D, Zhou L, Samanta M, Matsumoto M, Ebihara T, Seya T, et al. Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from toll-like receptor 3. *J Exp Med* (2009) **206**:2091–9. doi:10.1084/jem.20081761
66. Iwakiri D, Takada K. Role of EBERs in the pathogenesis of EBV infection. *Adv Cancer Res* (2010) **107**:119–36. doi:10.1016/S0065-230X(10)07004-1
67. Valente RM, Ehlers E, Xu D, Ahmad H, Steadman A, Blasznay L, et al. Toll-like receptor 7 stimulates the expression of Epstein-Barr virus latent membrane protein 1. *PLoS One* (2012) **7**:e43317. doi:10.1371/journal.pone.0043317
68. Severa M, Giacomini E, Gafa V, Anastasiadou E, Rizzo F, Corazzari M, et al. EBV stimulates TLR- and autophagy-dependent pathways and impairs maturation in plasmacytoid dendritic cells: implications for viral immune escape. *Eur J Immunol* (2013) **43**:147–58. doi:10.1002/eji.201242552
69. van Gent M, Braem SG, de Jong A, Delaglic N, Peeters JG, Boer IG, et al. Epstein-Barr virus large tegument protein BPLF1 contributes to innate immune evasion through interference with toll-like receptor signaling. *PLoS Pathog* (2014) **10**:e1003960. doi:10.1371/journal.ppat.1003960
70. Fathallah I, Parroche P, Gruffat H, Zannetti C, Johansson H, Yue J, et al. EBV latent membrane protein 1 is a negative regulator of TLR9. *J Immunol* (2010) **185**:6439–47. doi:10.4049/jimmunol.0903459
71. Ablasser A, Bauernfeind F, Hartmann G, Latz E, Fitzgerald KA, Hornung V. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nat Immunol* (2009) **10**:1065–72. doi:10.1038/ni.1779
72. Moore PS, Chang Y. Kaposi's sarcoma-associated herpesvirus immunoevasion and tumorigenesis: two sides of the same coin? *Annu Rev Microbiol* (2003) **57**:609–39. doi:10.1146/annurev.micro.57.030502.090824
73. West JA, Damania B. Kaposi's sarcoma-associated herpesvirus and innate immunity. *Future Virol* (2010) **5**:185–96. doi:10.2217/fvl.10.5
74. Dittmer DP, Damania B. Kaposi sarcoma associated herpesvirus pathogenesis (KSHV) – an update. *Curr Opin Virol* (2013) **3**:238–44. doi:10.1016/j.coviro.2013.05.012
75. Sathish N, Yuan Y. Evasion and subversion of interferon-mediated antiviral immunity by Kaposi's sarcoma-associated herpesvirus: an overview. *J Virol* (2011) **85**:10934–44. doi:10.1128/JVI.00687-11
76. Lagos D, Vart RJ, Gratrix F, Westrop SJ, Emuss V, Wong PP, et al. Toll-like receptor 4 mediates innate immunity to Kaposi sarcoma herpesvirus. *Cell Host Microbe* (2008) **4**:470–83. doi:10.1016/j.chom.2008.09.012
77. West J, Damania B. Upregulation of the TLR3 pathway by Kaposi's sarcoma-associated herpesvirus during primary infection. *J Virol* (2008) **82**:5440–9. doi:10.1128/JVI.02590-07
78. Gregory SM, West JA, Dillon PJ, Hilscher C, Dittmer DP, Damania B. Toll-like receptor signaling controls reactivation of KSHV from latency. *Proc Natl Acad Sci U S A* (2009) **106**:11725–30. doi:10.1073/pnas.0905316106
79. West JA, Gregory SM, Sivaraman V, Su L, Damania B. Activation of plasmacytoid dendritic cells by Kaposi's sarcoma-associated herpesvirus. *J Virol* (2011) **85**:895–904. doi:10.1128/JVI.01007-10
80. Ahmad H, Gubbels R, Ehlers E, Meyer F, Waterbury T, Lin R, et al. Kaposi sarcoma-associated herpesvirus degrades cellular toll-interleukin-1 receptor domain-containing adaptor-inducing beta-interferon (TRIF). *J Biol Chem* (2011) **286**:7865–72. doi:10.1074/jbc.M110.191452
81. Meyer F, Ehlers E, Steadman A, Waterbury T, Cao M, Zhang L. TLR-TRIF pathway enhances the expression of KSHV replication and transcription activator. *J Biol Chem* (2013) **288**:20435–42. doi:10.1074/jbc.M113.487421
82. Gregory SM, Davis BK, West JA, Taxman DJ, Matsuzawa S, Reed JC, et al. Discovery of a viral NLR homolog that inhibits the inflammasome. *Science* (2011) **331**:330–4. doi:10.1126/science.1199478
83. Kerur N, Veetil MV, Sharma-Walia N, Bottero V, Sadagopan S, Otageri P, et al. IFI16 acts as a nuclear pathogen sensor to induce the inflammasome in response to Kaposi Sarcoma-associated herpesvirus infection. *Cell Host Microbe* (2011) **9**:363–75. doi:10.1016/j.chom.2011.04.008
84. Singh VV, Kerur N, Bottero V, Dutta S, Chakraborty S, Ansari MA, et al. Kaposi's sarcoma-associated herpesvirus latency in endothelial and B cells activates gamma interferon-inducible protein 16-mediated inflammasomes. *J Virol* (2013) **87**:4417–31. doi:10.1128/JVI.03282-12
85. West JA, Wicks M, Gregory SM, Chugh P, Jacobs SR, Zhang Z, et al. An important role for MAVS in the KSHV lifecycle. *J Virol* (2014) **88**:5778–87. doi:10.1128/JVI.03226-13
86. Frazer IH, Leggett GR, Mattarollo SR. Prevention and treatment of papillomavirus-related cancers through immunization. *Annu Rev Immunol* (2011) **29**:111–38. doi:10.1146/annurev-immunol-031210-101308
87. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer* (2002) **2**:342–50. doi:10.1038/nrc798
88. Amador-Molina A, Hernandez-Valencia JF, Lamoyi E, Contreras-Paredes A, Lizano M. Role of innate immunity against human papillomavirus (HPV) infections and effect of adjuvants in promoting specific immune response. *Viruses* (2013) **5**:2624–42. doi:10.3390/v5112624
89. Roden R, Wu TC. How will HPV vaccines affect cervical cancer? *Nat Rev Cancer* (2006) **6**:753–63. doi:10.1038/nrc1973
90. Raff AB, Woodham AW, Raff LM, Skeate JG, Yan L, Da Silva DM, et al. The evolving field of human papillomavirus receptor research: a review of binding and entry. *J Virol* (2013) **87**:6062–72. doi:10.1128/JVI.00330-13
91. Zhou Q, Zhu K, Cheng H. Toll-like receptors in human papillomavirus infection. *Arch Immunol Ther Exp* (2013) **61**:203–15. doi:10.1007/s00005-013-0220-7
92. Hasan UA, Zannetti C, Parroche P, Goutagny N, Malfroy M, Roblot G, et al. The human papillomavirus type 16 E7 oncoprotein induces a transcriptional repressor complex on the toll-like receptor 9 promoter. *J Exp Med* (2013) **210**:1369–87. doi:10.1084/jem.20122394
93. Niebler M, Qian X, Hofler D, Kogosov V, Kaewprag J, Kaufmann AM, et al. Post-translational control of IL-1beta via the human papillomavirus type 16 E6 oncoprotein: a novel mechanism of innate immune escape mediated by the E3-ubiquitin ligase E6-AP and p53. *PLoS Pathog* (2013) **9**:e1003536. doi:10.1371/journal.ppat.1003536
94. Karim R, Tummers B, Meyers C, Biryukov JL, Alam S, Backendorf C, et al. Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1

- to suppress the keratinocyte's innate immune response. *PLoS Pathog* (2013) **9**:e1003384. doi:10.1371/journal.ppat.1003384
95. Kumar MM, Adurthi S, Ramachandran S, Mukherjee G, Joy O, Krishnamurthy H, et al. Toll-like receptors 7, 8, and 9 expression and function in primary human cervical cancer Langerhans cells: evidence of anergy. *Int J Gynecol Cancer* (2013) **23**:184–92. doi:10.1097/IGC.0b013e31827a2003
96. Verdonck K, Gonzalez E, Van Dooren S, Vandamme AM, Vanham G, Gotuzzo E. Human T-lymphotropic virus 1: recent knowledge about an ancient infection. *Lancet Infect Dis* (2007) **7**:266–81. doi:10.1016/S1473-3099(07)70081-6
97. Kannagi M, Hasegawa A, Takamori A, Kinpara S, Utsunomiya A. The roles of acquired and innate immunity in human T-cell leukemia virus type 1-mediated diseases. *Front Microbiol* (2012) **3**:323. doi:10.3389/fmicb.2012.00323
98. Cook LB, Elemans M, Rowan AG, Asquith B. HTLV-1: persistence and pathogenesis. *Virology* (2013) **435**:131–40. doi:10.1016/j.virol.2012.09.028
99. Journo C, Mahieux R. HTLV-1 and innate immunity. *Viruses* (2011) **3**:1374–94. doi:10.3390/v3081374
100. Hajj HE, Nasr R, Kfouri Y, Dassouki Z, Nasser R, Kchour G, et al. Animal models on HTLV-1 and related viruses: what did we learn? *Front Microbiol* (2012) **3**:333. doi:10.3389/fmicb.2012.00333
101. Zimmerman B, Niewiesk S, Lairmore MD. Mouse models of human T lymphotoxic virus type-1-associated adult T-cell leukemia/lymphoma. *Vet Pathol* (2010) **47**:677–89. doi:10.1177/0300985810370009
102. Colisson R, Barblu L, Gras C, Raynaud F, Hadj-Slimane R, Pique C, et al. Free HTLV-1 induces TLR7-dependent innate immune response and TRAIL relocalization in killer plasmacytoid dendritic cells. *Blood* (2010) **115**:2177–85. doi:10.1182/blood-2009-06-224741
103. Dalianis T, Hirsch HH. Human polyomaviruses in disease and cancer. *Virology* (2013) **437**:63–72. doi:10.1016/j.virol.2012.12.015
104. DeCaprio JA, Garcea RL. A cornucopia of human polyomaviruses. *Nat Rev Microbiol* (2013) **11**:264–76. doi:10.1038/nrmicro2992
105. Shahzad N, Shuda M, Gheit T, Kwun HJ, Cornet I, Saidj D, et al. The T antigen locus of Merkel cell polyomavirus downregulates human toll-like receptor 9 expression. *J Virol* (2013) **87**:13009–19. doi:10.1128/JVI.01786-13

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Cancer: a tale of aberrant PRR response

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INTRODUCTION

Cancer is a disease of complex etiology and multistep progression, manipulating the regular routes to homeostasis. Any deviation from homeostasis alerts the innate immune system and provokes inflammation. Inflammation is generated by the signaling cascades launched by the pattern recognition receptors (PRRs), the germline encoded molecules dedicated to sense pathogen, or danger-associated molecular patterns (PAMPs or DAMPs) in case of pathogen/foreign matter invasion and intrinsic disturbances, respectively (1–3). Through inflammation, PRRs eliminate stress signals and re-establish homeostasis in the body, via drawing the required cellular machinery to the inflammatory sites. However, the same lympho-reticular infiltrate has been linked with incidence of cancer at the site of chronic inflammation, since 1863, by Rudolf Virchow (4). From 1990s vast amount of literature has accumulated associating soluble and cellular factors of innate immune system with prevalence and progression of cancer. Furthermore, in the past decade, several pathogens have been linked with cancer as well [Ref. (5, 6) and references therein].

Fascinatingly, it is remarkable how the tightly regulated sensory system for stress removal and maintenance of homeostasis functions anomalously and promotes occurrence and progression of cancers (7–9).

PRR-MEDIATED RESPONSES AND CANCER PROGRESSION

All PRR-dependent pathways activate a particular set of transcription factors to generate appropriate responses. The same factors govern cellular proliferation, apoptosis, tissue remodeling, or angiogenesis, and exhibit a perturbed activity during

cancer. One such key protein is nuclear factor κ B (NF κ B); up-regulation of which leads to production of pro-inflammatory cytokines. Additionally, it induces anti-apoptotic proteins like Bcl2 or inhibitors of apoptotic proteins (IAPs) and angiogenic proteins, such as angiopoietin or vascular endothelial growth factor (VEGF). NF κ B also induces nitrous oxide synthase-2 (NOS-2), thus producing nitrous oxide (NO) in the immune cells, which along with reactive oxygen species (ROS) eradicates infected cells by lipid per-oxidation and DNA damage (10–14). Conversely, genomic instability and free radicals thus produced act as DAMPs, leading to sensitization of neighboring PRRs and further immune activation, for instance, the DNA fragments released can activate local DNA sensors, resulting in production of Type I IFN by DAI-TBK1, and activate KRAS pathway of cellular proliferation via TBK1-Sec5 complex, which leads to further activation of NF κ B and production of anti-apoptotic proteins (15). That is, detouring regular anti-cancer pathway toward proliferation. Also, RONS induce DNA methylases, which lead to methylation and silencing of tumor suppressor and DNA damage repair genes (2, 16–18).

Another pathway crucial in immunity and cancer is the Janus kinases (JAK)-signal transducers and activators of transcription (STAT) pathway. Triggered primarily by interferons and some other mediators, this pathway stimulates various proliferative genes, such as IL-6-mediated induction of myc and CyclinD1/D2 through JAK; also TNF α -mediated up-regulation of STAT-3 leading to activation of Ras-mitogen activated protein kinase (MAPK) pathway, which leads to the expression of transcription factor activating protein (AP)-1, and epidermal

growth factor (EGFs) along with eukaryotic initiation factor (eIF)-4. AP-1 couples with NF κ B, inducing matrix metalloproteinase (MMP)-9, a protein involved in tissue remodeling required during angiogenesis (19). Thus, the pro-inflammatory signal culminates in the production of proteins aiding tumor survival, proliferation, and development of tumor-associated vasculature (18).

Furthermore, NF κ B is also involved in the expression of NLRP3, which assembles with apoptosis-associated speck-like protein containing a CARD (ASC) caspase-1 to form multi-protein complexes, the inflammasomes, and responds to DAMPs, especially nucleotides released from damaged or necrotic tissue (due to cytotoxicity of free radicals) (20). Likewise, absent in myeloma (AIM)-2 inflammasomes also organize in response to the formation of DNA adducts (DNA and cytosolic protein HMGB-1) from the dying tissue (21). These assemblies lead to activation of IL-1 β –IL-1 β R pair; a system found commonly over-activated in many cancers (2, 22). Additionally, NF κ B also generates cyclo-oxygenase-2 (COX-2) enzyme, which converts arachidonic acid into prostaglandin-E2 (PGE-2), one of the dual (pro-inflammatory and/or anti-inflammatory) mediators of immune response. PGE-2 enhances T-cell activation and represses B-cell activity (23, 24). Another common enzyme, activation-induced deaminase (AID), also induced by NF κ B, involved in somatic hypermutation and class switch recombination in B-cells, causes genome instability and releases additional DAMPs into the microenvironment (25). Thus, the immune mediators produced for protection can divert inflammation toward pro-tumor facet (26).

A set of pro-inflammatory cytokines consisting of TNF- α and IL-1 and 6 is essentially tumor directing. TNF- α promotes tumor initiation and DNA damage. It also up-regulates hypoxia-inducible factor (HIF)-1 α (attributed to the increasingly low oxygen levels due to multiplying cells) aiding in angiogenesis (27). IL-1 β aids in tumor invasiveness and adhesion required during metastasis to new sites. IL-1 α , the membrane bound form, induces IL-1 expression, associated with tissue damage, compensatory cell proliferation, and activation of JAK-STAT pathway, as seen in hepatocellular carcinomas and colitis-associated cancers (22, 28).

Cigarette smoking has long been associated with incidence of cancer. Cigarette smoke contains numerous compounds with known cytotoxicity, mutagenicity, and carcinogenicity, most of which are particulate. Stable ROS present in the smoke damage DNA and cause lipid per-oxidation, sensitizing the PRRs present from the buccal cavity to lungs leading to increased IL-8 and TNF- α (11). In addition, both, NF κ B and AP-1 are up-regulated exaggerating the pro-inflammatory signal, at the same time homeostatic activity of both is reduced, compensating normal immune response. Such a response coupled with prolonged exposure can spontaneously lead to cellular transformations and their expansion (29, 30).

ROLE OF CELLULAR COMPONENTS OF INNATE IMMUNITY IN CANCER PROGRESSION

Specialized cells of the immune system are equipped with PRRs, and are responsible for clearance of diseased/damaged cells. Pro-inflammatory cytokines draw these cells toward the inflammatory site and direct them for removal of pathogens, particulates, or immune debris. These populations recede as the signal resolves. Since Virchow proposed their role at the site of chronic inflammation and cancer, a number of cellular populations and their effector responses have been ascertained for the same. In cases of prolonged exposure to PAMPs/DAMPs, infiltrating cells fail to withdraw and differentiate into M2 macrophages, identified as tumor-associated macrophages (TAMs), an integral population programed

for tissue remodeling and tumor progression. Upon activation of their PRRs, TAMs promote various properties of cancer by releasing a range of inflammatory and angiogenic bio-chemicals. These cells stimulate proliferation of stromal tissue and macrophages by growth factors such as platelet-derived growth factor (PDGF) and colony stimulating factor (CSF)-1 respectively (31). Moreover, they organize a route to metastasis by digesting the substratum, basal lamina and release inactive growth factors via, MMPs. In addition, they assist in cellular movements by releasing cell adhesion molecules such as intercellular adhesion molecule (ICAM)-1 (32–34).

Another such population, the NK cells meant to carry out cytotoxic clearance of all the cells which do not express human leukocyte antigen (HLA) A/B/C and thus fail to activate the membrane-expressed inhibitory receptors (NKp30/44/46). NK cells are also responsible for killing any cell, irrespective of HLA tag, which presents them with stress/abnormality/tumor-associated antigens, via, activating receptor (NKG2D). In addition, they also participate in antibody-dependent cell-mediated cytotoxicity (ADCC), on cells tagged through FCR γ III (35, 36). A number of cytokines activate NK cells and turn them into lymphokine-activated killers, causing them to display their killing property at the site of recruitment (37). Tumor cells escape NK cells by blocking the activating receptor. Also, even if recruited, NK cells can only bring about killing of the outer cells in a solid tumor (38, 39). Furthermore, a reduction in NK cell cytotoxic function as well as NK cell dependent tumor surveillance is evident as a tumor directing effect of cigarette smoke (29, 30).

Another newly characterized population of cells called myeloid-derived suppressor cells (MDSCs) are recruited by inflammatory mediators, which inhibit the anti-tumor responses and release pro-tumor molecules, like, NOS-2 and TGF- β . Arginase-1 and indolamine-2,3-dioxygenase produced by MDSCs are involved in silencing the anti-tumor immunity by reducing Th1 activation (40, 41).

Soluble and cellular factors work in union. Cellular proliferation at an

enhanced rate at tumor sites gives rise to hypoxia, low concentration of oxygen that stabilizes HIF-1 α , which activates NF κ B to secrete an angiogenic protein, VEGF and also induces expression of IL-12 and TNF- α . These mediators collectively induce STAT-3 production that up-regulates PGE-2 which further recruits NK cells and their cytotoxic function releases DAMPs in vicinity attracting TAMs which aid in shaping neo-vasculature and creating area for growing cell mass. Deregulation of Th1 responses by PGE-2 and MDSC/TAMs creates an imbalance (42–44). HIF-1 α which causes glycolytic environment, such conditions may tip the balance into a state when IL-12 production is replaced by IL-23, that is, a shift from anti-tumor to pro-tumor (45, 46). In this manner, the PRR triggered pathways to eliminate PAMPs/DAMPs, are rerouted in a manner that leads to exaggeration of the initial signal causing chronic inflammation; moreover eliciting other pathways concluding in tumor growth and metastasis (1, 47, 48).

REGULATORY MECHANISMS AND PROGRESSION OF CANCER

To maintain homeostasis in the body, all cellular processes, including PRR generated immune responses are regulated by various mechanisms. This control is exercised at various levels. At transcriptional level certain cytokines can inhibit transcription factors, such as IL-4/13 which hinder NF κ B. Alternatively; some cytokines directly inhibit other cytokines at protein level, such as inhibition of TNF- α by IL-10. At post-transcriptional level microRNAs play a crucial role by either resolving inflammation or potentiating pro-tumor effects of cytokines (18). For instance, TNF- α /IL-1 β induced mir-146a inhibits IRAK/TRAF6, that is, the downstream signaling of TLR pathway, thus resolving inflammation (23). In contrast IL-6 induced mir-21 targets tumor suppressor genes, such as phosphatase and tensin homolog (PTEN), programmed cell death (PDCD)-4, and others, thereby hampering the anti-tumor effects (49–52). Another microRNA found commonly up-regulated in cancer, miR-155, induces NOS-2 and inhibits apoptosis by down-regulating TP53INP1 (a downstream molecule of p53 signaling)

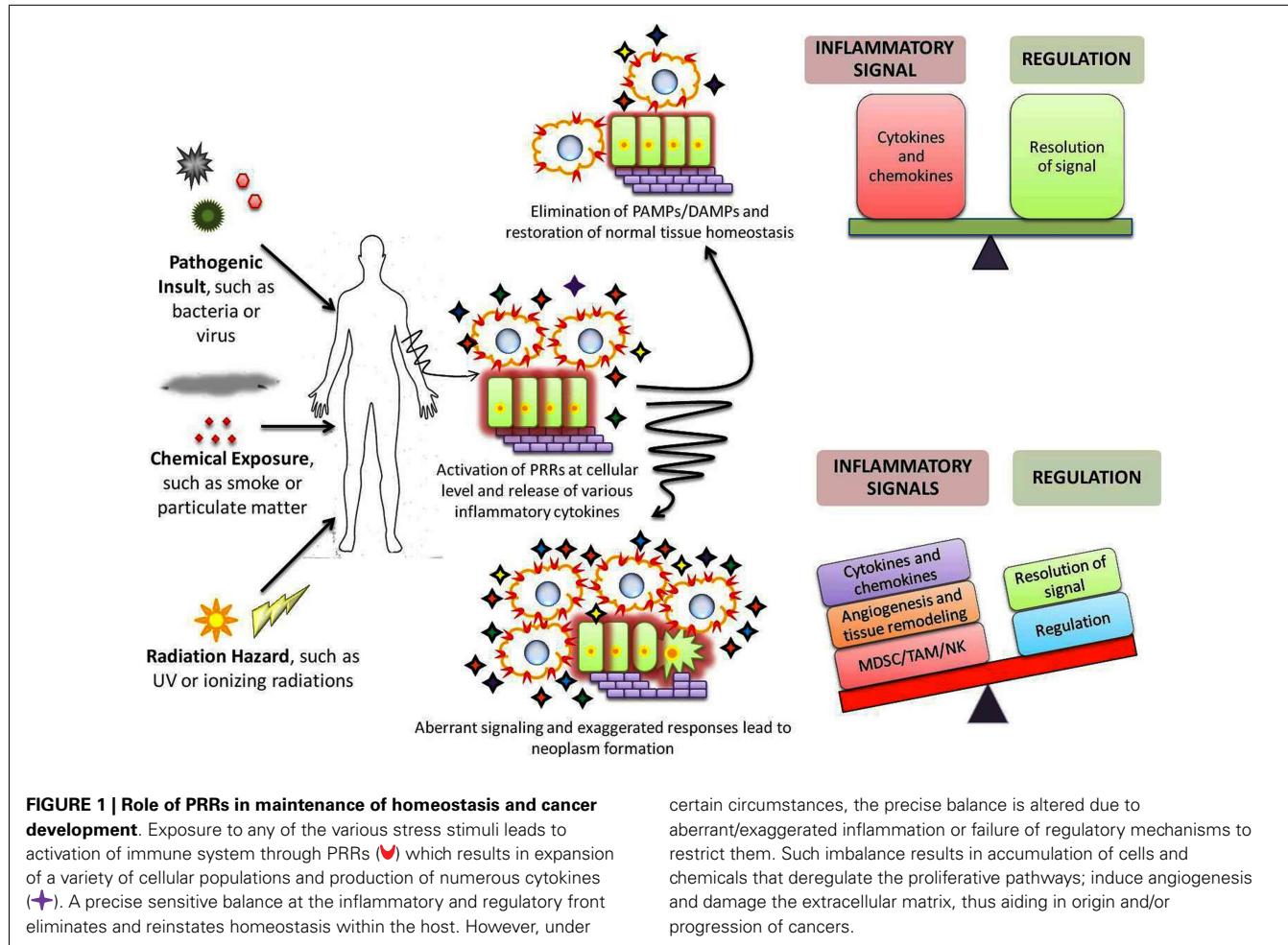


FIGURE 1 | Role of PRRs in maintenance of homeostasis and cancer development. Exposure to any of the various stress stimuli leads to activation of immune system through PRRs (●) which results in expansion of a variety of cellular populations and production of numerous cytokines (✚). A precise sensitive balance at the inflammatory and regulatory front eliminates and reinstates homeostasis within the host. However, under

certain circumstances, the precise balance is altered due to aberrant/exaggerated inflammation or failure of regulatory mechanisms to restrict them. Such imbalance results in accumulation of cells and chemicals that deregulate the proliferative pathways; induce angiogenesis and damage the extracellular matrix, thus aiding in origin and/or progression of cancers.

(53). Thus, a slight imbalance in the pro-inflammatory and anti-inflammatory signals can shift the equilibrium toward oncogenic transformations (18).

Pattern recognition receptor elicited signaling cascades induce synthesis of several zinc-finger proteins that help in regulating the inflammatory signals. For example, ZC3H12a/c and Zfp36 proteins degrade the cellular mRNA coding for pro-inflammatory cytokines; while ZAP proteins directly degrade the viral RNA, to be precise, the PAMP itself (54, 55). Thus, these proteins degrade the PAMPs or signal generated by them to curb inflammation. In addition, certain PRRs themselves restrain the downstream signaling such as inhibition of IPS-1, adaptor molecule of RLR, by NLRX-1. Mutations within these genes or their regulatory elements or imbalance at cellular level can skew the balance toward tumorigenesis (6, 55) (Figure 1).

NEW FACET IN THE FIELD

A relatively new but noteworthy field in analyzing cancer biology is polymorphisms. Single nucleotide polymorphisms (SNPs) are single base changes in the DNA, which may produce totally drastic effects on the structure and function of the encoded molecules. PRRs and the associated machinery, such as the adaptors or receptors are also susceptible to such polymorphisms and many such SNPs have been reported. SNPs could promote the anti-tumor effect if they prevent the PRR cascade from commencing, or support the pro-tumor effect, if they cause spontaneous induction of signaling without any stimulus. The cytoplasmic domains of the PRRs and cytokine receptors are of prime importance in this context as they form the docking site for progression of inflammatory response. Many polymorphisms have been identified to be associated with cancers of various origins.

Mostly CLRs and RLRs have been associated with sensing PAMPs of oncogenic origin, and polymorphisms in their genes have been correlated with cancers of mesoderm, endoderm, and also ectoderm origin. Also several genes and mutations have been correlated with cigarette smoke in association with cancers (56). Still, a rather comprehensive endeavor would be required to establish the integral role of these SNPs at the molecular level to outline their part in incidences and progression of cancers [Ref. (6) and references therein, Ref. (56)].

CONCLUSION

Recognition of pathogen/stress is one of the essential processes of the host. Tumor cells are in fact, abnormal cells which are steadily eliminated through PRR-mediated pathways. However, hyper or anomalous behavior of same pathways can divert the protective route

toward malignancy, by contributing to abnormal proliferation, angiogenesis, or modifying tissue architecture. The origin of anomalous behavior maybe external and internal, such as pathogen/foreign insults tissue damage/necrosis or mutations and polymorphisms in vital signaling components, respectively. Unfolding the root of these irregularities and malfunctions shall help in better understanding of the disease and thus, create new and personalized prospects for treatment.

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REFERENCES

- Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. *Mol Cancer Res* (2006) **4**:221–33. doi:10.1158/1541-7786.MCR-05-0261
- Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* (2010) **140**:805–20. doi:10.1016/j.cell.2010.01.022
- Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. *Int Rev Immunol* (2011) **30**:16–34. doi:10.3109/08830185.2010.529976
- Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* (2001) **357**:539–45. doi:10.1016/S0140-6736(00)04046-0
- Chen K, Huang J, Gong W, Iribarren P, Dunlop NM, Wang JM. Toll-like receptors in inflammation, infection and cancer. *Int Immunopharmacol* (2007) **7**:1271–85. doi:10.1016/j.intimp.2007.05.016
- Kutikhin AG, Yuzhalin AE. Inherited variation in pattern recognition receptors and cancer: dangerous liaisons? *Cancer Manag Res* (2012) **4**:31–8. doi:10.2147/CMAR.S28688
- Cristofori G. New signals from the invasive front. *Nature* (2006) **441**:444–50. doi:10.1038/nature04872
- Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? *Science* (2013) **339**:286–91. doi:10.1126/science.1232227
- de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev Cancer* (2006) **6**:24–37. doi:10.1038/nrc1782
- Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation and cancer: how hot is the link? *Biochem Pharmacol* (2006) **72**:1605–21. doi:10.1016/j.bcp.2006.06.029
- Closa D, Folch-Puy E. Oxygen free radicals and the systemic inflammatory response. *IUBMB Life* (2004) **56**:185–91. doi:10.1080/15216540410001701642
- Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, et al. NF-κB functions as a tumour promoter in inflammation-associated cancer. *Nature* (2004) **431**:461–6. doi:10.1038/nature02924
- Ying L, Hofseth AB, Browning DD, Nagarkatti M, Nagarkatti PS, Hofseth LJ. Nitric oxide inactivates the retinoblastoma pathway in chronic inflammation. *Cancer Res* (2007) **67**:9286–93. doi:10.1158/0008-5472.CAN-06-2149
- Meira LB, Bugni JM, Green SL, Lee CW, Pang B, Borenshtein D, et al. DNA damage induced by chronic inflammation contributes to colon carcinogenesis in mice. *J Clin Invest* (2008) **118**:2516–25. doi:10.1172/JCI35073
- Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature* (2009) **462**:108–12. doi:10.1038/nature08460
- Karin M. Nuclear factor-κB in cancer development and progression. *Nature* (2006) **441**:431–6. doi:10.1038/nature04870
- Sato Y, Goto Y, Narita N, Hoon DS. Cancer cells expressing toll-like receptors and the tumor microenvironment. *Cancer Microenviron* (2009) **2**(Suppl 1):205–14. doi:10.1007/s12307-009-0022-y
- Schetter AJ, Heegaard NH, Harris CC. Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis* (2010) **31**:37–49. doi:10.1093/carcin/bgp272
- Giraudo M, Inoue M, Hanahan D. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. *J Clin Invest* (2004) **114**:623–33. doi:10.1172/JCI200422087
- Chen GY, Nunez G. Inflammasomes in intestinal inflammation and cancer. *Gastroenterology* (2011) **141**:1986–99. doi:10.1053/j.gastro.2011.10.002
- Yanai H, Ban T, Wang Z, Choi MK, Kawamura T, Negishi H, et al. HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. *Nature* (2009) **462**:99–103. doi:10.1038/nature08512
- Song X, Voronov E, Dvorkin T, Fima E, Cagnano E, Benharroch D, et al. Differential effects of IL-1 alpha and IL-1 beta on tumorigenicity patterns and invasiveness. *J Immunol* (2003) **171**:6448–56.
- Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol* (2010) **22**:231–7. doi:10.1016/j.coi.2010.01.009
- Simmons DL, Botting RM, Hla T. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol Rev* (2004) **56**:387–437. doi:10.1124/pr.56.3.3
- Robbiani DF, Bothmer A, Callen E, Reina-San-Martin B, Dorsett Y, Difilippantonio S, et al. AID is required for the chromosomal breaks in c-myc that lead to c-myc/IgH translocations. *Cell* (2008) **135**:1028–38. doi:10.1016/j.cell.2008.09.062
- Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* (2010) **140**:883–99. doi:10.1016/j.cell.2010.01.025
- Rius J, Guma M, Schachtrup C, Akassoglou K, Zinkernagel AS, Nizet V, et al. Karin: NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α. *Nature* (2008) **453**:807–11. doi:10.1038/nature06905
- Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* (2006) **441**:437–43. doi:10.1038/nature04871
- Lee J, Taneja V, Vassallo R. Cigarette smoking and inflammation: cellular and molecular mechanisms. *J Dent Res* (2012) **91**:142–9. doi:10.1177/0022034511421200
- Mehta H, Nazzal K, Sadikot RT. Cigarette smoking and innate immunity. *Inflamm Res* (2008) **57**:497–503. doi:10.1007/s00011-008-8078-6
- Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* (2001) **193**:727–40. doi:10.1084/jem.193.6.727
- Chen JJW, Lin YC, Yao PL, Yuan A, Chen HY, Shun CT, et al. Tumor-associated macrophages: the double-edged sword in cancer progression. *J Clin Oncol* (2005) **23**:953–64. doi:10.1200/JCO.2005.12.172
- Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* (2008) **454**:436–44. doi:10.1038/nature07205
- Quatromoni JG, Eruslanov E. Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. *Am J Transl Res* (2012) **4**(4):376–89.
- Nakagomi H, Petersson M, Magnusson I, Juhillin C, Matsuda M, Mellstedt H, et al. Decreased expression of the signal-transducing zeta chains in tumor-infiltrating T-cells and NK cells of patients with colorectal carcinoma. *Cancer Res* (1993) **53**(23):5610–2.
- Lutz CT, Kurago ZB. Human leukocyte antigen class I expression on squamous cell carcinoma cells regulates natural killer cell activity. *Cancer Res* (1999) **59**:5793–9.
- Glas R, Franksson L, Une C, Eloranta ML, Ohlen C, Orn A, et al. Recruitment and activation of natural killer (NK) cells in vivo determined by the target cell phenotype. An adaptive component of NK cell-mediated responses. *J Exp Med* (2000) **191**:129–38. doi:10.1084/jem.191.1.129
- Chouaib S, Thiery J, Gati A, Guerra N, El Behi M, Dorothee G, et al. Tumor escape from killing: role of killer inhibitory receptors and acquisition of tumor resistance to cell death. *Tissue Antigens* (2002) **60**:273–81. doi:10.1034/j.1399-0039.2002.600401.x
- Jewett A, Tseng HC. Tumor induced inactivation of natural killer cell cytotoxic function; implication in growth, expansion and differentiation of cancer stem cells. *J Cancer* (2011) **2**:443–57. doi:10.7150/jca.2.443
- Muller AJ, Sharma MD, Chandler PR, Duhadaway JB, Everhart ME, Johnson BA III, et al. Chronic inflammation that facilitates tumor progression

- creates local immune suppression by inducing indoleamine 2,3 dioxygenase. *Proc Natl Acad Sci U S A* (2008) **105**:17073–8. doi:10.1073/pnas.0806173105
41. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* (2009) **9**:162–74. doi:10.1038/nri2506
42. Kusmartsev S, Gabrilovich DI. STAT1 signaling regulates tumor-associated macrophage-mediated T cell deletion. *J Immunol* (2005) **174**:4880–91.
43. Ochoa AC, Zea AH, Hernandez C, Rodriguez PC. Arginase, prostaglandins, and myeloid-derived suppressor cells in renal cell carcinoma. *Clin Cancer Res* (2007) **13**:721s–6s. doi:10.1158/1078-0432.CCR-06-2197
44. Sinha P, Clements VK, Fulton AM, Ostrand-Rosenberg S. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer Res* (2007) **67**:4507–13. doi:10.1158/0008-5472.CAN-06-4174
45. Shime H, Yabu M, Akazawa T, Kodama K, Matsumoto M, Seya T, et al. Tumor-secreted lactic acid promotes IL-23/IL-17 proinflammatory pathway. *J Immunol* (2008) **180**:7175–83.
46. Kortylewski M, Xin H, Kujawski M, Lee H, Liu Y, Harris T, et al. Regulation of the IL-23 and IL-12 balance by STAT3 signaling in the tumor microenvironment. *Cancer Cell* (2009) **15**:114–23. doi:10.1016/j.ccr.2008.12.018
47. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) **144**:646–74. doi:10.1016/j.cell.2011.02.013
48. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol* (2011) **31**:986–1000. doi:10.1161/ATVBAHA.110.207449
49. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. *J Biol Chem* (2008) **283**:1026–33. doi:10.1074/jbc.M707224200
50. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* (2007) **133**:647–58. doi:10.1053/j.gastro.2007.05.022
51. Löffler D, Brocke-Heidrich K, Pfeifer G, Stocsits C, Hackermüller J, Kretzschmar AK, et al. Interleukin-6 dependent survival of multiple myeloma cells involves the STAT3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood* (2007) **110**:1330–3. doi:10.1182/blood-2007-03-081133
52. Gironella M, Seux M, Xie MJ, Cano C, Tomasini R, Gommeaux J, et al. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci U S A* (2007) **104**:16170–5. doi:10.1073/pnas.0703942104
53. Jones MR, Quinton LJ, Blahna MT, Neilson JR, Fu S, Ivanov AR, et al. Zcchc11-dependent uridylation of microRNA directs cytokine expression. *Nat Cell Biol* (2009) **11**:1157–63. doi:10.1038/ncb1931
54. Matsushita K, Takeuchi O, Standley DM, Kumagai Y, Kawagoe T, Miyake T, et al. Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. *Nature* (2009) **458**:1185–90. doi:10.1038/nature07924
55. Kutikhin AG, Yuzhalin AE. C-type lectin receptors and RIG-I-like receptors: new points on the oncogenomics map. *Cancer Manag Res* (2012) **4**:39–53. doi:10.2147/CMAR.S28983
56. Oh SS, Chang SC, Cai L, Cordon-Cardo C, Ding BG, Greenland S, et al. Single nucleotide polymorphisms of 8 inflammation-related genes and their associations with smoking-related cancers. *Int J Cancer* (2010) **127**:2169–82. doi:10.1002/ijc.25214

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Pattern recognition receptors and autophagy

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The immune system senses exogenous threats or endogenous stress through specialized machinery known as pattern recognition receptors (PRRs). These receptors recognize conserved molecular structures and initiate downstream signaling pathways to control immune responses. Although various immunologic pathways mediated by PRRs have been described, recent studies have demonstrated a link between PRRs and autophagy. Autophagy is a specialized biological process involved in maintaining homeostasis through the degradation of long-lived cellular proteins and organelles. In addition to this fundamental function, autophagy plays important roles in various immunologic processes. In this review, we focus on the reciprocal influences of PRRs and autophagy in modulating innate immune responses.

Keywords: autophagy, toll-like receptors, RIG-I-like receptors, NOD-like receptors, inflammasomes, cytosolic DNA sensors

INTRODUCTION

Innate immune signaling pathways are initiated when microorganism-specific pathogen-associated molecular pattern (PAMP) molecules are recognized by host pattern recognition receptors (PRRs) (1). PRRs can be classified based on their site of localization (e.g., plasma membrane, endosomal vesicles, and cytoplasm) or by molecular structural similarities. PRRs classified by structural similarity include toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), and RIG-I-like receptors (RLRs).

The TLRs, which reside both within the cell surface membrane (TLR 1, 2, 4, 5, and 6) and in endosomal compartments (TLR 3, 7, 8, and 9), are the most well-characterized PRRs. After recognition of PAMPs, TLRs initiate downstream signaling pathways via myeloid differentiation primary response gene 88 (MyD88) or Toll/interleukin (IL)-1 receptor (TIR) domain-containing adapter-inducing interferon (IFN)- β (TRIF), ultimately activating the transcription factors nuclear factor (NF)- κ B and activator protein-1 (AP-1) or IFN regulatory factor 3 (IRF3). Activation of NF- κ B and AP-1 results in the production of proinflammatory cytokines, and activation of IRF3 results in the production of type I IFNs (2). NLRs are cytoplasmic members of the PRR family, and more than 20 NLRs have been identified in mammals. NOD1 and NOD2 – the first NLRs identified in mammals – recognize cytoplasmic bacterial cell wall components, eventually activating NF- κ B to induce the production of proinflammatory cytokines. In addition, NLRs act as sensory proteins in inflammasomes (which serve as platforms for protein complexes involved in innate immunity) and activate inflammasome-associated caspase-1 for pro-IL-1 β and pro-IL-18 processing. RLRs and other cytosolic sensors primarily recognize microbial nucleic acids in the cytosol.

RLRs composed of retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) are caspase-recruiting domain (CARD)-containing RNA helicases that recognize double-stranded RNA and signal through IFN- β promoter stimulator-1 [IPS-1; also known as mitochondrial antiviral signaling (MAVS), virus-induced signaling adaptor (VISA), or Cardif] to subsequently activate IRF3 and NF- κ B (3).

Autophagy is a highly conserved homeostatic process in eukaryotic cells that degrades long-lived cellular proteins and organelles. There are at least three types of autophagy: microautophagy, chaperone-mediated autophagy, and macroautophagy (4). During microautophagy, continuous degradation of cytosolic constituents close to the lysosomes occurs through budding of the lysosomal membrane. In chaperone-mediated autophagy, proteins containing a "KFERQ" motif are transported into the lysosomal lumen via Lamp2a for subsequent degradation. During this process, cytosolic chaperones such as HSC70 recognize the KFERQ motif and facilitate importation of substrates into the lysosomes (5). Macroautophagy, which is the primary route of degradation, involves the formation of a double-membrane vesicle known as an autophagosome. During this process, long-lived cellular components are first surrounded by an elongated cup-shaped membrane that forms the autophagosome, which then matures and fuses with lysosomes for degradation of the internalized materials (6). Recent research has suggested that autophagy is a selective process, in which specific adaptors such as p62 target ubiquitinated substrates for selective degradation (7).

The molecular processes involved in autophagy consist of three distinct stages. Initiation of isolation membrane formation requires complex interaction between autophagy-related gene (Atg) 6 (also known as beclin-1) and type III [phosphatidylinositol

3-kinase (PI3K). Elongation of the isolation membrane and termination of autophagosome formation are regulated by at least two ubiquitin-like molecules: microtubule-associated protein 1 light-chain 3 (LC3; mammalian homolog of yeast Atg8) and Atg12 (8, 9). Atg12 is conjugated to Atg5 through the sequential actions of the E1- and E2-like enzymes Atg7 and Atg10. Association of Atg12–Atg5 conjugates with Atg16 in turn facilitates elongation of the isolation membrane and catalyzes LC3 conjugation. The C-terminal amino acids of LC3 are cleaved by Atg4 and then transferred to phosphatidylethanolamine (PE) in the newly formed isolation membrane by the E1- and E2-like enzymes Atg7 and Atg3. Upon completion of the autophagosome, LC3 remains in the autophagosomal lumen (thus serving as an autophagosomal marker), whereas the Atg12–Atg5–Atg16 complex dissociates from the outer autophagosomal membrane. The outer membrane of the autophagosome eventually fuses with the lysosome for degradation of the autophagosomal contents and membrane (10).

Autophagy was originally identified as a mechanism for maintaining homeostasis through the degradation of long-lived proteins and recycling of intracellular organelles (11). However, autophagy is now recognized as playing multiple roles in various biological processes. For example, dysregulation of autophagy has been linked to many diseases, including cancer. Recent studies have revealed that PRRs activate autophagy to enhance immune responses against pathogens and that PRR-induced signaling pathways are regulated by autophagy to prevent excessive inflammation. In this review, we focus on the interactive role of PRRs and autophagy in controlling innate immune responses.

TLRs AND AUTOPHAGY

Toll-like receptors, which bind to conserved microbial molecular structures and initiate downstream signaling pathways, are the most thoroughly characterized type of PRR (1). Xu et al. (12) were the first to report that TLR4 stimulation activates autophagy to enhance elimination of phagocytosed mycobacteria. The authors found that stimulation of TLR4 with lipopolysaccharide (LPS) induces autophagosome formation in primary human macrophages and RAW 264.7 murine macrophages. This pathway is mediated by the TRIF–p38 axis rather than MyD88 (**Figure 1A**). In their study, Xu et al. provide an evidence of close relationship between autophagy and TLR-mediated innate immunity. In addition to LPS-induced autophagy, ligands of TLR3 and TLR7 also activate autophagy. Two different ligands of TLR7, single-stranded RNA (ssRNA) and imiquimod, induce autophagosome formation, characterized by LC3 puncta formation in murine macrophages [**Figure 1A**; Ref. (13)]. This process occurs via MyD88 and ultimately results in the killing of intracellular mycobacteria, even though mycobacteria are normally not associated with TLR7 signaling.

Recently, several studies reported that TLR2 stimulation by various pathogens induces autophagy (14, 15). In response to *Listeria monocytogenes*, macrophages deficient in the TLR2 and NOD/receptor-interacting protein 2 (RIP2) pathways show defective autophagy induction and fail to colocalize bacteria within autophagosomes [**Figure 1B**; Ref. (14)]. Autophagy induction in this process was found to be dependent on the extracellular signal-regulated kinase (ERK) pathway. Another study showed

that *Staphylococcus aureus*-mediated stimulation of TLR2 in RAW 264.7 mouse macrophages induces phagocytosis and autophagy. In particular, knockdown of TLR2 was shown to attenuate *S. aureus*-induced phosphorylation of macrophage c-Jun N-terminal kinase (JNK) but not phosphorylation of p38 or ERK (15). Collectively, these data indicate that TLR2 stimulated by invading microbes could mediate autophagy induction and promote the clearance of pathogens, despite the different pathways involved. Shi and Kehrl (16) revealed that various TLR agonists, including TLR1, TLR3, TLR5, TLR6, and TLR7, trigger autophagy induction through MyD88 and TRIF, which interacts with beclin-1. Beclin-1 is critical for the initiation of autophagosome formation. Interaction of beclin-1 with TLR-signaling pathway adaptor molecules partially inhibits the binding of beclin-1 to B cell lymphoma 2 (Bcl-2).

In addition to its role in autophagy induction, TLR-signaling is also utilized by Atg proteins to mediate phagosome maturation. Phagocytosis of the fungal cell wall component zymosan promotes the rapid recruitment of LC3 to phagosomes and facilitates their fusion with lysosomes (17). In RAW 264.7 cells, phagocytosis of Pam₃CSK₄-coated latex beads involves recruitment of LC3 to the phagosomes. This process is dependent on TLR2 but not MyD88 and requires both Atg5 and Atg7. However, LC3 translocation to phagosomal membranes is not associated with double-membrane structures, which is a unique feature of autophagosomes. Collectively, these results demonstrate a novel way in which the autophagic machinery is utilized for phagocytosis after TLR activation. Another recent study characterized the role of non-canonical autophagy in type I IFN secretion in response to DNA-immune complexes (DNA-ICs) (18). Upon stimulation of TLR9, which responds to double-stranded DNA (dsDNA) and facilitates the production of proinflammatory cytokines and type I IFNs, IFN- α is produced by the convergence of the phagocytic and autophagic pathways, a process termed LC3-associated phagocytosis (LAP). LAP occurs in response to DNA-ICs but not soluble ligands. In addition, LAP requires Fc γ R engagement, which controls TLR9 and LC3 recruitment (**Figure 1C**). The study of Henault et al. revealed the function of non-conventional autophagy in regulating type I IFN signaling in phagosomes. Moreover, their results suggest a mechanism for the uncontrolled production of type I IFNs induced by pathogenic DNA-ICs in systemic lupus erythematosus, which may lead to development of new therapeutic targets for treating this disease.

As previously discussed, induction of autophagy through TLR activation directly promotes pathogen clearance to enhance host protection. However, autophagy also enhances antiviral defenses by facilitating delivery of cytosolic viral PAMPs to endosomal TLRs. Viral nucleic acids endocytosed by host cells are recognized by endosomal TLR7 and TLR9. After recognition, signaling through NF- κ B and IRF7 induces production of proinflammatory cytokines and type I IFNs, respectively. In response to vesicular stomatitis virus (VSV) infection in plasmacytoid dendritic cells (pDCs), endosomal TLR7 recognizes the replicating virus in the cytosol rather than the viral genome. How these cytosolic replication intermediates gain access to endosomal TLR7 was demonstrated by Lee and colleagues. These authors showed that autophagy facilitates the delivery of cytosolic PAMPs to the lysosomes, activating TLR7 signaling [**Figure 1D**; Ref.

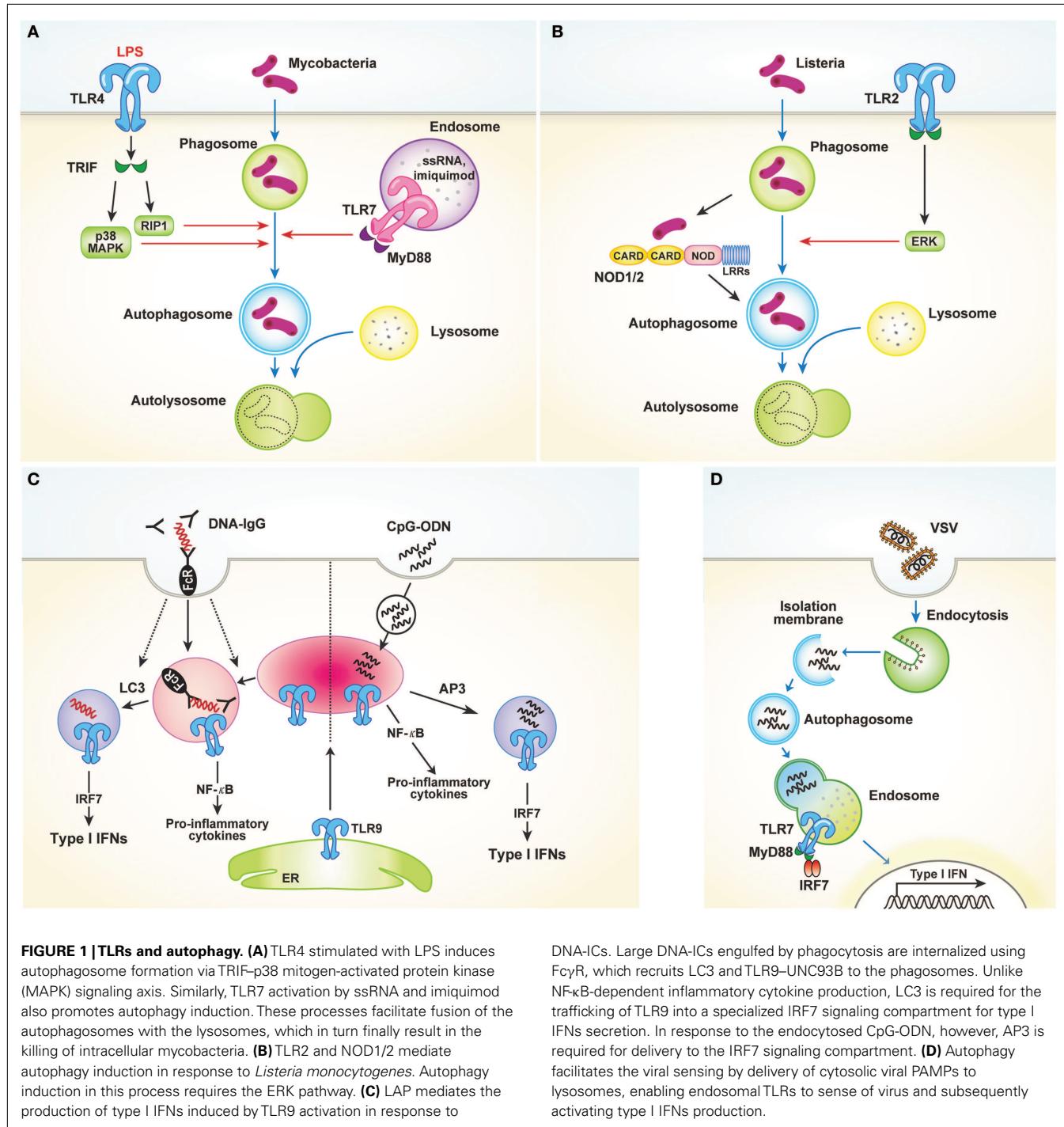


FIGURE 1 | TLRs and autophagy. (A) TLR4 stimulated with LPS induces autophagosome formation via TRIF-p38 mitogen-activated protein kinase (MAPK) signaling axis. Similarly, TLR7 activation by ssRNA and imiquimod also promotes autophagy induction. These processes facilitate fusion of the autophagosomes with the lysosomes, which in turn finally result in the killing of intracellular mycobacteria. (B) TLR2 and NOD1/2 mediate autophagy induction in response to *Listeria monocytogenes*. Autophagy induction in this process requires the ERK pathway. (C) LAP mediates the production of type I IFNs induced by TLR9 activation in response to

DNA-ICs. Large DNA-ICs engulfed by phagocytosis are internalized using Fc γ R, which recruits LC3 and TLR9-UNC93B to the phagosomes. Unlike NF- κ B-dependent inflammatory cytokine production, LC3 is required for the trafficking of TLR9 into a specialized IRF7 signaling compartment for type I IFNs secretion. In response to the endocytosed CpG-ODN, however, AP3 is required for delivery to the IRF7 signaling compartment. (D) Autophagy facilitates the viral sensing by delivery of cytosolic viral PAMPs to lysosomes, enabling endosomal TLRs to sense of virus and subsequently activating type I IFNs production.

(19)]. Consequently, pDCs lacking Atg5 cannot secrete IFN- α or IL-12p40 following VSV infection. Atg5-deficient mice are also susceptible to systemic VSV infection *in vivo*. Interestingly, in pDCs infected with herpes simplex virus-1 (HSV-1), which is recognized by TLR9, Atg5-deficient cells fail to produce IFN- α , whereas the IL-12 response of these cells is not affected. Thus, the precise mechanisms by which the NF- κ B and IFN- α signaling pathways are controlled by autophagy remain to be determined (20).

NLRs AND AUTOPHAGY

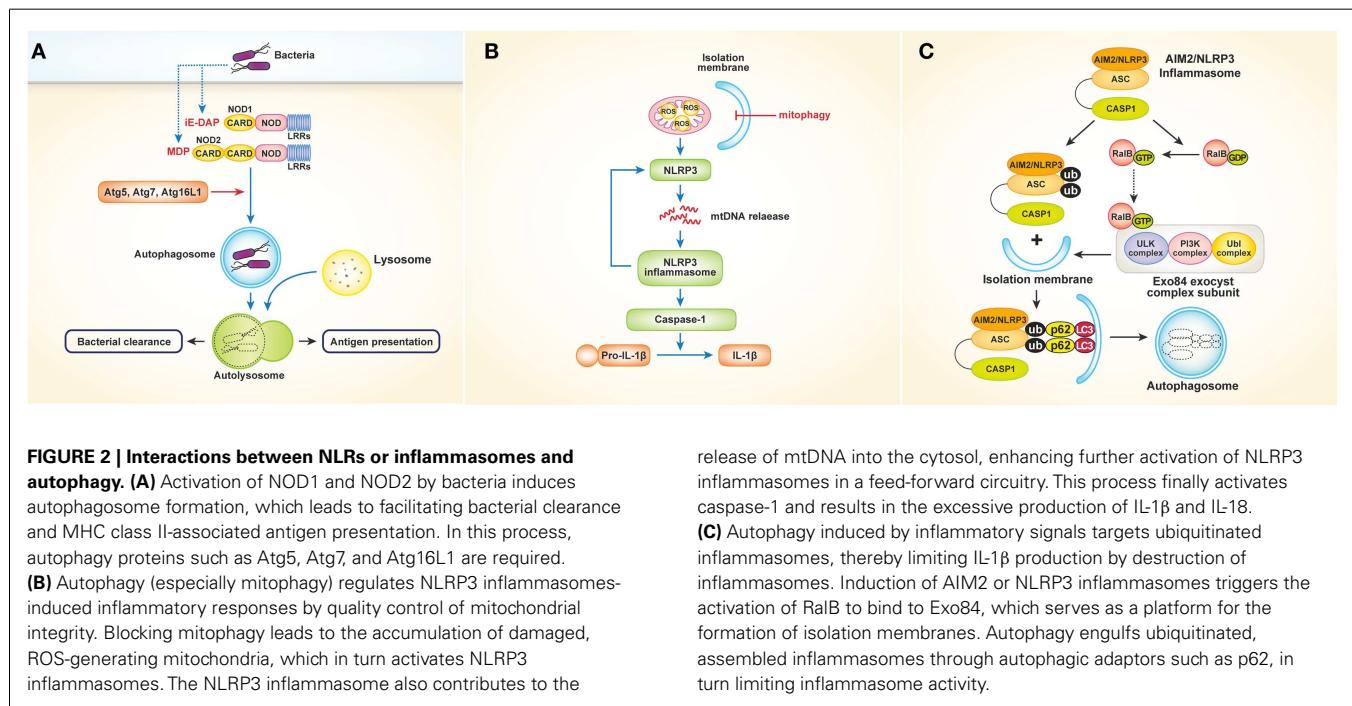
NOD-like receptors, which recognize bacterial cell wall components such as peptidoglycan in the cytosol, also play an important role in autophagy. Studies have shown that NOD1 and NOD2 activate autophagy in response to bacterial invasion (21, 22). In mouse embryonic fibroblasts (MEFs), NOD2 recruits Atg16L1 to the plasma membrane at the site of bacterial entry, in turn facilitating bacterial trafficking to the autophagosomes and fusion of the autophagosomes with the lysosomes to promote bacterial

clearance and antigen presentation via MHCII [Figure 2A; Ref. (22)]. Another study using human DCs showed that stimulation of NOD2 with muramyl dipeptide induces autophagosome formation and consequently enhances MHCII-associated antigen presentation. In this process, autophagic proteins such as Atg5, Atg7, Atg16L1, and receptor-interacting serine–threonine kinase 2 are required [Figure 2A; Ref. (21)]. The intracellular bacterial sensors NOD1 and NOD2 link the autophagic machinery via Atg16L1, thereby enhancing both bacterial clearance and protective immunity. However, the role of Atg16L1 in NOD-derived inflammation remains unclear. A recent study demonstrated that Atg16L1 suppresses NOD-induced inflammatory responses in an autophagy-independent manner (23). Atg16L1 blocks the activation of RIP2 by reducing the level of RIP2 polyubiquitination and diminishing the incorporation of RIP2 into NOD signaling complexes. This process appears to be specific to Atg16L1, given that knockdown of *Atg5* or *Atg9a* does not affect the NOD response. In addition, autophagy-incompetent truncated forms of Atg16L1 retain the capacity to regulate NOD-driven cytokine responses. Interestingly, NOD2 mutations and single-nucleotide polymorphisms in *Atg16L1* are well-known features of Crohn's disease. Collectively, the above-mentioned studies suggest that a functional relationship exists between NOD2 and Atg16L1 in Crohn's disease.

Inflammasomes are protein complexes in which NLRs serve as sensory proteins that promote innate immunity by enabling the maturation of pro-IL-1 β and pro-IL-18 through activation of pro-caspase-1. Many studies have described regulation of inflammasomes by autophagy and vice versa. Suppression of inflammasomes by autophagy was first reported in 2008 by Saitoh et al. (24), who showed that Atg16L1-deficiency results in increased production of IL-1 β and IL-18 following LPS stimulation. Atg16L1 is an

essential component of the autophagosome, forming a complex with Atg5–Atg12 conjugates, resulting in LC3–PE conjugation. Thus, Atg16L1-deficient macrophages impaired in autophagosome formation induce TRIF-dependent activation of caspase-1, leading to excessive production of IL-1 β in response to LPS. Considering that *Atg16L1* is an important gene in the development of Crohn's disease, endotoxin-induced inflammasome activation in Atg16L1-deficiency could be involved in the occurrence of Crohn's disease. Although the above-mentioned data suggest that inflammatory responses are regulated by autophagy, the mechanism by which autophagy regulates cytokine secretion is not clear. Two fascinating studies have provided evidence indicating that mitochondria play a critical role controlling innate immunity mediated by NLRP3 inflammasomes (25, 26). Zhou and colleagues demonstrated that blocking autophagy, especially mitophagy (mitochondrial autophagy), results in the accumulation of damaged, reactive oxygen species (ROS)-generating mitochondria, which in turn activate NLRP3 inflammasomes. Of note, inhibition of mitochondrial activity suppresses both ROS generation and inflammasome activation [Figure 2B; Ref. (26)]. Similarly, Nakahira et al. (25) showed that depletion of the autophagic proteins LC3B and beclin-1 induce excessive secretion of IL-1 β and IL-18, which is mediated by accumulation of dysfunctional mitochondria and cytosolic translocation of mitochondrial DNA (mtDNA) following LPS and adenosine triphosphate (ATP) stimulation. The NALP3 inflammasome, which is critical for the activation of caspase-1 in response to LPS and ATP stimulation, contributes to the release of mtDNA into the cytosol [Figure 2B; Ref. (25)]. Together, these studies indicate that regulation of NLRP3-induced inflammatory processes by autophagy is dependent on mitochondrial integrity.

Autophagy also limits the inflammatory responses resulting from inflammasome activation in another way. A recent study



showed that autophagy induced by inflammatory signals targets ubiquitinated inflammasomes, thereby limiting IL-1 β production through inflammasome destruction (27). Induction of absent in melanoma 2 (AIM2) or NLRP3 inflammasomes triggers nucleotide exchange on RalB and autophagosome assembly through Exo84, which serves as a platform for the formation of isolation membranes (28). During autophagy, ubiquitinated assembled inflammasomes are engulfed through autophagic adaptors such as p62, which contain both ubiquitin-associated domains and LC3-interacting regions that recognize ubiquitinated molecules and assist their entry into the autophagy pathway (Figure 2C). Thus, activation of inflammasomes induces autophagy, which in turn limits inflammasome activity via autophagic engulfment in order to maintain homeostasis as it pertains to inflammation.

Conversely, NLRs also negatively regulate autophagy. NLRC4, NLRP3, NLRP4, and NLRP10 interact with beclin-1, and NLRP4 in particular has a strong affinity for beclin-1. Following invasion by bacteria such as group A streptococci (GAS), NLRP4 recruits GAS-containing phagosomes and transiently dissociates from beclin-1, enabling the initiation of beclin-1-mediated autophagy. Moreover, NLRP4 physically interacts with the class C vacuolar protein-sorting complex, resulting in inhibition of autophagosome and endosome maturation (29). Taken together, the available data indicate that homeostasis is maintained through reciprocal regulation of NLR activation and autophagy.

OTHER CYTOSOLIC SENSORS AND AUTOPHAGY

Viral recognition in most cell types is mediated by cytosolic sensors such as RIG-I and MDA-5. RIG-I and MDA-5, both of which are RLRs, signal through IPS-1 to activate the transcription factors IRF3 and NF- κ B, leading to cytokine production.

Several studies have revealed that the RLR signaling pathway might be controlled by autophagy (30, 31). In Atg5- or Atg7-deficient MEFs, which lack Atg5-Atg12 conjugates, type I IFNs are overproduced following VSV infection. In contrast, overexpression of Atg5 or Atg12 results in suppression of IFN signaling. The Atg5-Atg12 conjugates directly interact with the CARD domains of RIG-I and IPS-1, inhibiting subsequent RLR signaling [Figure 3A; Ref. (30)]. These data indicate that autophagy-related proteins act as negative regulators of RLR-mediated antiviral responses. Similarly, Tal and colleagues revealed that Atg5-deficient cells overproduce IFNs through enhanced RLR signaling in response to VSV infection (31). However, the authors explained that dysfunctional mitochondria and mitochondria-associated IPS-1 that accumulate in the absence of autophagy enhance RLR signaling. Data suggest that ROS associated with dysfunctional mitochondria are the primary inducers of these responses, as increased mitochondrial ROS production following treatment with rotenone, which is independent of autophagy, also results in amplification of RLR signaling [Figure 3A; Ref. (31)]. Consequently, autophagy contributes to homeostatic regulation of antiviral responses through control of RLR signaling pathways.

The cytosolic DNA sensor stimulator of IFN genes (STING) is also associated with autophagy. In a study to determine the mechanism of mycobacterial clearance, ubiquitin-mediated autophagy targeting *M. tuberculosis* was shown to be activated by the STING-dependent cytosolic sensing pathway (32). In case of wild-type *M. tuberculosis*, which expresses the virulence factor extra-embryonic spermatogenic homeobox 1 (ESX-1) secretion system, mycobacterial DNA may be exposed to the host through ESX-1-mediated permeabilization of the phagosomal membrane.

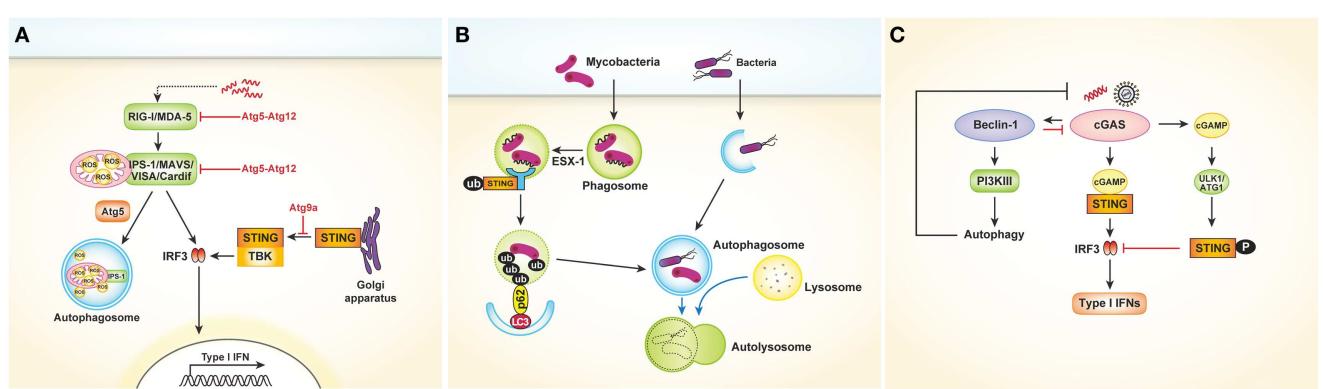


FIGURE 3 | Cytosolic nucleic acids sensors and autophagy.

(A) Autophagy negatively regulates type I IFNs production after viral infection. The Atg5-Atg12 conjugates directly interact with the CARD domains of RIG-I and IPS-1, inhibiting subsequent RLR signaling pathway and type I IFNs production. In another way, autophagy regulates RLR signaling by acting as a scavenger of dysfunctional mitochondria as well as mitochondria-associated IPS-1. Following dsDNA stimulation, STING is translocated from the ER to the Golgi apparatus and assembled with TBK1, which phosphorylates the transcription factor IRF3. During this process, Atg9a colocalizes with STING in the Golgi apparatus and controls the assembly of STING. (B) During mycobacterial clearance, ubiquitin-mediated autophagy targeting *M. tuberculosis* is shown to be activated by the STING-dependent cytosolic sensing pathway. Mycobacterial extracellular

DNA, which is exposed to the host through ESX-1-mediated permeabilization of the phagosomal membrane, is recognized by the STING-dependent cytosolic pathway. The ubiquitinated bacterial DNA, which binds to the autophagosome-associated protein LC3 via adaptor protein p62 and NDP52, is targeted to the selective autophagy pathway. (C) Cytosolic DNA-sensing cGAS produces cGAMP, which binds to and activates the adaptor protein STING, thus leading to the production of type I IFNs. Direct interaction between cGAS and Beclin-1 suppresses cGAMP synthesis. Moreover, this interaction activates PI3K III-induced autophagy, enhancing the autophagy-mediated degradation of pathogen DNA. cGAMP generated by cGAS initially activate STING-dependent type I IFN responses. However, they subsequently trigger negative-feedback control of STING activity through phosphorylation of STING by serine/threonine ULK1 (ATG1).

The released DNA may in turn be recognized by the STING-dependent cytosolic pathway. The bacteria are consequently surrounded by ubiquitin chains, and the ubiquitin and LC3-binding autophagic adaptors p62 and nuclear dot protein 52 recruit autophagy components that target the bacilli to the selective autophagy pathway (**Figure 3B**). Other studies involving dsDNA viruses such as HSV-1 or human cytomegalovirus revealed that STING plays a role in autophagy induction (33, 34).

Conversely, autophagy may also negatively regulate STING-dependent IFN responses. After dsDNA stimulation, Atg9a colocalizes with STING in the Golgi apparatus, where it controls the assembly of STING (35). The loss of Atg9a, but not that of Atg7, promotes the translocation of STING from the Golgi apparatus and its assembly with TBK1, thus inducing aberrant activation of type I IFN responses (**Figure 3A**). Collectively, these findings demonstrate the reciprocal regulation of autophagy and STING-dependent cytosolic pathways.

Recently, cyclic guanosine monophosphate–adenosine monophosphate (GMP–AMP) synthase (cGAS) was shown to be a cytosolic DNA sensor that activates the type I IFN pathway (36). Cytosolic DNA-sensing cGAS produces cyclic GMP–AMP (cGAMP), which binds to and activates the adaptor protein STING, thus leading to the production of type I IFNs. A very recent study showed that direct interaction between cGAS and beclin-1 suppresses cGAMP synthesis, leading to dampened type I IFN responses following dsDNA stimulation or HSV-1 infection. Moreover, this interaction activates PI3K III-induced autophagy through release of Rubicon, a negative regulator of autophagy, thus enhancing the autophagy-mediated degradation of pathogen DNA to prevent excessive immune stimulation [**Figure 3C**; Ref. (37)]. Similarly, cyclic dinucleotides contribute to the negative regulation of the STING pathway by activating UNC-51-like kinase (ULK1/Atg1). Cyclic dinucleotides generated by cGAS initially activate STING-dependent type I IFN responses; however, they subsequently trigger negative-feedback control of STING activity through phosphorylation of STING by serine/threonine ULK1/Atg1 [**Figure 3C**; Ref. (38)]. Taken together, these data suggest that autophagy controls the excessive and persistent immune responses mediated by cytosolic DNA-sensing pathways.

CONCLUSION

In this review, we describe the close interaction between PRRs and autophagy in various immunologic conditions. PRRs are not only involved in autophagy induction but also in the promotion of phagosomal maturation mediated by Atg proteins when pathogenic bacteria invade host cells. In addition, autophagy facilitates the delivery of viral PAMPs and TLR9 trafficking for type I IFN production. Autophagy regulates PRR-induced inflammation in various ways to prevent excessive inflammatory responses, and conversely, PRR signaling also controls autophagy. Collectively, the available data indicate that targeting autophagy would allow us to enhance pathogen clearance or suppress PRR-mediated inflammatory conditions, such as those associated with autoimmune diseases. Therefore, a more detailed analysis of how we could control autophagy is recommended.

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REFERENCES

- Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* (2004) 5:987–95. doi:10.1038/ni1112
- Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* (2004) 4:499–511. doi:10.1038/nri1391
- Lee MS, Kim YJ. Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annu Rev Biochem* (2007) 76:447–80. doi:10.1146/annurev.biochem.76.060605.122847
- Mizushima N, Klionsky DJ. Protein turnover via autophagy: implications for metabolism. *Annu Rev Nutr* (2007) 27:19–40. doi:10.1146/annurev.nutr.27.061406.093749
- Massey AC, Zhang C, Cuervo AM. Chaperone-mediated autophagy in aging and disease. *Curr Top Dev Biol* (2006) 73:205–35. doi:10.1016/S0070-2153(05)73007-6
- Mizushima N, Ohsumi Y, Yoshimori T. Autophagosome formation in mammalian cells. *Cell Struct Funct* (2002) 27:421–9. doi:10.1247/csf.27.421
- Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. *Autophagy* (2011) 7:279–96. doi:10.4161/auto.7.3.14487
- Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, et al. A protein conjugation system essential for autophagy. *Nature* (1998) 395:395–8. doi:10.1038/26506
- Ohsumi Y. Molecular dissection of autophagy: two ubiquitin-like systems. *Nat Rev Mol Cell Biol* (2001) 2:211–6. doi:10.1038/35056522
- Levine B, Deretic V. Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol* (2007) 7:767–77. doi:10.1038/nri2161
- Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. *Science* (2000) 290:1717–21. doi:10.1126/science.290.5497.1717
- Xu Y, Jagannath C, Liu XD, Sharafkhaneh A, Kolodziejska KE, Eissa NT. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity* (2007) 27:135–44. doi:10.1016/j.immuni.2007.05.022
- Delgado MA, Elmaouad RA, Davis AS, Kyei G, Deretic V. Toll-like receptors control autophagy. *EMBO J* (2008) 27:1110–21. doi:10.1038/emboj.2008.31
- Anand PK, Tait SWG, Lamkanfi M, Amer AO, Nunez G, Pages G, et al. TLR2 and RIP2 pathways mediate autophagy of *Listeria monocytogenes* via extracellular signal-regulated kinase (ERK) activation. *J Biol Chem* (2011) 286:42981–91. doi:10.1074/jbc.M111.310599
- Fang L, Wu H-M, Ding P-S, Liu R-Y. TLR2 mediates phagocytosis and autophagy through JNK signaling pathway in *Staphylococcus aureus*-stimulated RAW264.7 cells. *Cell Signal* (2014) 26:806–14. doi:10.1016/j.cellsig.2013.12.016
- Shi C-S, Kehrl JH. MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. *J Biol Chem* (2008) 283:33175–82. doi:10.1074/jbc.M804478200
- Sanjuan MA, Dillon CP, Tait SW, Moshiach S, Dorsey F, Connell S, et al. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. *Nature* (2007) 450:1253–7. doi:10.1038/nature06421
- Henault J, Martinez J, Riggs JM, Tian J, Mehta P, Clarke L, et al. Noncanonical autophagy is required for type I interferon secretion in response to DNA-immune complexes. *Immunity* (2012) 37:986–97. doi:10.1016/j.jimmuni.2012.09.014
- Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science* (2007) 315:1398–401. doi:10.1126/science.1136880
- Tal MC, Iwasaki A. Autophagy and innate recognition systems. *Curr Top Microbiol Immunol* (2009) 335:107–21. doi:10.1007/978-3-642-00302-8_5
- Cooney R, Baker J, Brain O, Danis B, Pichulik T, Allan P, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med* (2010) 16:90–7. doi:10.1038/nm.2069
- Travassos LH, Carneiro LA, Ramjeet M, Hussey S, Kim YG, Magalhaes JG, et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma

- membrane at the site of bacterial entry. *Nat Immunol* (2010) **11**:55–62. doi:10.1038/ni.1823
23. Sorbara MT, Ellison LK, Ramjeet M, Travassos LH, Jones NL, Girardin SE, et al. The protein ATG16L1 suppresses inflammatory cytokines induced by the intracellular sensors Nod1 and Nod2 in an autophagy-independent manner. *Immunity* (2013) **39**:858–73. doi:10.1016/j.jimmuni.2013.10.013
24. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature* (2008) **456**:264–8. doi:10.1038/nature07383
25. Nakahira K, Haspel JA, Rathinam VA, Lee SJ, Dolinay T, Lam HC, et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* (2011) **12**:222–30. doi:10.1038/ni.1980
26. Zhou R, Yazdi AS, Menu P, Tschoopp J. A role for mitochondria in NLRP3 inflammasome activation. *Nature* (2011) **469**:221–5. doi:10.1038/nature09663
27. Shi CS, Shenderov K, Huang NN, Kabat J, Abu-Asab M, Fitzgerald KA, et al. Activation of autophagy by inflammatory signals limits IL-1beta production by targeting ubiquitinated inflammasomes for destruction. *Nat Immunol* (2012) **13**:255–63. doi:10.1038/ni.2215
28. Bodenmann BO, Orvedahl A, Cheng T, Ram RR, Ou YH, Formstecher E, et al. RalB and the exocyst mediate the cellular starvation response by direct activation of autophagosome assembly. *Cell* (2011) **144**:253–67. doi:10.1016/j.cell.2010.12.018
29. Jounai N, Kobiyama K, Shiina M, Ogata K, Ishii KJ, Takeshita F. NLRP4 negatively regulates autophagic processes through an association with beclin1. *J Immunol* (2011) **186**:1646–55. doi:10.4049/jimmunol.1001654
30. Jounai N, Takeshita F, Kobiyama K, Sawano A, Miyawaki A, Xin KQ, et al. The Atg5 Atg12 conjugate associates with innate antiviral immune responses. *Proc Natl Acad Sci U S A* (2007) **104**:14050–5. doi:10.1073/pnas.0704014104
31. Tal MC, Sasai M, Lee HK, Yordy B, Shadel GS, Iwasaki A. Absence of autophagy results in reactive oxygen species-dependent amplification of RLR signaling. *Proc Natl Acad Sci U S A* (2009) **106**:2770–5. doi:10.1073/pnas.0807694106
32. Watson RO, Manzanillo PS, Cox JS. Extracellular *M. tuberculosis* DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell* (2012) **150**:803–15. doi:10.1016/j.cell.2012.06.040
33. McFarlane S, Aitken J, Sutherland JS, Nicholl MJ, Preston VG, Preston CM. Early induction of autophagy in human fibroblasts after infection with human cytomegalovirus or herpes simplex virus 1. *J Virol* (2011) **85**:4212–21. doi:10.1128/JVI.02435-10
34. Rasmussen SB, Horan KA, Holm CK, Stranks AJ, Mettenleiter TC, Simon AK, et al. Activation of autophagy by α -herpesviruses in myeloid cells is mediated by cytoplasmic viral DNA through a mechanism dependent on stimulator of IFN genes. *J Immunol* (2011) **187**:5268–76. doi:10.4049/jimmunol.1100949
35. Saitoh T, Fujita N, Hayashi T, Takahara K, Satoh T, Lee H, et al. Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. *Proc Natl Acad Sci U S A* (2009) **106**:20842–6. doi:10.1073/pnas.0911267106
36. Sun L, Wu J, Du F, Chen X, Chen ZJ. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* (2013) **339**:786–91. doi:10.1126/science.1232458
37. Liang Q, Seo GJ, Choi YJ, Kwak MJ, Ge J, Rodgers MA, et al. Crosstalk between the cGAS DNA sensor and beclin-1 autophagy protein shapes innate antimicrobial immune responses. *Cell Host Microbe* (2014) **15**:228–38. doi:10.1016/j.chom.2014.01.009
38. Konno H, Konno K, Barber GN. Cyclic dinucleotides trigger ULK1 (ATG1) phosphorylation of STING to prevent sustained innate immune signaling. *Cell* (2013) **155**:688–98. doi:10.1016/j.cell.2013.09.049

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Pattern recognition receptors and DNA repair: starting to put a jigsaw puzzle together

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The group of pattern recognition receptors (PRRs) includes families of toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), and AIM2-like receptors (ALRs) (1–7). Conceptually, receptors constituting these families are united by two general features. Firstly, they directly recognize common antigen determinants of virtually all classes of pathogens (so-called pathogen-associated molecular patterns, PAMPs) and initiate immune response against them via specific intracellular signaling pathways (1–7). Secondly, they also recognize endogenous ligands released in cells under stress, which are known as damage-associated molecular patterns (DAMPs). Therefore, a subset of PRR-mediated immune response can be activated without an influence of infectious agents (1–7).

Long-standing data implicate that PRRs play a key role in innate and adaptive immune responses (1–7). Besides their effect on immunity, many PRRs may have a crucial impact on almost all vital cellular processes, such as cell growth, survival, apoptosis, cell cycle control, cell proliferation and differentiation, autophagy, angiogenesis, cell motility, and migration (8–14). In recent years, the evidence of the involvement of PRRs in the processes of DNA repair started to emerge. A recent comprehensive review by Harberts and Gaspari (15) has shed light on this issue;

nevertheless, a number of newer investigations were performed after the publication of their paper.

One of the most investigated TLRs is TLR4, which is a transmembrane protein with an ectodomain located on the cell surface (16). The two most known TLR4 ligands are lipopolysaccharide (LPS), one of the main components of Gram-negative bacteria outer membrane, and high-mobility group protein B1 (HMGB1), which is known to be an important chromatin protein (16). It is well known that X-ray repair cross-complementing group (XRCC)5/KU80 and XRCC6/KU70 are the key non-homologous end-joining (NHEJ) repair pathway proteins (17, 18). Wang et al. observed that a diminishment of TLR4-mediated immune response may lead to reduced expression of XRCC5/KU80 and XRCC6/KU70 in mouse liver tissue and cells in response to the diethylnitrosamine, therefore, being a cause of the DNA repair impairment and reactive oxygen species (ROS) accumulation (17, 18). However, when TLR4^{−/−} mice and wild-type mice were locally exposed to ultraviolet B (UVB, shortwave radiation), the expression of DNA repair gene xeroderma pigmentosum, complementation group A (*XPA*), and production of interleukins (ILs) 12 and 23 were significantly higher (19). Further, cyclobutane pyrimidine dimers were repaired more efficiently in the skin and bone

marrow-derived dendritic cells (DCs) of TLR4^{−/−} mice (19). The addition of anti-IL-12 and anti-IL-23 antibodies to bone marrow-derived DCs of TLR4^{−/−} mice before UVB exposure inhibited repair of cyclobutane pyrimidine dimers along with a decline of *XPA* gene expression; similarly, the addition of TLR4 agonist to wild-type bone marrow-derived DCs lowered *XPA* gene expression and diminished repair of cyclobutane pyrimidine dimers (19). Hence, the activation of TLR4 signaling by ultraviolet radiation may launch a specific pathway and result in decrease of IL-12 and/or IL-23 production, thereby reducing the expression of genes encoding DNA repair enzyme such as *XPA* (19). According to these studies (17–19), TLR4 may both upregulate and downregulate distinct DNA repair proteins, and possibly does it in different ways in distinct cell types, so its exact role in DNA repair remains unclear.

Certain TLRs are located on the endoplasmic reticulum membrane (in a resting state) or on the endosomal/lysosomal membrane (upon ligand stimulation and trafficking) (20). Among these are TLR7, TLR8, and TLR9 (20). The main ligands for TLR7 and TLR8 are imidazoquinolines, ssRNA, and antiphospholipid antibodies, while the main ligands for TLR9 are bacterial and viral CpG DNA and IgG-chromatin complexes (20). However, all these receptors signal via the protein encoded by myeloid differentiation primary response

gene 88 (*MyD88*) (20). Tsukamoto et al. found that 8-mercaptopguanosine (8SGuo) induces the activation-induced cytidine deaminase (AID) expression and double-strand breaks (DSBs) through TLR7–*MyD88*-dependent pathway in cluster of differentiation (CD)38- or B cell receptor (BCR)-activated B cells (21). Nevertheless, imiquimod, a TLR7/8 agonist, which is used in the treatment of certain non-melanoma skin cancer, increased an expression and nuclear localization of *XPA* gene and other DNA repair genes in a *MyD88*-dependent manner (22). In addition, as it was detected by Fishelevich et al. imiquimod enhanced DNA repair and accelerated the resolution of cyclobutane pyrimidine dimers after an exposure of bone marrow-derived cells to ultraviolet light (22). Imiquimod-activated cutaneous antigen presenting cells were characterized by better DNA repair in comparison with resting antigen presenting cells under the exposure to both non-ionizing and ionizing radiation (22). Moreover, topical application of imiquimod before the exposure to ultraviolet light had a protective effect and reduced the number of cyclobutane pyrimidine dimers-positive antigen presenting cells (22). Therefore, the role of TLR7 and TLR8 in DNA repair may differ depending on their influence on the specific DNA repair proteins or on the cell type, as in the case with TLR4.

In the study of Zheng et al., TLR9-stimulated CD4 T cells demonstrated an increased capacity to repair ionizing radiation-induced DSBs, whereas the treatment of irradiated CD4 T cells with TLR9 ligands along with checkpoint kinase (Chk)1/2 inhibitors or along with ataxia telangiectasia mutated/ataxia telangiectasia and Rad3 related (ATM/ATR) inhibitor wortmannin abrogated the improvement of DNA repair observed previously (23). In addition, TLR9 stimulation did not elevate DNA repair rates after an exposure to ionizing radiation in TLR9^{−/−} and *MyD88*^{−/−} CD4 T cells; thus, TLR9-induced DNA repair may be mediated by Chk1/2 and ATM/ATR via *MyD88*-dependent pathway (23). Klaschik et al. performed a global gene expression analysis on mouse splenic cells and revealed that CpG DNA, a ligand for TLR9, may cause the activation of genes responsible for DNA repair 3–5 days after an intraperitoneal injection, so

the long-term enhancement of DNA repair after TLR9 stimulation is possible (24). Sommariva et al. carried out an *in silico* analysis of DNA repair genes in data sets obtained from murine colon carcinoma cells in mice injected intratumorally with synthetic oligodeoxynucleotides expressing CpG motifs (CpG-ODN, a TLR9 agonist) and from splenocytes in mice treated intraperitoneally with CpG-ODN (25). According to their results, CpG-ODN downregulated DNA repair genes in tumors, but upregulated them in immune cells (25). Moreover, «CpG-like» expression pattern of CpG-ODN modulated DNA repair genes was associated with a better outcome of patients with breast and ovarian cancer treated by DNA-damaging agents than «CpG-untreated-like» expression pattern, so these genes may determine tumor cell response to genotoxic drugs (25). It seems to be that the exact role of TLR9 in DNA repair substantially depends on the cell type.

It was found that *MyD88* mediates the optimal activation of the Ras/mitogen-activated protein kinase (MAPK) pathway by binding to extracellular signal-regulated kinase (ERK) and protecting it from dephosphorylation (26–29). In accordance with the data obtained by Kfouri et al., *MyD88* inhibition may lead to defective excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1)-dependent DNA repair and to accumulation of DNA damage (29, 30). In addition, abrogation of *MyD88* gene expression sensitizes cancer cells to genotoxic agents such as platinum salts *in vitro* and *in vivo* (29, 30). It is worthy of note that platinum-based chemotherapeutic agents (cisplatin, carboplatin, and oxaliplatin) cause DNA damage that is preferentially repaired by the nucleotide excision repair (NER) pathway, which is implicated in the repair of DNA single-strand breaks (SSBs), and ERCC1 predominantly functions as NER enzyme via Ras-MAPK pathway (29, 30). So, *MyD88*-dependent Ras-MAPK-mediated activation of ERCC1 may play a major role in DNA repair (29, 30). However, Lai and Egan reported that early induction of DSBs in mouse colonic epithelial cells by ionizing radiation was independent of the presence and absence of *MyD88* gene expression (31). Notwithstanding, they observed a later loss of DSBs

and an enhanced activation of DSB repair pathways in *MyD88*^{−/−} mice compared to control mice (31). It seems to be that *MyD88* has no specific inhibitory effects regarding the pathways of DSB repair since both the NHEJ and homologous recombination (HR) repair pathways were over-activated in the absence of *MyD88* (31). Possibly, *MyD88*-mediated signaling pathway may regulate the repair of SSBs and DSBs in a distinct way via activation or inhibition of the proteins specific for each of pathways responsible for the repair of SSBs and DSBs.

The only study investigating the role of NLRs in DNA repair was carried out by Licandro et al. regarding NLR family, pyrin domain containing 3 (*Nlrp3*) gene (32). The ectodomain of NLRP3 recognizes certain DAMPs that may lead to the assembly of inflammasome and, hence, to the development of aseptic inflammation (33). The authors exposed murine DCs to monosodium urate, rotenone, and γ-radiation, and detected a lesser level of DNA fragmentation in *Nlrp3*^{−/−} DCs compared to wild-type DCs (32). Moreover, *Nlrp3*^{−/−} DCs experienced significantly less ROS-mediated DNA damage, and a significantly lower expression of several genes involved in DSB and base excision repair (BER) was revealed in wild-type DCs (32). These genes included *XRCC1*, *RAD51*, 8-oxoguanine–DNA glycosylase 1 (*OGG1*), breast cancer 1, early onset (*BRCA1*), DNA polymerase beta (*POLB*), and thymidylate synthase (*TYMS*) (32). It was demonstrated that DSB and BER enzymes responsible for repair of 8-oxoguanine, which is a DNA adduct formed as a result of oxidation, and therefore, is considered a marker of oxidative stress, were more active in *Nlrp3*^{−/−} cells in comparison with wild-type DCs (32). In addition, Nijmegen breakage syndrome 1 (*NBS1*), another protein involved in DNA repair, was highly phosphorylated in *Nlrp3*^{−/−} DCs compared with wild-type DCs, indicating greater efficacy of DNA repair in the absence of *Nlrp3* gene expression (32).

Taken together, these reports strongly implicate PRRs, in particular TLRs (TLR4, TLR7, TLR8, and TLR9) and NLRs (NLRP3), as major regulators of DNA repair (Table S1 in Supplementary Material). According to the above-mentioned

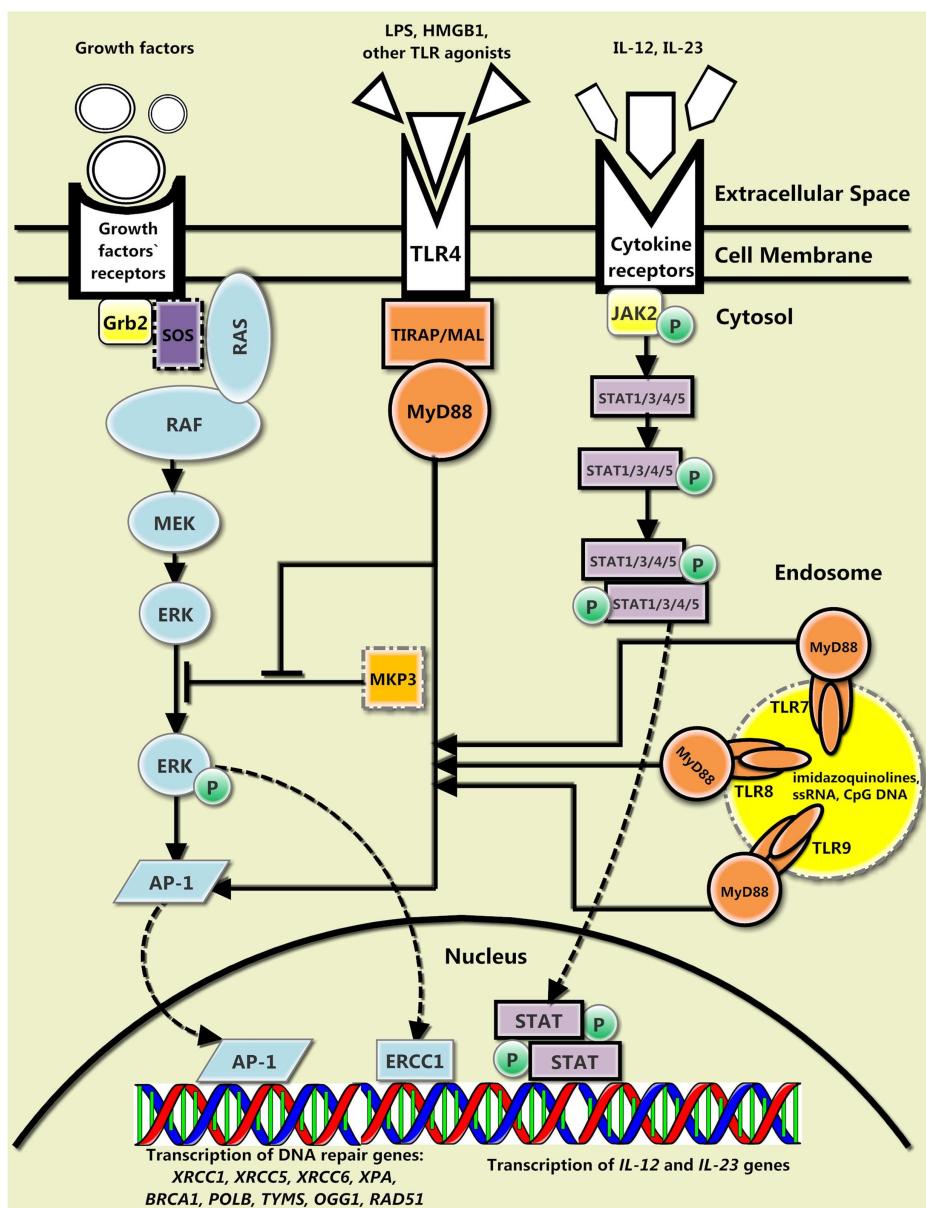


FIGURE 1 |The general interplay between the canonical TLR signaling pathway, the cytokine-mediated DNA repair feedback loop, and the growth factors-mediated signaling pathway. There are three main TLR-mediated pathways of DNA repair. The protein encoded by myeloid differentiation primary response gene 88 (MyD88) and its downstream signaling proteins (not shown) may inhibit mitogen-activated protein kinase phosphatase 3 (MKP3), which hinders phosphorylation of extracellular signal-regulated kinase (ERK), and therefore, prevents further

signaling via Ras-MAPK pathway. In addition, MyD88 and its downstream signaling proteins (not shown) along with pERK activate AP-1 transcription factor, which promotes transcription of certain DNA repair genes. Finally, IL-12 and IL-23, which enhance DNA repair and whose transcription is also amplified by MyD88-regulated transcription factors, bind to their receptors, activate Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway, and increase further transcription of their own encoding genes.

findings, these five receptors may affect the expression of at least eight enzymes (XRCC1, XRCC5, XRCC6, XPA, BRCA1, POLB, TYMS, OGG1, and RAD51) and two ILs (IL-12 and IL-23) involved in various mechanisms of DNA repair. Further, PRRs are responsible not only for

the initiation of one specific DNA repair pathway, but a number of such pathways repairing different types of DNA damage, i.e., oxidation, alkylation, and hydrolysis of bases, bulky adducts, SSBs, DSBs, and crosslinks. Interestingly, the effect of PRRs on DNA repair may vary between cell

types and cell lines, which address a number of questions to be answered in future studies.

Nowadays, we are only beginning to put the pieces of this puzzle together. Current vision of this topic is blurred, although a preliminary picture based on

recent research can be drawn (**Figure 1**). Both TLRs located on the cell surface and thus responsible for the recognition of the pathogen envelope molecular patterns (TLR4) and TLRs located on the endoplasmic reticulum, endosomal, or lysosomal membrane, and therefore, responsible for the recognition of pathogen nucleic acids (TLR7, TLR8, and TLR9) are involved in DNA repair. Therefore, other TLRs belonging to any of these groups may also participate in such processes. Definitely, the cytokine-mediated DNA repair feedback loop is not restricted to IL-12 and IL-23, and might consist of much greater number of cytokines, possibly TLR-regulated cytokines [IL-1, IL-2, IL-6, IL-8, IL-10, IL-13, IL-27, macrophage inflammatory protein-1 (MIP-1), monocyte chemotactic protein-1 (MCP-1), regulated on activation, normal T-cell expressed and secreted (RANTES), suppressor of cytokine signaling (SOCS), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor- α (TNF- α), interferon (IFN)- α , IFN- β , IFN- γ , and IFN-inducible proteins]. Furthermore, the exact composition of the growth factors-mediated DNA repair signaling pathway is still elusive; importantly, this pathway may have a particular importance since it includes both MyD88 and Ras-MAPK pathways, representing an interesting example of a crosstalk between canonical TLR MyD88-mediated signaling pathway and Ras-MAPK signaling pathway. In addition, there are no studies on the feasible influence of CLRs, RLRs, and ALRs on DNA repair. The improvement of our understanding of the role of PRRs in DNA repair may find implications for clinical medicine; peculiarities of PRRs functioning should definitely be considered when assessing the possibility of the use of PRR agonists in therapy of various diseases such as cancer. No doubt, further in-depth investigations are needed for deciphering the role of PRRs in sophisticated mechanisms of DNA repair.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00343/abstract>

REFERENCES

- Kutikhin AG, Yuzhalin AE. Inherited variation in pattern recognition receptors and cancer: dangerous liaisons? *Cancer Manag Res* (2012) **4**:31–8. doi:10.2147/CMAR.S28688
- Kutikhin AG, Yuzhalin AE. C-type lectin receptors and RIG-I-like receptors: new points on the oncogenomics map. *Cancer Manag Res* (2012) **4**:39–53. doi:10.2147/CMAR.S28983
- Osorio F, Reis e Sousa C. Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity* (2011) **34**:651–64. doi:10.1016/j.immuni.2011.05.001
- Elinav E, Strowig T, Henao-Mejia J, Flavell RA. Regulation of the antimicrobial response by NLR proteins. *Immunity* (2011) **34**:665–79. doi:10.1016/j.immuni.2011.05.007
- Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* (2011) **34**:637–50. doi:10.1016/j.immuni.2011.05.006
- Loo YM, Gale M Jr. Immune signaling by RIG-I-like receptors. *Immunity* (2011) **34**:680–92. doi:10.1016/j.immuni.2011.05.003
- Ratsimandresy RA, Dorfleutner A, Stehlik C. An update on PYRIN domain-containing pattern recognition receptors: from immunity to pathology. *Front Immunol* (2013) **4**:440. doi:10.3389/fimmu.2013.00440
- Troutman TD, Bazan JF, Pasare C. Toll-like receptors, signaling adapters and regulation of the pro-inflammatory response by PI3K. *Cell Cycle* (2012) **11**:3559–67. doi:10.4161/cc.21572
- Zhao X, Ai M, Guo Y, Zhou X, Wang L, Li X, et al. Poly I:C-induced tumor cell apoptosis mediated by pattern-recognition receptors. *Cancer Biother Radiopharm* (2012) **27**:530–4. doi:10.1089/cbr.2012.1226
- Hua Z, Hou B. TLR signaling in B-cell development and activation. *Cell Mol Immunol* (2013) **10**:103–6. doi:10.1038/cmi.2012.61
- Hammer GE, Ma A. Molecular control of steady-state dendritic cell maturation and immune homeostasis. *Annu Rev Immunol* (2013) **31**:743–91. doi:10.1146/annurev-immunol-020711-074929
- Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunol Rev* (2012) **249**:158–75. doi:10.1111/j.1600-065X.2012.01146.x
- Yang S, Xu L, Yang T, Wang F. High-mobility group box-1 and its role in angiogenesis. *J Leukoc Biol* (2014) **95**:563–74. doi:10.1189/jlb.0713412
- Kamba A, Lee IA, Mizoguchi E. Potential association between TLR4 and chitinase 3-like 1 (CHI3L1/YKL-40) signaling on colonic epithelial cells in inflammatory bowel disease and colitis-associated cancer. *Curr Mol Med* (2013) **13**:1110–21. doi:10.2174/1566524011313070006
- Harberts E, Gaspari AA. TLR signaling and DNA repair: are they associated? *J Invest Dermatol* (2013) **133**:296–302. doi:10.1038/jid.2012.288
- Kutikhin AG. Impact of toll-like receptor 4 polymorphisms on risk of cancer. *Hum Immunol* (2011) **72**:193–206. doi:10.1016/j.humimm.2010.11.003
- Wang Z, Yan J, Lin H, Hua F, Wang X, Liu H, et al. Toll-like receptor 4 activity protects against hepatocellular tumorigenesis and progression by regulating expression of DNA repair protein Ku70 in mice. *Hepatology* (2013) **57**:1869–81. doi:10.1002/hep.26234
- Wang Z, Lin H, Hua F, Hu ZW. Repairing DNA damage by XRCC6/KU70 reverses TLR4-deficiency-worsened HCC development via restoring senescence and autophagic flux. *Autophagy* (2013) **9**:925–7. doi:10.4161/auto.24229
- Ahmad I, Simanyi E, Gurojii P, Tamimi IA, Delarosa HJ, Nagar A, et al. Toll-like receptor-4 deficiency enhances repair of UVR-induced cutaneous DNA damage by nucleotide excision repair mechanism. *J Invest Dermatol* (2014) **134**:1710–7. doi:10.1038/jid.2013.530
- Kutikhin AG. Association of polymorphisms in TLR genes and in genes of the toll-like receptor signaling pathway with cancer risk. *Hum Immunol* (2011) **72**:1095–116. doi:10.1016/j.humimm.2011.07.307
- Tsukamoto Y, Nagai Y, Kariyone A, Shibata T, Kaisho T, Akira S, et al. Toll-like receptor 7 cooperates with IL-4 in activated B cells through antigen receptor or CD38 and induces class switch recombination and IgG1 production. *Mol Immunol* (2009) **46**:1278–88. doi:10.1016/j.molimm.2008.11.022
- Fishelevich R, Zhao Y, Tuchinda P, Liu H, Nakazono A, Tammaro A, et al. Imiquimod-induced TLR7 signaling enhances repair of DNA damage induced by ultraviolet light in bone marrow-derived cells. *J Immunol* (2011) **187**:1664–73. doi:10.4049/jimmunol.1100755
- Zheng L, Asprodites N, Keene AH, Rodriguez P, Brown KD, Davila E. TLR9 engagement on CD4 T lymphocytes represses gamma-radiation-induced apoptosis through activation of checkpoint kinase response elements. *Blood* (2008) **111**:2704–13. doi:10.1182/blood-2007-07-104141
- Klaschik S, Tross D, Shirota H, Klinman DM. Short- and long-term changes in gene expression mediated by the activation of TLR9. *Mol Immunol* (2010) **47**:1317–24. doi:10.1016/j.molimm.2009.11.014
- Sommariva M, De Cecco L, De Cesare M, Sfondrini L, Ménard S, Melani C, et al. TLR9 agonists oppositely modulate DNA repair genes in tumor versus immune cells and enhance chemotherapy effects. *Cancer Res* (2011) **71**:6382–90. doi:10.1158/0008-5472.CAN-11-1285
- Cataisson C, Salcedo R, Hakim S, Moffitt BA, Wright L, Yi M, et al. IL-1R-MyD88 signaling in keratinocyte transformation and carcinogenesis. *J Exp Med* (2012) **209**:1689–702. doi:10.1084/jem.20101355
- Coste I, Le Corf K, Kfouri A, Hmitou I, Druillennec S, Hainaut P, et al. Dual function of MyD88 in RAS signaling and inflammation, leading to mouse and human cell transformation. *J Clin Invest* (2010) **120**:3663–7. doi:10.1172/JCI42771
- Sharrocks AD, Yang SH, Galanis A. Docking domains and substrate-specificity determination for MAP kinases. *Trends Biochem Sci* (2000) **25**:448–53. doi:10.1016/S0968-0004(00)01627-3
- Kfouri A, Le Corf K, El Sabeh R, Journeaux A, Badran B, Hussein N, et al. MyD88 in DNA repair

- and cancer cell resistance to genotoxic drugs. *J Natl Cancer Inst* (2013) **105**:937–46. doi:10.1093/jnci/djt120
30. Kfouri A, Virard F, Renno T, Coste I. Dual function of MyD88 in inflammation and oncogenesis: implications for therapeutic intervention. *Curr Opin Oncol* (2014) **26**:86–91. doi:10.1097/CCO.000000000000037
31. Lai XY, Egan LJ. Suppression of radiation-induced DNA double-strand break repair by MyD88 is accompanied by apoptosis and crypt loss in mouse colon. *Oncogenesis* (2013) **2**:e62. doi:10.1038/oncsis.2013.22
32. Licandro G, Ling Khor H, Beretta O, Lai J, Derkx H, Laudisi F, et al. The NLRP3 inflammasome affects DNA damage responses after oxidative and genotoxic stress in dendritic cells. *Eur J Immunol* (2013) **43**:2126–37. doi:10.1002/eji.201242918
33. Chen GY, Nuñez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol* (2010) **10**:826–37. doi:10.1038/nri2873

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Toll-like receptor 4 in inflammation and angiogenesis: a double-edged sword

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Toll-like receptors (TLRs) primarily known for the pathogen recognition and subsequent immune responses are being investigated for their pathogenic role in various chronic diseases. The recent reports correlating the microbial infections with chronic disorders such as atherosclerosis have led to questions in relation to the role of microbial sensors such as TLR4 in an intriguing phenomenon of the *inflammation-induced angiogenesis*. This article focuses on the possible mechanisms involved in it.

Toll-like receptors comprise a large family of the pathogen-pattern recognition receptors (PPRR) originally identified in *Drosophila* in the mid 1990s as a *Toll protein* (1). In *Drosophila*, it was found to be involved in the resistance against fungal infections (2). The first human homolog for the Toll protein was described in 1997 (3). Since then, 13 mammalian homologs of the TLR family have been identified; including 12 in mice (TLR1-9 and TLR11-13) and 10 in humans (TLR1-10). TLR 10 is a pseudogene in mice, but is functional in humans (4). The membrane expressed TLRs recognize the pathogen-associated molecular patterns (PAMPs) either directly on the plasma membrane or within the endosomal compartment after the phagocytosis. In addition to the foreign molecules, a range of various endogenous ligands are also detected by TLRs, which suggests a role beyond that of simple pathogen recognition. Endogenous ligands released from the damaged, apoptotic, or fibrotic cells during inflammation, are termed *danger-associated molecular patterns* (DAMPs). A significant number of DAMPs have been reported for TLR4 (5, 6).

TLR4 is one of the best characterized and the first member of the TLR family to be discovered as a PPRR. TLR4 signaling is implicated in the innate immune responses against a wide-range of microbes, including Gram-negative and -positive bacteria, mycobacteria, spirochetes, yeasts, and some viruses such as respiratory syncytial viruses (RSV) and mammary tumor viruses (4). TLR4 is a type I transmembrane protein characterized by an extracellular domain containing leucine-rich repeats (LRRs) and a cytoplasmic tail harboring a conserved region known as Toll/IL-1 receptor (TIR) domain. TLR4, along with its two co-receptors, the myeloid differentiation antigen (MD2) and the LRR protein CD14, forms a trimeric receptor that is involved in the recognition of lipopolysaccharide (LPS). The TLR4 ligand binding causes the C termini of the ectodomains to move close to each other, thus triggering signaling and inflammation. The diverse interactions between TLRs with their ligands converge into either the MyD88-dependent or MyD88-independent pathways, resulting in the: (1) activation of lymphocytes, (2) up-regulation/expression of co-stimulatory signals, and (3) release of pro-inflammatory cytokines/chemokines (7). As sentinels in the innate immunity, TLR expression was thought to be confined to the immune cells such as macrophages, monocytes, and dendritic cells. However, an increasing number of reports show a more diverse expression of TLRs; including epithelial cells, endothelial cells (8), neural and glial cells, thereby playing an important role in tissue-specific inflammation (9).

TLR4 is implicated in a diverse range of pathological processes associated with or induced by angiogenesis including autoimmune diseases such as psoriasis, diabetic retinopathy, thrombosis, and inflammatory disorders including arthritis and atherosclerosis and cancer (10, 11). It has been proposed that TLR4 contributes to these diseases through *inflammation-induced angiogenesis*. The recent association between bacterial infections and atherosclerosis has intensified the search for the biological functions of TLRs especially TLR4 in blood vessel formation (12). The exact mechanism needs to be elucidated.

Angiogenesis is the normal process required for the development of an extensive vasculature. With its over 60 trillion endothelial cells, the vascular network is the first and the largest organ to develop in the human body (13). It mainly occurs during embryonic development. In adults, angiogenesis is a highly regulated process only occurring during the retinal development, in the adult intestinal villi and in the female reproductive organs (14). The postnatal angiogenesis may take place through one of the two possible mechanisms; (1) vasculogenesis – the *de novo* generation of blood vessels from endothelial progenitor cells (EPCs) or mesoderm and more commonly (2) angiogenesis, which is the sprouting/branching of the pre-existing blood vessels – together they are called neoangiogenesis. Angiogenesis is a highly complex series of sequential events orchestrating various molecular events involving multiple cell populations, cytokines, and chemokines. It takes place in two important steps; (1) formation of a nascent

vascular network and (2) its subsequent maturation. The degradation of extracellular matrix (ECM) allows the sprouting of EPCs from old vessel into an avascular space and differentiation into nascent vasculature under the influence of pro-angiogenic factors. The maturation process involves the recruitment of supporting cells (mural cells) and vessel remodeling. Mural cells include vascular smooth-muscle cells (VSMC) in arteries, arterioles, and veins; pericytes in capillaries (15, 16). They provide structural integrity to the developing vasculature and may also interact with the endothelial cells, through paracrine signaling. Pro-angiogenic factors such as the vascular endothelial growth factor (VEGF); the basic fibroblast growth factor (bFGF); the transforming growth factor beta (TGF- β); the platelet-derived growth factor (PDGF); the tumor necrosis factor alpha (TNF- α); the insulin-like growth factor-1 (IGF-1); the monocyte chemotactic protein (MCP)-1; interleukin (IL)-6 and 8 all help in the recruitment of cells, ECM degradation, and with vessel development and maturity (14). An important empirical role played by TLR4 in the lymphocytic activation, recruitment, and release of cytokines is evident in TLR4-deficient mice. Such mice are reported to display significantly impaired expression of pro-inflammatory cytokines after reperfusion triggered by retinal ischemia injury (17). The process of lymphangiogenesis was shown to be affected in TLR4-deficient mice through lack of macrophage recruitment by TLR4 $^+$ lymphatic endothelial cells (LEC) (7).

As one of the two main sources of cytokines, macrophages play a critical role in the leukocyte trafficking and the postnatal angiogenesis. TLR4-mediated LPS-activated macrophages have been shown to be an important source of pro-angiogenic factors. Accumulating evidence shows that antigenic stimulation and the surrounding cytokine environment can have profound effects on the activation status and the functional capabilities of macrophages. Although there are various schools of thought regarding the macrophage activation status, here, we focus on two; the M1 and M2 phenotypes. The classical activation or M1 phenotype of macrophages contributes substantially toward anti-microbial immune

responses via the production of pro-inflammatory cytokines such as IL-6, IL-8, IL-12, inducible nitric oxide synthase (iNOS), and interferons (IFNs) (18) (**Figure 1**). The alternate activation of macrophages may lead to the M2 phenotype, which is reported to be involved in the wound repair and fibrosis by contributing toward angiogenesis through the VEGF production (19). The strong mitogenic effect on the endothelial cells and the induction of vascular permeability are the pro-angiogenic effects, which makes VEGF the most potent simulator of angiogenesis. In murine macrophages and other TLR4 $^+$ cell populations, a strong synergism is reported to significantly influence the production of VEGF. Endotoxins (including LPS) together with the growth factors and cytokines such as IFN- γ , TGF- β , IL-1, and IL-6 have been implicated in a significant augmentation in VEGF levels (20–24). In this regard, the synergism reported between TLR4 and adenosine receptor 2A (A_{2A}R) in the murine macrophages (M2) is noteworthy (**Figure 1**) (25). Adenosine receptor signaling plays an important role in inflammation. Adenosine is produced by many different cell types and is elevated in conditions such as hypoxia, ischemic conditions, and stress. So far, four adenosine receptors have been reported, i.e., the A₁, A_{2A}, A_{3B}, and A₃ receptors (26). The synergistic effect of A_{2A}R is not restricted to TLR4, but TLR2, 7, and 9 also lead to high VEGF production in the presence of adenosine signaling (22). Both TLR4 and A_{2A}R were shown to signal through hypoxia inducible factor (HIF)-1 α and hypoxia response element (HRE) (27). Although the TLR4 along with its co-receptors are known to be expressed on the endothelial cells, it is not yet known whether the endothelial cells share the synergistic effect of TLR4 with A_{2A}R. The transcriptional expression of A_{2A}R has been reported on the endothelial cells; however, there are limited number of studies in this context. Many groups have demonstrated potent endothelial responses to LPS *in vitro* (28–32). However, there are reports supporting the *in vivo* role of LPS in postnatal angiogenesis. A study conducted in murine tumor model (metastatic) demonstrated the pro-angiogenic effects of LPS. The LPS-induced

growth and metastasis of 4T1 experimental lung metastases model was shown to take place through increased angiogenesis, vascular permeability, and tumor cell migration (33). The LPS-mediated angiogenic effects can be reversed through TLR4 downregulation. While studying the anti-inflammatory affects of a compound known as *Baicalein*, its anti-angiogenic effects were shown to be carried out through the downregulation of TLR4 and its downstream mitogen-activated phosphate kinase (MAPK) pathway (34).

The ubiquitous and abundantly expressed DAMPs are often found in association with different anomalies. One such commonly expressed protein is high mobility group chromatin protein B1 (HMGB-1). It is a nuclear DNA binding protein released by injured or necrotic cells. Resting, non-activated inflammatory cells, such as monocytes or macrophages, contain HMGB-1 in their nuclei. When these cells are activated by LPS or inflammatory cytokines, HMGB-1 translocates in the cytoplasm, undergoes acetylation, and is exocytosed. It is evident that excreted HMGB-1 acts like a pro-inflammatory cytokine, therefore, HMGB-1 can be regarded as a signal of tissue injury and a mediator of inflammation (35). Macrophage-derived HMGB-1 has been shown to increase the endothelial cell proliferation, sprouting, and chemotaxis by stimulating the migration of adherent cells, such as fibroblasts and smooth-muscle cells. In a recent study, HMGB-1-TLR4 signaling was reported to be an important mediator in retinal neoangiogenesis in an oxygen-induced retinopathy murine model (36). HMGB-1 is an important marker for tumor endothelial cells and was shown to be necessary for the sustained expression of pro-angiogenic genes. A positive feedback mechanism has been suggested for the HMGB-1 expression and that of its cognate receptors, i.e., TLR4 and receptor for advanced glycation end products (RAGE) on the endothelial cells. Thus HMGB-1 may prove to be a promising target for interfering with cancer-related angiogenesis (37). However, there is some disagreement in relation to the HMGB-1 as an endogenous ligand for TLR4. The lack of an LPS-free *in vitro* system makes it difficult to study the signaling resulting exclusively

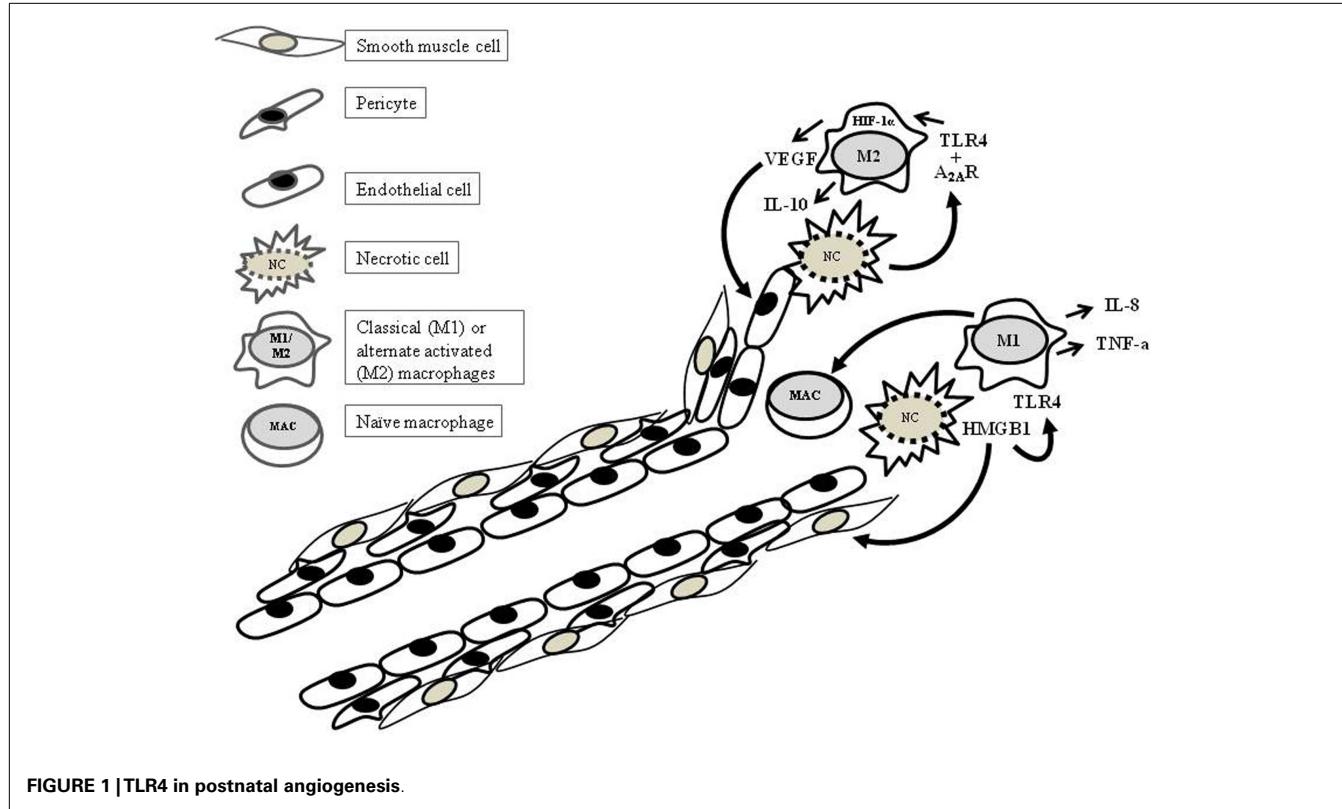


FIGURE 1 | TLR4 in postnatal angiogenesis.

from the TLR4-ligands other than LPS. Even small traces of LPS can upregulate TLR4 and can affect the interpretation of results.

Ischemic diseases are one of the major causes of morbidity and mortality. Treatment of such disorders requires angiogenesis. It is therefore the prime goal of *therapeutic angiogenesis* to achieve this. However, the close association between angiogenesis and inflammation presents an obstacle to the success of the therapy. Most of the pro-angiogenic factors are also pro-inflammatory. Therefore, the reperfusion of ischemic tissues often results in injury due to the microvascular dysregulations and inflammation (edema) associated with it. The activated endothelial cells lead to an imbalance between oxygen radicals and nitric oxide causing the release of inflammatory mediators (38, 39). The TLR4-deficient mice have been a valuable tool for studying the role of TLR4 in tissue-related ischemia–reperfusions *in vivo*. A recent study reported the role of TLR4-mediated responses contributing to the oxygen-induced neovascularization in ischemic neural tissue (retina).

The TLR4-dependent responses, proposed to be mediated through HMGB-1 release in the ischemic neural tissue were found to be impaired in TLR4-deficient mice, revealing an important angiogenic role of TLR4 in neural tissues (36). On the other hand, there are several studies highlighting the inflammatory role of TLR4 in various reperfusion–ischemic models in tissues such as liver, lung, and intestine. Most of these studies showed reduced inflammation in relation to the injury induced by the reperfusion of various organs after a period of ischemia in TLR4-deficient mice, thus, highlighting the inflammatory role of TLR4 in reperfusion-related injury models, without significant compromise in angiogenesis (40–43). Considering these reports, the dual role of TLR4 in angiogenesis and inflammation comes to light, which seems to be governed by an intricate balance between the inhibitory or stimulatory factors that may be tissue-specific. Nevertheless, TLR4 remains a promising target for suppressing the undesired and prolonged inflammatory responses. In this regard, various synthetic and plant-derived therapies are currently being tested.

TLR4-blocking through small molecule inhibitors and antibodies are being evaluated in pre-clinical trials for their efficacy in various inflammatory conditions. Novimmune is a humanized counterpart of rat anti-TLR4 monoclonal antibody; 1A6, found to reduce inflammation in a murine colitis model. It is undergoing pre-clinical evaluation for the treatment of the inflammatory bowel diseases (44–46). Various plant-derived drugs such as wogonoside and celastrol have shown promising results against TLR4-mediated LPS-induced angiogenesis in pre-clinical drug testing (47, 48).

In conclusion, it can be said that the close association between inflammation and angiogenesis makes the therapeutic modulation of TLR4 somewhat challenging and can lead to potential side effects. Therefore, the fine tuning of TLR4 and its associating proteins is required in order to circumvent the undesired inflammatory or angiogenic responses associated with TLR4 targeting in various pathologies. For that purpose, further insight into its *in vivo* networking and the effects of TLR4 targeting in various pathologies through the use

of closely related animal disease models is required.

REFERENCES

- Taguchi T, Mitcham JL, Dower SK, Sims JE, Testa JR. Chromosomal localization of TIL, a gene encoding a protein related to the *Drosophila* transmembrane receptor Toll, to human chromosome 4p14. *Genomics* (1996) **32**:486–8. doi:10.1006/geno.1996.0150
- Fehlbaum P, Bulet P, Michaud L, Lagueux M, Broeckaert W, Hetru C, et al. Insect immunity: septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. *J Biol Chem* (1994) **269**:33159–33163.
- Medzhitov R, Preston-Hurlburt P, Janeway C. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* (1997) **388**:394–397. doi:10.1038/41131
- Pichlmair A, Reis e Sousa C. Innate recognition of viruses. *Immunity* (2007) **27**:370–83. doi:10.1016/j.jimmuni.2007.08.012
- Brikos C, O'Neill LA. Signalling of Toll-like receptors. *Handb Exp Pharmacol* (2008) **183**:21–50. doi:10.1007/978-3-540-72167-3_2
- Yu L, Wang L, Chen S. Endogenous Toll-like receptor ligands and their biological significance. *J Cell Mol* (2010) **14**:2592–603. doi:10.1111/j.1582-4934.2010.01127.x
- Kang S, Lee SP, Kim KE, Kim HZ, Mémet S, Koh GY. Toll-like receptor 4 in lymphatic endothelial cells contributes to LPS-induced lymphangiogenesis by chemotactic recruitment of macrophages. *Blood* (2009) **113**(11):2605–13. doi:10.1182/blood-2008-07-166934
- Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytniuk L, Raharjo E, et al. Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *J Clin Invest* (2003) **111**:1011–20. doi:10.1172/JCI16510
- Zhou H, Lapointe BM, Clark SR, Zbytniuk L, Kubes P. A requirement for microglial TLR4 in leukocyte recruitment into brain in response to lipopolysaccharide. *J Immunol* (2006) **177**(11):8103–10. doi:10.4049/jimmunol.177.11.8103
- Satoh M, Ishikawa Y, Minami Y, Takahashi Y, Nakamura M. Role of Toll like receptor signaling pathway in ischemic coronary artery disease. *Front Biosci* (2008) **13**:6708–15. doi:10.2741/3183
- Ehsan N, Murad S, Ashiq T, Mansoor MU, Gul S, Khalid S, et al. Significant correlation of TLR4 expression with the clinicopathological features of invasive ductal carcinoma of the breast. *Tumour Biol* (2013) **34**(2):1053–9. doi:10.1007/s13277-013-0645-y
- Frantz S, Ertl G, Bauersachs J. Mechanisms of disease: Toll-like receptors in cardiovascular disease. *Nat Clin Pract Cardiovasc Med* (2007) **4**:444–54. doi:10.1038/ncpcardio0938
- Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res* (2007) **100**(2):158–73. doi:10.1161/01.RES.0000255691.76142.4a
- Carmeliet P. Angiogenesis in life, disease and medicine. *Nature* (2005) **438**:932–6. doi:10.1038/nature04478
- Sato TN. Vascular development: molecular logic for defining arteries and veins. *Curr Opin Hematol* (2003) **10**:131–5. doi:10.1097/00062752-200303000-00006
- Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. *Nature* (2000) **407**:242–8. doi:10.1038/35025215
- Dvoriantchikova G, Barakat DJ, Hernandez E, Shestopalov VI, Ivanova D. Liposome-delivered ATP effectively protects the retina against ischemia-reperfusion injury. *Mol Vis* (2010) **16**:2882–90.
- Pinhal-Enfield G, Ramanathan M, Hasko G, Vogel SN, Salzman AL, Boons GJ, et al. An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A(2A) receptors. *Am J Pathol* (2003) **163**(2):711–21. doi:10.1016/S0002-9440(10)63698-X
- Wu WK, Llewellyn OP, Bates DO, Nicholson LB, Dick AD. IL-10 regulation of macrophage VEGF production is dependent on macrophage polarisation and hypoxia. *Immunobiology* (2010) **215**(9–10):796–803. doi:10.1016/j.imbio.2010.05.025
- Pertojaara L, Kaipainen A, Mustonen T, Orpana A, Ferrara N, Saksela O, et al. Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem* (1994) **269**:6271–4.
- Levy AP, Levy NS, Wegner S, Goldberg MA. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* (1995) **270**:13333–40. doi:10.1074/jbc.270.22.13333
- Cohen T, Nahari D, Cerem IW, Neufeld G, Levi BZ. Interleukin 6 induces the expression of vascular endothelial growth factor. *J Biol Chem* (1996) **271**:736–741. doi:10.1074/jbc.271.2.736
- Gille J, Swerlick RA, Caughman SW. Transforming growth factor-alpha-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. *EMBO J* (1997) **16**:750–759. doi:10.1093/emboj/16.4.750
- Xiong M, Elson G, Legarda D, Leibovich SJ. Production of vascular endothelial growth factor by murine macrophages: regulation by hypoxia, lactate, and the inducible nitric oxide synthase pathway. *Am J Pathol* (1998) **153**:587–98. doi:10.1016/S0002-9440(10)65601-5
- Leibovich SJ, Chen JF, Pinhal-Enfield G, Belem PC, Elson G, Rosania A, et al. Synergistic up-regulation of vascular endothelial growth factor expression in murine macrophages by adenosine A(2A) receptor agonists and endotoxin. *Am J Pathol* (2002) **160**(6):2231–44. doi:10.1016/S0002-9440(10)61170-4
- Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, et al. Nomenclature and classification of purinoreceptors. *Pharmacol Rev* (1994) **46**:143–56.
- Ramanathan M, Pinhal-Enfield G, Hao I, Leibovich SJ. Synergistic up-regulation of vascular endothelial growth factor (VEGF) expression in macrophages by adenosine A2A receptor agonists and endotoxin involves transcriptional regulation via the hypoxia response element in the VEGF promoter. *Mol Biol Cell* (2007) **18**:14–23. doi:10.1091/mbc.E06-07-0596
- Fan J, Frey RS, Malik AB. TLR4 signaling induces TLR2 expression in endothelial cells via neutrophil NADPH oxidase. *J Clin Invest* (2003) **112**:1234–43. doi:10.1172/JCI200318696
- Li X, Tupper JC, Bannerman DD, Winn RK, Rhodes CJ, Harlan JM. Phosphoinositide 3 kinase mediates Toll-like receptor 4-induced activation of NF-kappa B in endothelial cells. *Infect Immun* (2003) **71**(8):4414–20. doi:10.1128/IAI.71.8.4414-4420.2003
- Lloyd K, Kubes P. GPI-linked endothelial CD14 contributes to the detection of LPS. *Am J Physiol Heart Circ Physiol* (2006) **291**(1):H473. doi:10.1152/ajpheart.01234.2005
- Faure E, Equils O, Sieling PA, Thomas L, Zhang FX, Kirschning CJ, et al. Bacterial lipopolysaccharide activates NF-kappaB through Toll-like receptor 4 (TLR-4) in cultured human dermal endothelial cells. Differential expression of TLR-4 and TLR-2 in endothelial cells. *J Biol Chem* (2000) **275**(15):11058–63. doi:10.1074/jbc.275.15.11058
- Zhang FX, Kirschning CJ, Mancinelli R, Xu XP, Jin Y, Faure E, et al. Bacterial lipopolysaccharide activates nuclear factor-kappaB through interleukin-1 signaling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. *J Biol Chem* (1999) **274**(12):7611–4. doi:10.1074/jbc.274.12.7611
- Ling Y, Wang L, Chen Y, Feng F, You Q, Lu N, et al. Baicalein inhibits angiogenesis induced by lipopolysaccharide through TRAF6 mediated Toll-like receptor 4 pathway. *Biomed Prev Nutr* (2011) **1**:172–9. doi:10.1016/j.bionut.2011.06.013
- Harmey IH, Bucana CD, Lu W, Byrne AM, McDonnell S, Lynch C, et al. Lipopolysaccharide-induced metastatic growth is associated with increased angiogenesis, vascular permeability and tumor cell invasion. *Int J Cancer* (2002) **101**:415–22. doi:10.1002/ijc.10632
- Wu CX, Sun H, Liu Q, Guo H, Gong JP. LPS induces HMGB1 relocation and release by activating the NF-κB-CBP. Signal transduction pathway in the murine macrophage-like cell line RAW264.7. *J Surg Res* (2012) **175**(1):88–100. doi:10.1016/j.jss.2011.02.026
- He C, Sun Y, Ren X, Lin Q, Hu X, Huang X, et al. Angiogenesis mediated by Toll-like receptor 4 in ischemic neural tissue. *Arterioscler Thromb Vasc Biol* (2013) **33**(2):330–8. doi:10.1161/ATVBAHA.112.300679
- Van Beijnum JR, Nowak-Sliwinska P, van den Boezem E, Hautvast P, Buurman WA, Griffioen AW. Tumor angiogenesis is enforced by autocrine regulation of high-mobility group box 1. *Oncogene* (2013) **32**(3):363–74. doi:10.1038/onc.2012.49
- Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. *J Pathol* (2000) **190**(3):255–66. doi:10.1002/(SICI)1096-9896(200002)190:3<255::AID-PATH526>3.0.CO;2-6
- Hori M, Nishida K. Toll-like receptor signaling defensive or offensive for the heart? *Circ Res* (2008) **102**:137–9. doi:10.1161/CIRCRESAHA.107.170225
- Oyama J, Blais CJr, Liu X, Pu M, Kobzik L, Kelly RA, et al. Reduced myocardial ischemia-reperfusion

- injury in Toll-like receptor 4-deficient mice. *Circulation* (2004) **109**(6):784–9. doi:10.1161/01.CIR.0000112575.66565.84
41. Cao CX, Yang QW, Lv FL, Cui J, Fu HB, Wang JZ. Reduced cerebral ischemia-reperfusion injury in Toll-like receptor 4 deficient mice. *Biochem Biophys Res Commun* (2007) **353**(2):509–14. doi:10.1016/j.bbrc.2006.12.057
42. Tatum PM Jr, Harmon CM, Lorenz RG, Dimmitt RA. Toll-like receptor 4 is protective against neonatal murine ischemia-reperfusion intestinal injury. *J Pediatr Surg* (2010) **45**(6):1246–55. doi:10.1016/j.jpedsurg.2010.02.093
43. Ellett JD, Evans ZP, Atkinson C, Schmidt MG, Schnellmann RG, Chavin KD. Toll-like receptor 4 is a key mediator of murine steatotic liver warm ischemia/reperfusion injury. *Liver Transpl* (2009) **15**(9):1101–9. doi:10.1002/lt.21782
44. Ungaro R, Fukata M, Hsu D, Hernandez Y, Breglio K, Chen A, et al. A novel Toll-like receptor 4 antagonist antibody ameliorates inflammation but impairs mucosal healing in murine colitis. *Am J Physiol Gastrointest Liver Physiol* (2009) **296**:G1167–79. doi:10.1152/ajpgi.90496.2008
45. Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of Toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation* (2002) **105**(10):1158–61.
46. Simpson JL, Grissell TV, Douwes J, Scott RJ, Boyle MJ, Gibson PG. Innate immune activation in neutrophilic asthma and bronchiectasis. *Thorax* (2007) **62**:211–8. doi:10.1136/thx.2006.061358
47. Chen Y, Lu N, Ling Y, Gao Y, Wang L, Sun Y, et al. Wogonoside inhibits lipopolysaccharide-induced angiogenesis in vitro and in vivo via Toll-like receptor 4 signal transduction. *Toxicology* (2009) **259**(1–2):10–7. doi:10.1016/j.tox.2009.01.010
48. Ni H, Zhao W, Kong X, Li H, Ouyang J. Celastrol inhibits lipopolysaccharide-induced angiogenesis by suppressing TLR4-triggered nuclear factor-kappa B activation. *Acta Haematol* (2014) **131**(2):102–11. doi:10.1159/000354770

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Toll-like receptors in esophageal cancer

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INTRODUCTION

Toll-like receptors (TLRs) are evolutionarily conserved receptors of the innate immune system (1). The 13 TLRs that have been identified so far recognize their unique pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide (LPS) (TLR4), DNA (TLR9), or flagellin (TLR5) (1, 2). TLR stimulation induces down-stream activation of various signaling molecules and this ultimately results in the innate immune response, which also activates the adaptive immune system (1–3). The aim of this review is to explore the role and function of TLRs in esophageal adenocarcinoma (EAC) and in squamous cell carcinoma.

ESOPHAGEAL CANCER

Esophageal cancer is the eighth most common cancer in the world with estimated 482,000 new cases worldwide in 2008. The incidence of esophageal cancer was 70/100,000 in 2008 in the world. The majority of esophageal cancers are esophageal squamous cell carcinomas (ESCC), but the incidence of EAC is rising rapidly (4, 5).

As with oral squamous cell cancer, tobacco and alcohol, low socioeconomic status, poor oral health, and betel nuts, as well as the autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED)-syndrome have been listed as risk factors for ESCC (6–10). With regard to pathologic anatomy, esophagus could be considered as an extension of the oral cavity, as it is lined by squamous epithelium and it encounters swallowed oral bacteria before they enter the stomach.

Esophageal squamous cell carcinoma and esophageal adenocarcinoma are cancers of high mortality. EAC develops through Barrett's esophagus (BE) and columnar dysplasia, preceded by gastro-esophageal reflux disease. The risk of esophageal squamous cell carcinoma is increased by smoking and alcohol consumption. New treatment options for esophageal cancer are desperately needed. Toll-like receptors (TLRs) play a central role in mammalian immunity and cancer. TLRs are activated by microbial components, such as lipopolysaccharide, flagellin, DNA, and RNA, as well as endogenous ligands, including heat-shock proteins and endogenous DNA. This review summarizes the studies on TLRs in esophageal squamous cell carcinoma and EAC. It has been shown that TLRs 1–10 are expressed in the normal esophagus. In esophageal squamous cell carcinoma, TLRs 3, 4, 7, and 9 have been studied, showing associations to aggressive disease properties. In BE and EAC, only TLRs 4, 5, and 9 have been studied. In the review, we discuss the implications of TLRs in esophageal cancer.

Keywords: Toll-like receptors, microbiome, esophageal cancer, esophageal adenocarcinoma, esophageal squamous cell carcinoma

For EAC, the most important risk factor is Barrett's esophagus (BE), determined by columnar metaplastic cells, which replace the normal squamous epithelium after long-lasting gastro-esophageal reflux, or gastro-esophageal reflux disease (GERD). Patients with BE have a 30- to 125-fold risk for EAC compared to normal population (11, 12). A recent study, however, concluded that only 0.12% of patients with BE develop EAC (13). Other minor risk factors include obesity, smoking, hiatal hernia, and low socioeconomic status (10, 14–18). Furthermore, both types of esophageal cancers develop through dysplasia to cancer via genetic alterations (19, 20).

The 5-year survival rate for esophageal cancer varies between 10 and 16% (4). After esophageal resection, the 5-year survival rate was 20.6% in a meta-analysis of Western population (21). Furthermore, these cancers are often diagnosed late because at the time of the diagnosis, more than half of the patients have an inoperable disease (22).

The most important prognostic determinant for both esophageal cancers is the WHO TNM-classification (23). The histologically defined grade of differentiation is also a predictor of prognosis (24).

TOLL-LIKE RECEPTORS IN NORMAL ESOPHAGUS

Esophageal epithelial cells have been shown to express TLRs. The human esophageal epithelial cell-line TE-1 was shown to express TLRs 2, 3, 4, and 7, with up-regulation of beta-defensin 2 as a response to stimulation with their cognate, synthetic ligands (25). In 2009, Lim and colleagues demonstrated the expression of TLRs 1–10, but not TLR4 at the mRNA level in the normal human

esophageal epithelial cell-line EPC-2. Furthermore, they demonstrated TLR1, 2, 3, and 5 mRNA expression in biopsies taken from esophageal mucosa. IL-8 was up-regulated in the EPC-2 cells by stimulation of the respective TLR-ligands. TLR3 stimulation was the most effective in inducing IL-8 expression synergistically with TLR2 and this effect was dependent on NF- κ B activation (26).

TLR3 was later demonstrated also to mediate the induction of IL-8 mRNA via NF- κ B by necrotic cell supernatants in the EPC-2 cells (27). TLR2 and TLR3 protein expression was demonstrated in esophageal epithelial cells, but not in cultured primary esophageal epithelial cells (28). The expression of TLR3, 4, 5, 7, and 9 proteins in normal esophagus has been characterized using immunohistochemistry in clinical samples (29–31). These studies have demonstrated that TLRs 1–10 are expressed in normal esophagus.

TOLL-LIKE RECEPTORS AND ESOPHAGEAL SQUAMOUS CELL CARCINOMA

Esophageal squamous cell carcinoma develops to squamous epithelium via dysplasia. A variety of TLRs, including TLR3, 4, 7, and 9, have been shown to be overexpressed in esophageal squamous cell carcinoma, when compared to normal esophagus (30, 31). We demonstrated an increased TLR9 expression in esophageal squamous dysplasia and in squamous cell carcinoma, suggesting a possible role for TLR9 in esophageal carcinogenesis (31).

High TLR3, 4, and 9 expression in esophageal squamous cell carcinoma cells have been associated with lymph node metastasis and TLR7 and 9 expression to worse histological grade (30, 31). TLR9 expression in the fibroblastoid cells of the tumor was, however, associated with decreased invasion depth and a smaller prevalence of lymph node metastasis at the time of diagnosis (30). TLR4 stimulation by LPS has been shown to increase migration and adhesive properties of esophageal squamous cell carcinoma cells via p38 and selectin (32). No studies thus far have evaluated the anti-cancer efficacy of TLR-agonists or inhibitors in the treatment of ESCC.

TOLL-LIKE RECEPTORS, BARRETT'S ESOPHAGUS, AND ESOPHAGEAL ADENOCARCINOMA

Esophageal adenocarcinoma is developed through the metaplasia–dysplasia–carcinoma sequence. Normal or inflamed esophageal epithelium is believed to transform to BE or columnar metaplasia through continuous exposure to acidic gastric contents, but also transformation of esophageal microbiome occurs during these changes (33, 34).

It was shown in BE and in normal human esophageal cell lines, that stimulation of TLR4 with LPS resulted in NF- κ B activation and an increase of IL-8 secretion, this response was more significant in BE. *Ex vivo* culture demonstrated increased cyclooxygenase-2 (COX-2) activation by LPS stimulation of TLR4 in BE (35). TLR5 was recently analyzed in the metaplasia–dysplasia–adenocarcinoma sequence, with high expression potentially differentiating between BE and columnar dysplasia (29).

The increased expression of TLR5 and 9 has been shown in EAC. TLR5 expression had no associations to clinico-pathological variables or prognosis, but TLR9 expression was associated with metastasis, poor grade of differentiation and poor prognosis in EAC (29, 36). Stimulation of EAC cells with CpG-oligonucleotides

that either have the physiological phosphodiester DNA-backbone or the nuclease-resistant phosphothioate backbone, induced cellular invasion and matrix metalloproteinase-9 and -13 mRNA expression (37).

At the current moment, there are no published clinical studies on TLRs in EAC.

TOLL-LIKE RECEPTOR GENETICS AND ESOPHAGEAL CANCER

Genetic studies have been performed on Toll-like receptor polymorphisms in esophageal cancer. Unlike in gastric cancer, polymorphisms in *TLR4 + 896A > G* and *TLR9-1237T/C* genes were not associated to esophageal cancer risk (38, 39). However, genetic up-regulation of CD14, a co-receptor of TLR4, was observed in families with history of esophageal cancer (40).

DISCUSSION

The treatment of esophageal cancer is overshadowed by its poor prognosis. New options for early diagnosis and treatment are desperately needed. The esophageal epithelium encounters bacteria from oral cavity and in the case of reflux disease, also from the stomach and possibly also from the duodenum. TLRs act by recognizing bacteria-derived molecular patterns which results in a pro-inflammatory reaction in the epithelium.

The role of TLRs in esophageal cancer has been studied sparsely. However, there is evidence that the function of TLRs is pro-carcinogenic and pro-inflammatory as the overexpression of many of the TLRs have been linked with esophageal cancer and with poor prognosis. Inflammation is a known important factor in the pathogenesis of various cancers. It was demonstrated by Yang et al. that the microbiome of distal esophagus frequently undergoes changes during esophagitis and BE. During these processes, the microbiome is switched from aerobic to gram-negative anaerobic bacteria (33, 34). This finding together with abnormal TLR expression, particularly those of TLRs4, 5, and 9, in esophageal cancer supports the hypothesis of bacteria contributing to the carcinogenesis of esophageal cancer. These findings further suggest that TLRs may be important mediators for bacteria in oncogenesis (37, 40, 41).

In addition to microbes, TLRs can also detect molecular patterns that are derived from the host itself. TLRs3, 4, and 9 are known to be activated by endogenous ligands from dead or damaged host cells (42, 43). The combination of cellular damage by alcohol, tobacco, and acidic contents of the stomach results in the loss of epithelial wall integrity, through epithelial cell death and by disruption of the cell-to-cell contacts. Especially TLR3 and TLR9 (but also other TLRs) can recognize particles from dead cells (43). This can result in an inflammatory wound reaction through the activation of interleukins, NF- κ B, and matrix metalloproteinases. This wound reaction could facilitate the passage of bacteria through epithelium and result in the loss of host-microbiome homeostasis, further leading to abnormal activation of for example TLR2, 4, 5, and 9 by bacterial components. Inflammation and wound reaction then could produce a vicious cycle of cellular damage, which might be a major player in esophageal metaplasia and carcinogenesis. This role of bacteria and TLR4 in genesis of BE has been discussed earlier by Yang et al. (33). Cell-to-cell junctions become dysfunctional in exogenous damage to the epithelium as discussed earlier. Thus, a similar effect can also

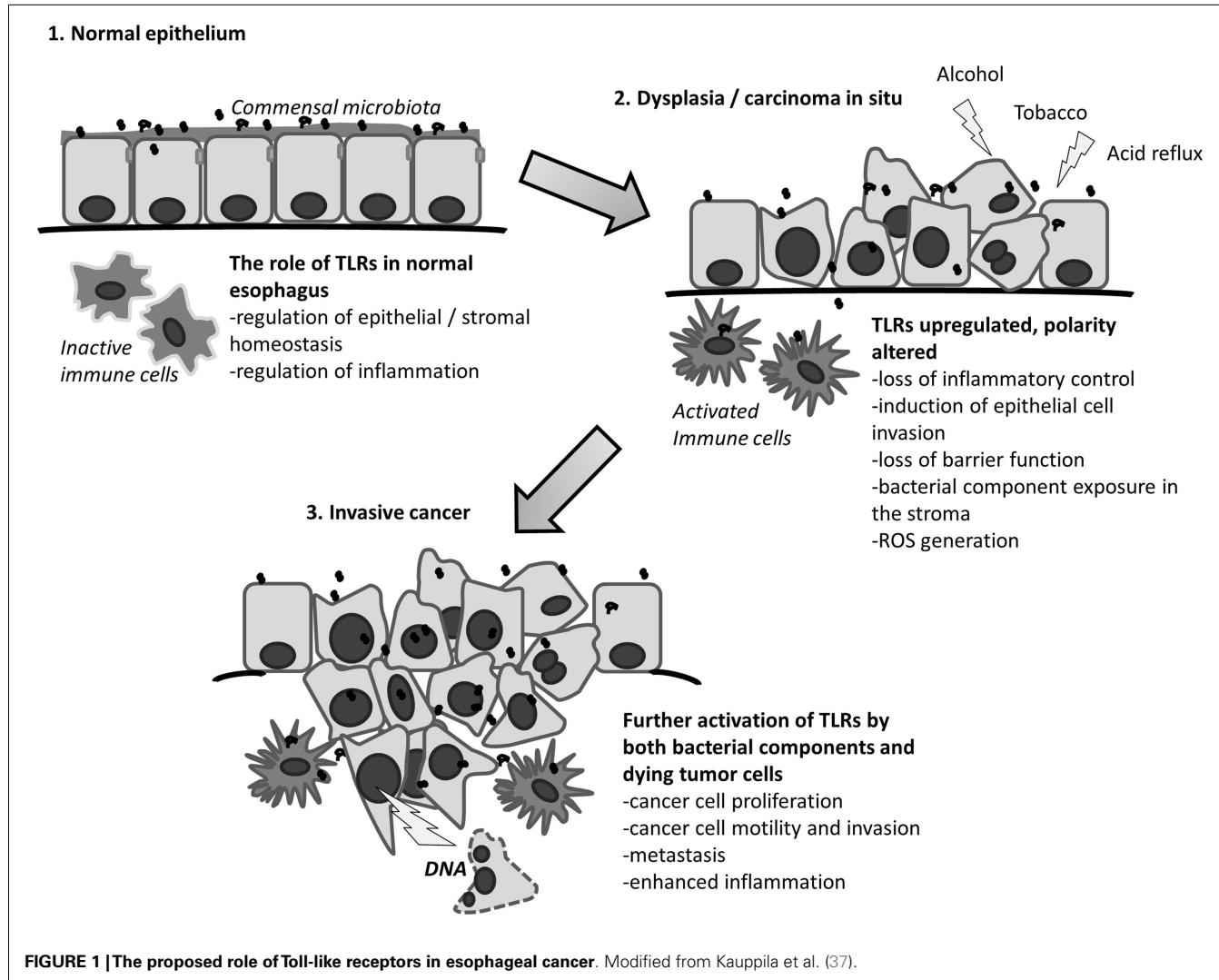


FIGURE 1 |The proposed role of Toll-like receptors in esophageal cancer. Modified from Kauppila et al. (37).

be observed in dysplasia and cancer (44). This dysfunction may lead to Toll-like receptor activation in cancer by exogenous and endogenous ligands. The hypothesis is summarized in Figure 1.

Finally, Toll-like receptor expression is up-regulated in both squamous cell carcinoma and adenocarcinoma of the esophagus. This may indicate that cancer cells are sensitized to bacteria- and host-derived ligands. Poor prognosis in strongly TLR-expressing tumors could then be an indicator of increased level of tumor-stroma interaction.

CONCLUSION

There seems to be a connection between TLRs and esophageal cancer development. The fact that bacterial flora changes during esophageal metaplasia and inflammation, as well as observed up-regulation of TLRs in esophageal cancers support the hypothesis that bacteria as well as TLRs have a role in esophageal cancer.

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REFERENCES

1. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* (2003) 21:335–76. doi:10.1146/annurev.immunol.21.120601.141126
2. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* (2004) 4(7):499–511. doi:10.1038/nri1391
3. Li M, Zhou Y, Feng G, Su SB. The critical role of Toll-like receptor signaling pathways in the induction and progression of autoimmune diseases. *Curr Mol Med* (2009) 9(3):365–74. doi:10.2174/1566524097847137
4. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* (2005) 55(2):74–108. doi:10.3322/canjclin.55.2.74
5. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* (2010) 127(12):2893–917. doi:10.1002/ijc.25516
6. Pickens A, Orringer MB. Geographical distribution and racial disparity in esophageal cancer. *Ann Thorac Surg* (2003) 76(4):S1367–9. doi:10.1016/S0003-4975(03)01202-5
7. Garavello W, Negri E, Talamini R, Levi F, Zambon P, Dal Maso L, et al. Family history of cancer, its combination with smoking and drinking, and risk of squamous cell carcinoma of the esophagus. *Cancer Epidemiol Biomarkers Prev* (2005) 14(6):1390–3. doi:10.1158/1055-9965.EPI-04-0911
8. Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *J Clin Oncol* (2006) 24(14):2137–50. doi:10.1200/JCO.2005.05.2308

9. Rautemaa R, Hietanen J, Niissalo S, Pirinen S, Perheentupa J. Oral and oesophageal squamous cell carcinoma – a complication or component of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, APS-I). *Oral Oncol* (2007) **43**(6):607–13. doi:10.1016/j.oraloncology.2006.07.005
10. Kamangar F, Chow WH, Abnet CC, Dawsey SM. Environmental causes of esophageal cancer. *Gastroenterol Clin North Am* (2009) **38**(1):27–57, vii. doi:10.1016/j.gtc.2009.01.004
11. Fitzgerald RC. Molecular basis of Barrett's oesophagus and oesophageal adenocarcinoma. *Gut* (2006) **55**(12):1810–20. doi:10.1136/gut.2005.089144
12. Sikkema M, de Jonge PJ, Steyerberg EW, Kuipers EJ. Risk of esophageal adenocarcinoma and mortality in patients with Barrett's esophagus: a systematic review and meta-analysis. *Clin Gastroenterol Hepatol* (2010) **8**(3):235–44;quiz32. doi:10.1016/j.cgh.2009.10.010
13. Hvid-Jensen F, Pedersen L, Drewes AM, Sorensen HT, Funch-Jensen P. Incidence of adenocarcinoma among patients with Barrett's esophagus. *N Engl J Med* (2011) **365**(15):1375–83. doi:10.1056/NEJMoa1103042
14. Jansson C, Johansson AL, Nyren O, Lagergren J. Socioeconomic factors and risk of esophageal adenocarcinoma: a nationwide Swedish case-control study. *Cancer Epidemiol Biomarkers Prev* (2005) **14**(7):1754–61. doi:10.1158/1055-9965.EPI-05-0140
15. Corley DA, Kubo A, Levin TR, Block G, Habel L, Zhao W, et al. Abdominal obesity and body mass index as risk factors for Barrett's esophagus. *Gastroenterology* (2007) **133**(1):34–41; quiz 311. doi:10.1053/j.gastro.2007.04.046
16. Holmes RS, Vaughan TL. Epidemiology and pathogenesis of esophageal cancer. *Semin Radiat Oncol* (2007) **17**(1):2–9. doi:10.1016/j.semradonc.2006.09.003
17. Abnet CC, Freedman ND, Hollenbeck AR, Fraumeni JF Jr, Leitzmann M, Schatzkin A. A prospective study of BMI and risk of oesophageal and gastric adenocarcinoma. *Eur J Cancer* (2008) **44**(3):465–71. doi:10.1016/j.ejca.2007.12.009
18. Corley DA, Kubo A, Zhao W. Abdominal obesity and the risk of esophageal and gastric cardia carcinomas. *Cancer Epidemiol Biomarkers Prev* (2008) **17**(2):352–8. doi:10.1158/1055-9965.EPI-07-0748
19. Koppert LB, Wijnhoven BP, van Dekken H, Tilanus HW, Dinjens WN. The molecular biology of esophageal adenocarcinoma. *J Surg Oncol* (2005) **92**(3):169–90. doi:10.1002/jso.20359
20. Cai YC, So CK, Nie AY, Song Y, Yang GY, Wang LD, et al. Characterization of genetic alteration patterns in human esophageal squamous cell carcinoma using selected microsatellite markers spanning multiple loci. *Int J Oncol* (2007) **30**(5):1059–67. doi:10.3892/ijo.30.5.1059
21. Hulscher JB, Tijssen JG, Obertop H, van Lanschot JJ. Transthoracic versus transhiatal resection for carcinoma of the esophagus: a meta-analysis. *Ann Thorac Surg* (2001) **72**(1):306–13. doi:10.1016/S0003-4975(00)02570-4
22. Shahbaz Sarwar CM, Luketich JD, Landreneau RJ, Abbas G. Esophageal cancer: an update. *Int J Surg* (2010) **8**(6):417–22. doi:10.1016/j.ijsu.2010.06.011
23. Sabin LH, Gospodarowicz MK, Wittekind C, editors. International union against cancer. *TNM Classification of Malignant Tumours*. 7th ed. Chichester: Wiley-Blackwell (2010). 309 p.
24. Liu J, Xie X, Zhou C, Peng S, Rao D, Fu J. Which factors are associated with actual 5-year survival of oesophageal squamous cell carcinoma? *Eur J Cardiothorac Surg* (2012) **41**(3):e7–11. doi:10.1093/ejcts/ezr240
25. Uehara A, Fujimoto Y, Fukase K, Takada H. Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines. *Mol Immunol* (2007) **44**(12):3100–11. doi:10.1016/j.molimm.2007.02.007
26. Lim DM, Narasimhan S, Michaylira CZ, Wang ML. TLR3-mediated NF- κ B signaling in human esophageal epithelial cells. *Am J Physiol Gastrointest Liver Physiol* (2009) **297**(6):G1172–80. doi:10.1152/ajpgi.00065.2009
27. Lim DM, Wang ML. Toll-like receptor 3 signaling enables human esophageal epithelial cells to sense endogenous danger signals released by necrotic cells. *Am J Physiol Gastrointest Liver Physiol* (2011) **301**(1):G91–9. doi:10.1152/ajpgi.00471.2010
28. Mulder DJ, Lobo D, Mak N, Justinich CJ. Expression of toll-like receptors 2 and 3 on esophageal epithelial cell lines and on eosinophils during esophagitis. *Dig Dis Sci* (2012) **57**(3):630–42. doi:10.1007/s10620-011-1907-4
29. Helminen O, Huhta H, Takala H, Lehenkari PP, Saarnio J, Kauppila JH, et al. Increased Toll-like receptor 5 expression indicates esophageal columnar dysplasia. *Virchows Arch* (2014) **464**(1):11–8. doi:10.1007/s00428-013-1505-2
30. Sheyhidin I, Nabi G, Hasim A, Zhang RP, Ainiwaer J, Ma H, et al. Overexpression of TLR3, TLR4, TLR7 and TLR9 in esophageal squamous cell carcinoma. *World J Gastroenterol* (2011) **17**(32):3745–51. doi:10.3748/wjg.v17.i32.3745
31. Takala H, Kauppila JH, Soini Y, Selander KS, Vuopala KS, Lehenkari PP, et al. Toll-like receptor 9 is a novel biomarker for esophageal squamous cell dysplasia and squamous cell carcinoma progression. *J Innate Immun* (2011) **3**(6):631–8. doi:10.1159/000329115
32. Rousseau MC, Hsu RY, Spicer JD, McDonald B, Chan CH, Perera RM, et al. Lipopolysaccharide-induced toll-like receptor 4 signaling enhances the migratory ability of human esophageal cancer cells in a selectin-dependent manner. *Surgery* (2013) **154**(1):69–77. doi:10.1016/j.surg.2013.03.006
33. Yang L, Francois F, Pei Z. Molecular pathways: pathogenesis and clinical implications of microbiome alteration in esophagitis and Barrett esophagus. *Clin Cancer Res* (2012) **18**(8):2138–44. doi:10.1158/1078-0432.CCR-11-0934
34. Yang L, Lu X, Nossa CW, Francois F, Peek RM, Pei Z. Inflammation and intestinal metaplasia of the distal esophagus are associated with alterations in the microbiome. *Gastroenterology* (2009) **137**(2):588–97. doi:10.1053/j.gastro.2009.04.046
35. Verbeek RE, Siersema PD, Ten Kate FJ, Fluiter K, Souza RF, Vleggaar FP, et al. Toll-like receptor 4 activation in Barrett's esophagus results in a strong increase in COX-2 expression. *J Gastroenterol* (2013). doi:10.1007/s00535-013-0862-6
36. Kauppila JH, Takala H, Selander KS, Lehenkari PP, Saarnio J, Karttunen TJ. Increased Toll-like receptor 9 expression indicates adverse prognosis in oesophageal adenocarcinoma. *Histopathology* (2011) **59**(4):643–9. doi:10.1111/j.1365-2559.2011.03991.x
37. Kauppila JH, Karttunen TJ, Saarnio J, Nyberg P, Salo T, Graves DE, et al. Short DNA sequences and bacterial DNA induce esophageal, gastric, and colorectal cancer cell invasion. *APMIS* (2013) **121**(6):511–22. doi:10.1111/apm.12016
38. Hold GL, Rabkin CS, Gammon MD, Berry SH, Smith MG, Lissowska J, et al. CD14-159C/T and TLR9-1237T/C polymorphisms are not associated with gastric cancer risk in Caucasian populations. *Eur J Cancer Prev* (2009) **18**(2):117–9. doi:10.1097/CEJ.0b013e3283101292
39. Hold GL, Rabkin CS, Chow WH, Smith MG, Gammon MD, Risch HA, et al. A functional polymorphism of toll-like receptor 4 gene increases risk of gastric carcinoma and its precursors. *Gastroenterology* (2007) **132**(3):905–12. doi:10.1053/j.gastro.2006.12.026
40. Chattopadhyay I, Phukan R, Singh A, Vasudevan M, Purkayastha J, Hewitt S, et al. Molecular profiling to identify molecular mechanism in esophageal cancer with familial clustering. *Oncol Rep* (2009) **21**(5):1135–46. doi:10.3892/or_00000333
41. Kauppila JH, Mattila AE, Karttunen TJ, Salo T. Toll-like receptor 5 and the emerging role of bacteria in carcinogenesis. *Oncoimmunology* (2013) **2**(4):e23620. doi:10.4161/onci.23620
42. Yu L, Wang L, Chen S. Endogenous toll-like receptor ligands and their biological significance. *J Cell Mol Med* (2010) **14**(11):2592–603. doi:10.1111/j.1582-4934.2010.01127.x
43. Tuomela J, Sandholm J, Kaakinen M, Patel A, Kauppila JH, Ilvesaro J, et al. DNA from dead cancer cells induces TLR9-mediated invasion and inflammation in living cancer cells. *Breast Cancer Res Treat* (2013) **142**(3):477–87. doi:10.1007/s10549-013-2762-0
44. Ding L, Lu Z, Lu Q, Chen YH. The claudin family of proteins in human malignancy: a clinical perspective. *Cancer Manag Res* (2013) **5**:367–75. doi:10.2147/CMAR.S38294

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Pattern-recognition receptors and gastric cancer

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Chronic inflammation has been associated with an increased risk of several human malignancies, a classic example being gastric adenocarcinoma (GC). Development of GC is known to result from infection of the gastric mucosa by *Helicobacter pylori*, which initially induces acute inflammation and, in a subset of patients, progresses over time to chronic inflammation, gastric atrophy, intestinal metaplasia, dysplasia, and finally intestinal-type GC. Germ-line encoded receptors known as pattern-recognition receptors (PRRs) are critical for generating mature pro-inflammatory cytokines that are crucial for both Th1 and Th2 responses. Given that *H. pylori* is initially targeted by PRRs, it is conceivable that dysfunction within genes of this arm of the immune system could modulate the host response against *H. pylori* infection, and subsequently influence the emergence of GC. Current evidence suggests that Toll-like receptors (TLRs) (TLR2, TLR3, TLR4, TLR5, and TLR9), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (NOD1, NOD2, and NLRP3), a C-type lectin receptor (DC-SIGN), and retinoic acid-inducible gene (RIG)-I-like receptors (RIG-I and MDA-5), are involved in both the recognition of *H. pylori* and gastric carcinogenesis. In addition, polymorphisms in genes involved in the TLR (TLR1, TLR2, TLR4, TLR5, TLR9, and CD14) and NLR (NOD1, NOD2, NLRP3, NLRP12, NLRX1, CASP1, ASC, and CARD8) signaling pathways have been shown to modulate the risk of *H. pylori* infection, gastric precancerous lesions, and/or GC. Further, the modulation of PRRs has been suggested to suppress *H. pylori*-induced inflammation and enhance GC cell apoptosis, highlighting their potential relevance in GC therapeutics. In this review, we present current advances in our understanding of the role of the TLR and NLR signaling pathways in the pathogenesis of GC, address the involvement of other recently identified PRRs in GC, and discuss the potential implications of PRRs in GC immunotherapy.

Keywords: stomach neoplasm, *Helicobacter pylori*, inflammation, pattern-recognition receptors, Toll-like receptors, NOD-like receptors, genetic polymorphism, therapeutics

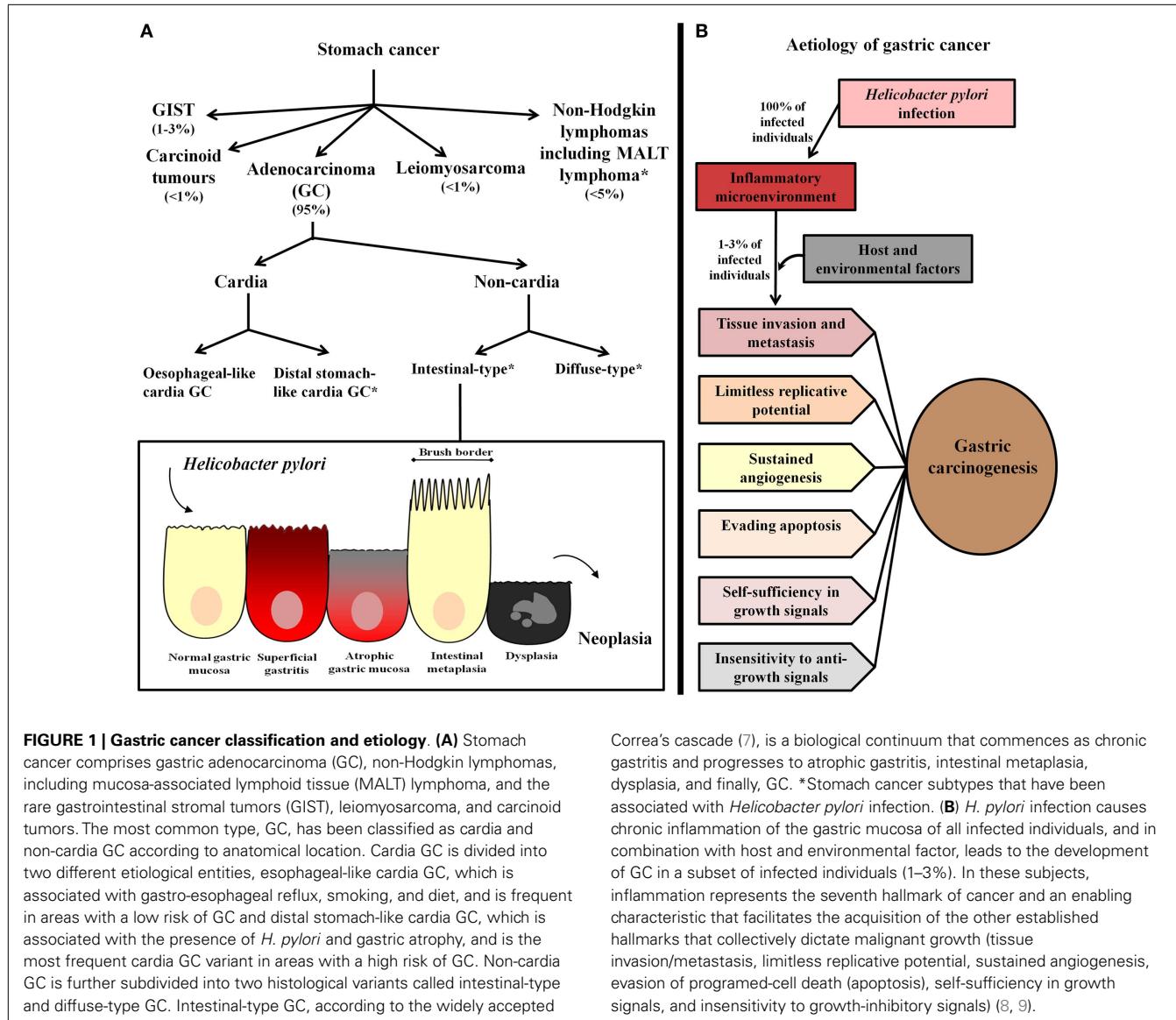
INTRODUCTION

Of the three main types of stomach cancer, gastric adenocarcinoma (GC), non-Hodgkin's lymphoma, and gastrointestinal stromal tumors, approximately 95% are GC, which remains one of the most commonly diagnosed cancers in the world (1). In 2012, stomach cancer was the fifth most common cancer worldwide, with 952,000 new cases diagnosed, accounting for 6.8% of the total cancer cases (1). Furthermore, it is the third leading cause of cancer-related deaths worldwide, accounting for 8.8% of total deaths from cancer, with 5-year relative survival rates lower than 30%, except in Japan where mass screening has been undertaken for several years (2).

Gastric cancer is a heterogeneous pathology with respect to anatomical location and histological subtypes (Figure 1A). In relation to location, GC may occur in the cardia or non-cardia region of the stomach. Cardia GC has been associated with gastro-esophageal reflux, *Helicobacter pylori* infection, and atrophic gastritis, male gender, smoking, and diet (3). Epidemiological studies assessing the worldwide incidence of GC by anatomical location have shown an increase in the incidence of cardia GC, however, in high GC risk areas, non-cardia GC remains the most frequent pathology (4). Further, even though

cardia and non-cardia GC have been considered etiologically different phenomena, it has been demonstrated that cancer of the cardia among individuals from areas with a high risk of GC represents a subset of cardia GC that is associated with *H. pylori*-related atrophic gastritis and resembles non-cardia GC pathogenesis (5, 6).

According to the Lauren Classification, non-cardia GC has been further subdivided into the two histological variants intestinal-type and diffuse-type. Intestinal-type GC is characterized by the formation of gland-like structures, distal stomach localization, and a predilection for older individuals. It is also more frequent in males (2:1 ratio) and in subjects of lower socioeconomic status (10). This type of GC is often preceded by a precancerous phase that starts with the transition of normal mucosa into multifocal atrophic gastritis. This initial histological alteration is followed by intestinal metaplasia, dysplasia, and finally adenocarcinoma (11). On the other hand, diffuse-type GC is poorly differentiated, affects younger individuals, and has been highly associated with genetic susceptibility (the variant hereditary diffuse GC, which is associated with germ-line mutations in *CDH1*, a gene encoding E-cadherin) (12, 13). Additionally, it is not associated with the formation of precancerous lesions and has been found to affect the



entire surface of the stomach. This type of GC is present equally between the two sexes and is associated with a worse prognosis in comparison to intestinal-type GC (10, 12).

Most GC cases are sporadic and arise due to the combination of a permissive environment interacting with a susceptible host. Several factors that contribute to the development of GC have been identified; these include bacterial (*H. pylori*), host, and environmental factors (12).

Helicobacter pylori is a Gram-negative bacterium that infects nearly 50% of the human population (14). In the gastric mucosa, the majority of *Helicobacter pylori* are found within the mucus layer but they can also be attached to epithelial cells leading to the maintenance, spread, and severity of the infection (15). *H. pylori* infection has been associated with the development of a range of diseases, including peptic ulcer disease (10%), non-cardia GC (1–3%), and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (<0.1%) (14, 16–18). Furthermore, this bacterium has

been associated with three distinct phenotypes in the infected host: (1) a corpus-predominant gastritis, which has the potential to lead to atrophic gastritis, hypochlorhydria, and to the development of GC; (2) a duodenal ulcer phenotype in which an antrum-predominant gastritis leads to increased gastric acid secretion; and (3) a benign phenotype in which the bacterial infection causes a mild mixed gastritis that has a minor effect on gastric acid production (19).

Helicobacter pylori infection is transmitted by direct human-to-human transmission, via either the oral–oral route, fecal–oral route, or both (14). *H. pylori* is acquired early in life, the majority of individuals being infected before the age of 10 years with close family members being a common source of infection (20–22). It has been postulated that early acquisition of infection might be associated with the broad pathological spectrum associated with *H. pylori* infection and the highly persistent GC incidence rates in genetically susceptible populations who have migrated to

developed countries. In the absence of antibiotic therapy, *H. pylori* infection generally persists for life (23).

Natural colonization by *H. pylori* is restricted to humans, primates, and domestic animals such as cats (23–25). *H. pylori* is considered to be the dominant microorganism in the human stomach as the majority of bacteria cannot survive in the low gastric pH (26). Several other factors make the human stomach an unfavorable environment for bacterial colonization including peristalsis, poor nutrient availability, and host innate and adaptive immunity (23). The ability of *H. pylori* to survive and colonize the stomach relates to a number of mechanisms. Most importantly *H. pylori*, unlike other bacteria, produces large amounts of the enzyme urease, which hydrolyzes urea to ammonia, which subsequently interacts with hydrogen ions in the stomach to form ammonium (27, 28). In addition, *H. pylori* is able to regulate gene expression in response to changes in pH (29). Further, *H. pylori* expresses multiple paralogous outer membrane proteins, including the blood-group antigen-binding adhesin (BabA), the sialic-acid binding adhesin (SabA), and the outer inflammatory protein (OipA), which appear to bind to receptors on the surface of gastric epithelial cells, which reduces the rate of bacterial elimination as a result of peristalsis (30, 31). *H. pylori* counteracts the lack of nutrients by inducing tissue inflammation and using specific systems that facilitate the transport and uptake of nutritional resources (23). In addition, *H. pylori* has been reported to produce antibacterial peptides that might decrease competition from other microorganisms (32).

Further, a number of other factors have been shown to help *H. pylori* evade the host immune system. For example, the vacuolating cytotoxin (VacA) produced by some strains of *H. pylori* has been shown to inhibit T-cell proliferation as well as antigen presentation by B cells and to alter the normal functions of CD8⁺ T cells, mast cells, and macrophages (33–36). In addition, gamma-glutamyl transpeptidase, another immunosuppressive factor of *H. pylori*, has been associated with inhibition of T-cell proliferation by induction of a cell cycle arrest in the G₁ phase (37). Furthermore, *H. pylori* has been shown to use arginase to down-regulate the production of inducible nitric oxide synthase by macrophages (38).

The fact that more than one *H. pylori* strain can colonize the gastric mucosa provides the opportunity for *H. pylori* to acquire new genetic sequences and to undergo recombination events (23). One of the most remarkable differences among *H. pylori* strains is the presence or absence of a 40-kb DNA insertion element known as the cytotoxin-associated gene pathogenicity island (*cag* PAI) (39). This region contains between 27 and 31 genes flanked by 31-bp repeats and encodes the most widely investigated *H. pylori* virulence factor, the cytotoxin-associated antigen A (CagA) (40, 41). *H. pylori* strains expressing CagA represent 60–70% of Western strains and approximately 100% of East Asian strains (39, 42). CagA is a 120- to 140-kDa protein that is translocated into host cells through a type IV secretion system following attachment to gastric epithelial cells (43). Following translocation, CagA is tyrosine phosphorylated at the EPIYA (glutamate–proline–isoleucine–tyrosine–alanine) motifs by members of the host cell kinase families known as proto-oncogene proteins Abl and Src

(18). In Western populations strains, EPIYA-A, EPIYA-B, and varying numbers of EPIYA-C motifs have been reported, while in *H. pylori* strains from East Asian populations, EPIYA-A and EPIYA-B with EPIYA-D motifs, are found (44). Both phosphorylated and non-phosphorylated CagA result in alterations in the gastric epithelium including: (1) the activation of the protein tyrosine phosphatase, non-receptor type 11 (SHP-2), (2) alterations in cell scattering and proliferation, (3) alterations in cell structure and cell motility, (4) perturbation of epithelial cell differentiation and polarity, (5) alteration of tight junctions, and (6) aberrant activation of β-catenin (45–47). Furthermore, numerous studies have shown that *cag* PAI-positive *H. pylori* strains are associated with an increased risk of gastric diseases including peptic ulcer disease, premalignant gastric lesions and GC (48–51). Further details of the interplay between *H. pylori* virulence factors and gastric epithelial cells and GC, can be found in an excellent review by Posselt et al. (44).

In the last two decades, a large number of epidemiological studies have established the association between *H. pylori* and the subsequent risk of developing both intestinal-type and diffuse-type GC (52–57). This finding has been consistent among different populations. For example, in the study by Parsonnet et al. (57), conducted in Caucasian, African-American, and Asian individuals, subjects infected with *H. pylori* who had antibodies against CagA were shown to be more likely than uninfected subjects to develop both intestinal-type and diffuse-type GC (OR: 5.1, 95% CI: 2.1–12.2 and OR: 10.1, 95% CI: 2.2–47.4, respectively). Consistently, a further study conducted in a Japanese population showed that, although the association was stronger in cases with intestinal-type GC (OR: 3.2, 95% CI: 1.8–5.8), there was also a positive association between *H. pylori* infection and diffuse-type GC (OR: 3.0, 95% CI: 1.0–8.8) (53). Further, a study conducted in a Spanish population showed no differences in *H. pylori* infection between the two GC histological subtypes (58). Similarly, a recent study in German individuals showed that *H. pylori* prevalence was comparable in patients with intestinal-type (82.1%) and diffuse-type (77.9%) GC (59).

Interestingly, more recent studies, assessing *H. pylori* infection through Western blot (CagA) for the detection of past infection, have shown an unprecedented association between *H. pylori* and GC that can be explained by a reduction of the misclassification that might take place when samples are analyzed with the enzyme-linked immunosorbent assay (ELISA) alone (60, 61). For example, Ekstrom et al. (60) conducted a population-based study, comprising 298 GC patients and 244 controls, in which the OR for *H. pylori* infection among non-cardia GC was 21.0 (95% CI: 8.3–53.4). Further, Siman et al. (61) showed that *H. pylori* significantly increased the risk of non-cardia GC showing an OR of 17.8 (95% CI: 4.2–74.8).

While *H. pylori* infection has been established as the most important risk factor for GC and was classified as a class 1 carcinogen by the World Health Organization in 1994, the etiology of GC also involves host and environmental factors. This is evidenced by the fact that only 1–3% of *H. pylori*-infected patients develop GC, and that progression to GC in some subjects occurs even after eradication of the bacterium (18).

Given that *H. pylori* is initially targeted by germ-line encoded receptors known as pattern-recognition receptors (PRRs), it is conceivable that dysfunction within genes of this arm of the immune system would affect the magnitude and direction of the host inflammatory response against the infection, resulting in an increased risk of GC development. Recent studies clearly show that PRRs are critical for generating mature pro-inflammatory cytokines that are crucial for both Th1 and Th2 responses during *H. pylori* infection, and these immune responses have been directly associated with gastric immunopathology. In this review, we present current advances in the understanding of the role of PRRs, mainly the Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) signaling pathways, in the pathogenesis of GC, and discuss future directions for continued research in this area. In the first section, we highlight the relevance of inflammation in GC. In subsequent sections, we address new developments in the TLR and NLR signaling pathways in GC, the role of other PRRs in GC, and the new frontier of therapeutic application of these concepts.

INFLAMMATION IN GASTRIC CANCER

It is well established that most cancer cell genotypes are the manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: (1) self-sufficiency in growth signals, (2) insensitivity to growth-inhibitory signals, (3) evasion of programmed-cell death (apoptosis), (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion/metastasis (8). Recently, inflammation has been considered the seventh hallmark of cancer and an enabling characteristic that facilitates the acquisition of the other hallmarks (**Figure 1B**). Inflammation initiated by innate immune cells, mainly macrophage subtypes, mast cells, myeloid progenitors, and neutrophils (62–65), designed to fight infections and heal wounds, can instead result in unintentional support of multiple cancer hallmark functions, thereby manifesting the widely accepted tumor-promoting consequences of inflammatory responses (9). In addition, active evasion by cancer cells from attack and elimination by immune cells, mainly CD8+ cytotoxic T lymphocytes, CD4+ Type 1 helper T cells, and natural killer (NK) cells, highlights the dual role of an immune system that both antagonizes and promotes cancer development and progression (9).

In the context of tumor enhancement, it has been proposed that once inflammation is initiated, tissue integrity is compromised leading to the multistage process of carcinogenesis by altering targets and pathways that are pivotal for normal tissue homeostasis (66). The mechanisms that are connected to these alterations include production of mutagenic reactive oxygen and nitrogen species as well as synthesis of cytokines and growth factors that favor tumor cell growth (67). In addition, inflammation provides a source of other bioactive molecules to the tumor microenvironment, including survival factors that limit cell death, pro-angiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and inductive signals that lead to activation of the epithelial–mesenchymal transition (a developmental regulatory program that enables epithelial cells to invade, resist apoptosis, and disseminate) (9). Interestingly, inflammation can be considered a “perigenetic alteration” of

cancer cells because it may promote growth, expansion, and invasion of tumors even without the involvement of further genetic mutations or epigenetic alterations (68).

In 1988, Correa proposed a human model of intestinal-type gastric carcinogenesis (7). The model hypothesized a sequence of events progressing from acute inflammation to chronic inflammation, to atrophy, to intestinal metaplasia, to dysplasia, to carcinoma *in situ*, and finally to invasive GC. A subsequent study by Correa evaluated the gastric precancerous process in a Colombian population (7). The results of this cross-sectional study led to the widely accepted conclusion that the severity of atrophy correlates with the prevalence of metaplasia and that the severity of metaplasia correlates with the prevalence of dysplasia, suggesting that the process is indeed a biological continuum (69).

Given that inflammation is a hallmark of gastric carcinogenesis, polymorphisms in genes encoding pro-inflammatory cytokines/chemokines have been the focus of much research in recent years. To date, polymorphisms in the interleukin (IL)-1 family genes have been the most widely studied, including polymorphisms in *IL1A*, *IL1B*, and *IL1RN* that encode IL-1 α , IL-1 β , and their endogenous receptor antagonist IL-1RA, respectively. In particular, IL-1 β , a potent endogenous pyrogen and an important component in the development of Th2-mediated immunity (70, 71), has been associated with lipid peroxidation, DNA damage, inhibition of gastric acid secretion, increased *H. pylori* colonization, and induction of gastric atrophy and dysplasia in the presence or absence of *H. pylori* (72). Global meta-analyses have shown that the *IL1B*-511 T allele is significantly associated with an increased risk of developing GC in Caucasians but not Asians or Mestizos (73, 74). Furthermore, IL-1 receptor signaling is known to induce the production of genes that not only stimulate tumor growth but are also involved in angiogenesis and metastasis such as matrix metalloproteinases, basic fibroblast growth factor, vascular endothelial growth factor, vascular cell adhesion molecule 1, intercellular adhesion molecule 1, monocytic chemotactic protein 1, and CXCL-2 (75). To date, only one study has addressed the role of *IL1R1* (also known as *CD121A*) in GC and *H. pylori* infection. The study, conducted in a Caucasian population, showed an increased risk of *H. pylori* infection in those harboring the *IL1R1* Hinfl A allele (OR: 2.01, *P*-value: 0.009) but failed to show an association with GC (76). In addition, a recent meta-analysis on the endogenous receptor antagonist IL-1RA has shown the *IL1RN**22 genotype to increase the risk of gastric precancerous lesions, supporting a role for this polymorphism in the early stages of gastric carcinogenesis (OR: 2.27, 95% CI: 1.40–3.70) (77). A further meta-analysis that included 39 case–control studies, showed statistically significant associations between the *IL1RN**22 genotype and both intestinal-type and diffuse-type GC, showing ORs of 1.83 and 1.72, respectively (78). Further examples of polymorphisms in pro-inflammatory cytokines/chemokines that play an essential role promoting inflammation in the context of gastrointestinal carcinogenesis are IL-4 (*IL4*-590C/T and -168T/C) (79), IL-6 (*IL6*-174 G/C) (80–82), IL-8 (*IL8*-251 A/T, +396 T/G, and +781 C/T) (79, 83), IL-10 (*IL10*-1082 A/G, -819 C/T, and -592 C/A) (84–86), IL-12 (*IL12A*-701 C/A, -798 T/A, +277 G/A, and -504 T/G) (87), IL-17 (*IL17*-197 G/A and +7488 T/C) (79), IL-18 (*IL18*-137 G/C) (88), and TNF- α (*TNFA* -238 G/A, -308 G/A,

and -857 C/T (89). In addition to this, a recent comprehensive review on this topic recommended the investigation of other polymorphisms in *IL1B* (3954 C/T and -1473 G/C), *IL4* (-168T/C), *IL6* (572 G/C and 597 G/A), and *IL17* ($+7488\text{A/G}$ and -197G/A), given their potential relevance in GC (79).

While extensive evidence supports the important role of pro-inflammatory cytokines/chemokines in gastric carcinogenesis, given that PRRs, mainly TLRs and NLRs, are important modulators of intestinal epithelial barrier function, epithelial repair, and immune homeostasis in the gastrointestinal tract (90), and that signal transduction from these receptors converges upon a common set of signaling molecules, including the activation of the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the activator protein 1 (AP-1) that lead to the production of pro-inflammatory cytokines/chemokines (e.g., IL-1 α , IL-1 β , IL-6, IL-8, IL-10, and TNF- α) as well as members of the interferon (IFN) regulatory transcription factor family that mediate type I IFN-dependent responses, defects in PRRs function could be even more important than defects in pro-inflammatory cytokines/chemokines *per se* in the instauration of an inflammation-related disorder such as GC.

PATTERN-RECOGNITION RECEPTORS IN GASTRIC CANCER

Innate immunity refers to responses that do not require previous exposure to an immune stimulus and represents the first line of host defense in the response to pathogens. PRRs are part of the innate immune system and are pivotal for the detection of invariant microbial motifs. PRRs have been divided into five distinct genetic and functional clades: TLRs, NLRs, C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and absent in melanoma 2 (AIM2)-like receptors (ALRs) (91, 92). PRRs are commonly expressed by cells of the innate immune system such as monocytes, macrophages, dendritic cells (DCs), neutrophils, and epithelial cells, as well as cells of the adaptive immune system (93).

Toll-like receptors and CLRs scan the extracellular milieu and endosomal compartments for pathogen-associated molecular patterns (PAMPs), which are highly conserved microbial structures that are essential for microbial survival (94), while intracellular PRRs, including NLRs, RLRs, and ALRs, cooperate to provide cytosolic surveillance (92, 93).

In *H. pylori* infection, the first physical–chemical barriers for the pathogen are the mucus layer, gastric epithelial cells, autophagy, and PRRs (TLRs, NLRs, CLRs, and RLRs) (Figure 2).

TOLL-LIKE RECEPTORS AND *HELICOBACTER PYLORI*-RELATED GASTRIC CANCER

TOLL-LIKE RECEPTORS RECOGNITION OF *HELICOBACTER PYLORI*

The involvement of the TLR signaling pathway in infectious, autoimmune, and inflammatory diseases is well accepted (95). During *H. pylori* infection, TLRs on gastric epithelial and immune cells recognize diverse PAMPs such as flagellin/unknown PAMP (TLR5), unmethylated CpG motifs (TLR9), and lipopolysaccharide (LPS) (TLR4 and TLR2).

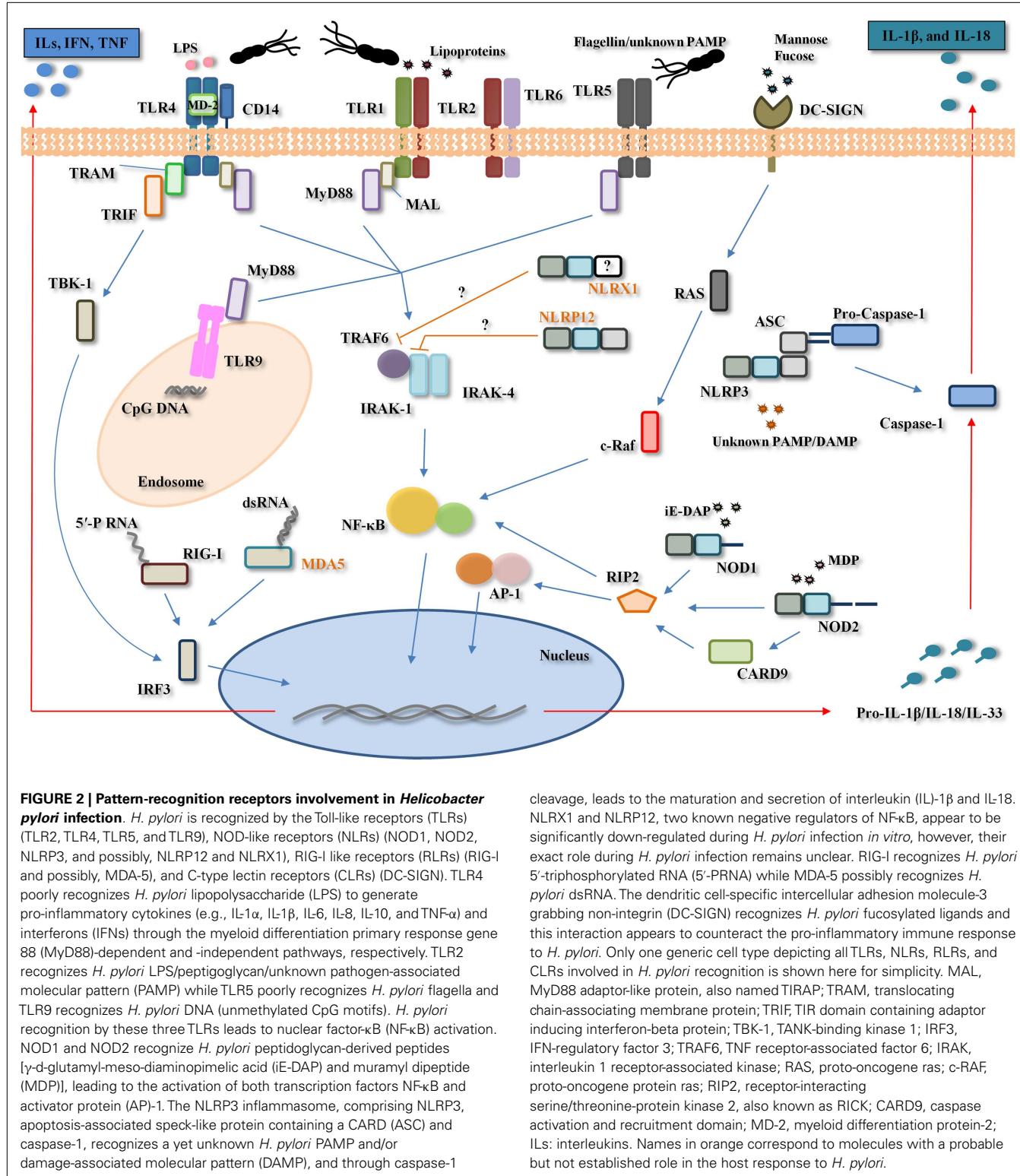
TLR4 was initially identified as the potential signaling receptor for *H. pylori* LPS on gastric epithelial cells (96–99). After forming a complex with the LPS-binding protein (LBP), LPS interacts with

the monocyte differentiation antigen CD14 (CD14), and subsequently with the myeloid differentiation protein-2 (MD-2) (100). Together with TLR4, this complex induces the TLR4-mediated MyD88-dependent signal transduction pathway, which leads to the rapid activation of transcription factors, mainly NF- κ B, and cytokines such as TNF- α , IL-1 β , IL-6, and IL-12 (95). On the other hand, stimulation of TLR4 by LPS also facilitates the activation of a Myd88-independent pathway that activates IFN-regulatory factor (IRF) 3 and involves the late phase of NF- κ B activation, both of which lead to the production of IFN- β and the expression of IFN-inducible genes (101, 102). In addition to LPS, the *H. pylori* secretory protein HP0175, through its ability to bind to TLR4, was shown to transactivate the epidermal growth factor receptor (EGFR) and stimulate the EGFR-dependent vascular endothelial growth factor production in the GC cell line AGS, which have been linked to *H. pylori*-associated gastroduodenal diseases, ulcerogenesis, and carcinogenesis (103).

Although early studies concluded that TLR4 is the first innate immune response against *H. pylori* (104, 105), later studies suggested that TLR4 had a limited role, given that *H. pylori* LPS appeared to bind poorly to LBP, resulting in it being inefficiently transferred to CD14 (106). Consequently, recent studies addressing the role of other TLRs during *H. pylori* infection, have found TLR2 to be the initial barrier against *H. pylori* infection (107–112). A potential explanation for these inter-study differences in relation to the TLRs response to *H. pylori* might be attributed to cell type (i.e., epithelial versus immune cells), origin of the cell studied (i.e., peritoneal versus bone marrow derived macrophages), and the type of inflammatory response measured (i.e., type of cytokines), and thus, currently any conclusions regarding the role of TLR4 must be treated with caution.

In contrast, there is strong evidence supporting an important role for TLR2 in *H. pylori* infection, with both animal and cell culture experiments suggesting that TLR2 ligands (LPS or other) exist in *H. pylori* and related *Helicobacter* species (112–114), and that TLR2 may be involved in the innate immune sensing of these bacteria by epithelial cells (113). Furthermore, an interesting publication by Smith et al. (115) showed that *H. pylori* LPS functions as a classic TLR2 ligand and induces a discrete pattern of chemokine expression in epithelial cells, which involves modulation of the expression of the signaling protein tribbles 3 (TRIB3), a molecule implicated in the regulation of NF- κ B.

Yet, the most likely scenario is that both TLR4 and TLR2 are involved in the early immune response against *H. pylori* as has been demonstrated by a number of investigators (116–118). For example, Obonyo et al. (116) showed that both TLR2 and TLR4 were crucial signaling receptors for *H. pylori* activation of the host immune response leading to the secretion of cytokines. Further, Yokota et al. (118) not only showed that *H. pylori* LPS was initially targeted by TLR2 as described by others, but, for the first time, showed that this TLR2 activation leads to cell proliferation and TLR4 expression via the MEK1/2-ERK1/2 pathway. The final outcome of this signaling pathway is increased proliferation of gastric epithelial cells and the instauration of a strong inflammatory reaction. Once this response is instaurated, *H. pylori* could then enhance inflammatory reactions mediated by TLR4 agonists such as other bacterial LPS, which would also contribute to gastric



inflammation and subsequent carcinogenesis (118). Further, the heat-shock protein 60, an immune-potent antigen of *H. pylori*, has been shown to activate NF- κ B and induce IL-8 production through TLR2 and TLR4 pathways in gastric epithelial cells, a

phenomenon that is likely to contribute to the development of gastric inflammation caused by *H. pylori* infection (117).

In addition, TLR9 appears to play an important role in *H. pylori* recognition. Interestingly, Rad et al. (112) identified

TLR9-mediated recognition of *H. pylori* DNA as a main *H. pylori*-induced intracellular TLR signaling pathway in DCs. Further, a study using a murine model of *H. pylori* infection has suggested that TLR9 signaling is involved in the suppression of *H. pylori*-induced gastritis in the early phase of infection via down-regulation of Th1-type cytokines modulated by IFN- α (119). In addition, a recent study has shown that the gastric epithelia of children respond to *H. pylori* infection by increasing the expression of TLR2, TLR4, TLR5, and TLR9, as well as the cytokines IL-8, IL-10, and TNF- α (120).

Although TLR5 interaction with *H. pylori* induces only weak receptor activation (121), TLR5 has been involved in the inflammatory response to *H. pylori*. An interesting publication by Smith et al. (107), using HEK293 cells transfected with specific TLR expression constructs and MKN45 cells expressing dominant negative versions of TLR2, TLR4, and TLR5, which block the activity of wild-type forms of these receptors, has demonstrated that live *H. pylori* induces NF- κ B activation and chemokine gene expression due to ligation of TLR2 and TLR5. A further study that aimed to explore the involvement of TLR2 and TLR5 in THP-1 cells and HEK293 cell lines (stably transfected with TLR2 or TLR5) during *H. pylori* infection, has indicated that *H. pylori*-induced expression of TLR2 and TLR5 can qualitatively shift *cag* PAI-dependent to *cag* PAI-independent pro-inflammatory signaling pathways with possible impact on the outcome of *H. pylori*-associated diseases (122). Given the established TLR5 evasion of α and ϵ *Proteobacteria* including *H. pylori* (123), the TLR5-mediated inflammatory responses during *H. pylori* infection described by Smith et al. (107) and Kumar Pachathundikandi et al. (122) are likely to be flagellin-independent, and therefore, a still unknown *H. pylori* factor might be responsible for this.

The importance of TLRs recognition during *H. pylori* infection and GC development is further supported by the acquired characteristics that enable *H. pylori* to survive in the human stomach and cause chronic inflammation. For example, *H. pylori* LPS is characterized by a modification of the lipid A component of LPS that makes it less pro-inflammatory (124) and has been reported to exhibit a 1000-fold reduction in bioactivity as compared to *Escherichia coli* LPS (125). Also, the flagellin of this bacterium has been shown to be poorly recognized due to modifications in the TLR5 recognition site of the N-terminal D1 domain of flagellin (123).

TOLL-LIKE RECEPTORS AND GASTRIC CARCINOGENESIS

While TLR2, TLR4, TLR5, and TLR9 appear to be important for *H. pylori* recognition, their role in the evolution of gastritis to more advanced lesions remains unclear. Interestingly, Schmausser et al. (126) showed that TLR9 was not detectable in intestinal metaplasia or dysplasia and was only focally detected in 6 out of 22 gastric carcinomas, while TLR4 and TLR5 were strongly expressed by gastric carcinomas. Consistently, a study by Pimentel-Nunes et al. (127) showed a statistically significant trend for a progressive increase of TLR2, TLR4, and TLR5 expression from normal mucosa to gastric dysplasia (mean expression in normal mucosa: 0.1, gastritis: 1.0, metaplasia: 2.2, and dysplasia: 2.8, P -value <0.01), with dysplasia presenting more than 90% positive epithelial cells showing strong expression (2.8, 95% CI: 2.7–3). In

addition, these authors showed a significant trend for decrease in TOLLIP and PPAR γ , two TLR signaling pathway inhibitors, which was associated with increasing levels of CDX-2, a marker for adenocarcinoma, from normal mucosa to carcinoma (P -value <0.05) (128). Fernandez-Garcia et al. (129) have also reported increased expression of TLR3, TLR4, and TLR9 in GC, and furthermore, these authors noted that TLR3 expression by cancer cells was significantly associated with a poor overall survival in patients with resectable tumors, which lead them to suggest that TLR3 might be an indicator of tumor aggressiveness. Similarly, Yakut et al. (130) investigating the association between serum IL-1 β , TLR4 levels, pepsinogen I and II, gastrin 17, vascular endothelial growth factor, and *H. pylori* CagA status in patients with a range of gastric precancerous lesions, concluded that serum TLR4 levels could be used as a biomarker to differentiate individuals presenting with dysplasia from those with other gastric precancerous lesions, the mean TLR4 level in patients with dysplasia (0.56 ± 0.098 ng/mL) being significantly higher than in patients with *H. pylori* positive chronic non-atrophic gastritis (0.10 ± 0.15 ng/mL), chronic atrophic gastritis (0.06 ± 0.07 ng/mL), and intestinal metaplasia (0.12 ± 0.18 ng/mL). Furthermore, while TLRs have been shown to be expressed at the apical and basolateral pole of both normal gastric epithelial cells and in *H. pylori* gastritis, in metaplasia, dysplastic, and neoplastic epithelial cells all TLRs are expressed diffusely and homogeneously throughout the cytoplasm, with no apparent polarization, which may suggest an increased activation of these diffusely over-expressed receptors during gastric carcinogenesis (126, 128).

In recent years, TLRs have been associated with tumor development and progression processes including cell proliferation, epithelial–mesenchymal transition, angiogenesis, metastasis, and immunosuppression. Interestingly, Chochi et al. (104) not only showed that *H. pylori* augmented the growth of GC via the LPS-TLR4 pathway but also found that this bacterium attenuated the antitumor activity and IFN- γ -mediated cellular immunity of human mononuclear cells. In addition, Song et al. (131) have suggested that flagellin-activated TLR5 enhances the proliferation of GC cells through an ERK-dependent pathway. Furthermore, Tye et al. (132) have proposed a novel role for TLR2 in promoting gastric tumorigenesis independent of inflammation, whereby up-regulation of TLR2 within epithelial tumor cells, rather than infiltrating inflammatory cells, by the uncontrolled activation of the oncogenic transcription factor STAT3, promoted gastric tumor cell proliferation, and survival via up-regulation of anti-apoptotic genes [e.g., BCL2-related protein A1 (*BCL2A1*), baculoviral IAP repeat containing 3 (*BIRC3*), and B-cell CLL/lymphoma 3 (*BCL3*)]. Further, two processes that facilitate carcinogenesis and involve TLRs have recently been described by Li et al. (133). Using LPS-treated CD14-knockdown GC cells, these authors showed that CD14, an important co-receptor in the TLR4 complex, promotes tumor cell epithelial–mesenchymal transition and invasion through TNF- α (133).

In addition, the expression of tumor-associated molecules known to be important in gastric carcinogenesis has been linked to the activation of the TLR signaling pathway. For example, prostaglandin-endoperoxide synthase 2 (PTGS2), which is also termed cyclooxygenase 2 (COX2), a key enzyme that catalyzes

the conversion of arachidonic acid to prostaglandins, has been shown to play a pivotal role in gastric inflammation and carcinogenesis (134). For example, a study by Chang et al. (108), using clinical *H. pylori* isolates, has shown that *H. pylori* acts through TLR2/TLR9 to activate both the PI-PLC γ /PKC α /c-Src/IKK α/β and NIK/IKK α/β pathways, resulting in the phosphorylation and degradation of I κ B α , which in turn leads to the stimulation of NF- κ B and the expression of PTGS2.

Further, as compared with normal cells, cancer cells are more metabolically active and generate more reactive oxygen species (ROS), which affects cell survival. Several studies have suggested that ROS can act as secondary messengers and control a range of signaling cascades, leading to sustained proliferation of cancer cells (135, 136). In the context of gastric carcinogenesis, *H. pylori*-infected gastric epithelial cells have been shown to generate ROS (137). Interestingly, Yuan et al. (138) recently suggested that TLR4 expression in GC correlated with tumor stage and that activation of TLR4 contributed to GC cell proliferation via mitochondrial ROS production through up-regulation of phosphorylated Akt and NF- κ B p65 activation and nuclear translocation.

However, the involvement of TLRs in GC might be more complex than initially suspected as TLRs not only recognize antigenic determinants of viruses, bacteria, protozoa, and fungi, but are also involved in the detection of damage-associated molecular patterns (DAMPs) (e.g., extracellular adenosine triphosphate, hyaluronan, extracellular glucose, monosodium urate crystals) (139). Release of DAMPs, which are especially targeted by TLR2 and TLR4 (140–145) during cancer progression may cause chronic inflammation leading to down-regulation of the ζ chain of the T-cell and NK cell activating receptors [for comprehensive information on this topic see the review by Baniyash et al. (146)], which entails T-cell and NK cell dysfunction, a phenomenon observed in some malignancies such as GC (147, 148), colon (149), prostate (150), cervical (151), and pancreatic cancer (152). In addition to immunosuppression, DAMPs appear to facilitate other processes during gastric carcinogenesis. For example, Wu et al. (153) have recently showed that hyaluronan, derived from malignant cells, induced long-lived tumor-associated neutrophils and subsequent malignant cell migration in gastric carcinomas via a TLR4/PI3K interaction.

Collectively, TLRs might be involved in both gastric carcinogenesis mediated by *H. pylori* infection (a tumor-promoting consequence of inflammatory responses) and in GC perpetuation associated with immunosuppression (active evasion by cancer cells from attack and elimination by immune cells) and increased metastasis.

GENETIC POLYMORPHISMS INVOLVED IN THE TOLL-LIKE RECEPTOR SIGNALING PATHWAY AND GASTRIC CANCER

In recent years, a number of investigations have attempted to establish the relationship between polymorphisms in molecules of the TLR signaling pathway and risk of GC. Recent studies, conducted in several populations, have shown associations between the polymorphisms *TLR1* rs5743618 (Ile602Ser) (154), *TLR2* –196 to –174del (155–158), *TLR2* rs3804099 (157), *TLR4* rs4986790 (Asp299Gly) (155, 157, 159), *TLR4* rs4986791 (Thr399Ile) (160), *TLR4* rs10116253 (161), *TLR4* rs10983755

(162), *TLR4* rs11536889 (+3725G/C) (155), *TLR4* rs1927911 (161), *TLR5* rs5744174 (158), *TLR9* rs187084 (–1486 T/C) (163), and *CD14* rs2569190 (–260 C/T) (155, 164–167), and risk of GC development in an ethnic-specific manner (Table 1). In addition, three polymorphisms located in the *TLR4* mRNA promoter region (sites –2081, –2026, and –1601) and *TLR4* Thr135Ala at the leucine-rich repeat (LRR), have been associated with poorly differentiated GC (168, 169).

Interestingly, some of these polymorphisms including *TLR4* Asp299Gly (159, 184), *TLR4* Thr399Ile (184, 185), *TLR4* rs10759932 (186), *CD14*-260 C/T (187), and *TLR2* –196 to –174del (157), appear to be involved in the biological continuum that results in intestinal-type GC as they have also been associated with gastric precancerous lesions (Table 2).

Given that some authors have failed to show specific associations between polymorphisms in the TLR signaling pathway, especially in *TLR2*, *TLR4*, and *CD14*, and gastric precancerous lesions/GC (157, 160, 162, 164, 172, 174–178, 180–183, 185, 188, 189), we performed the first global meta-analysis to assess the role of *TLR2*, *TLR4*, and *CD14* polymorphisms in gastric carcinogenesis (155), in an attempt to clarify the limited and current conflicting evidence, and to establish the true impact of the TLR signaling pathway in GC. Our meta-analysis, which included 18 case-control studies conducted in Caucasian, Asian, and Latin American populations, showed that *TLR4* Asp299Gly was a definitive risk factor for GC in Western populations (pooled OR: 1.87, 95% CI: 1.31–2.65). In addition, there was a potential association between *TLR2* –196 to –174 and GC in Japanese (pooled OR: 1.18, 95% CI: 0.96–1.45) (155). Interestingly, a recent meta-analysis on *TLR2* –196 to –174 and the risk of GC, conducted by Cheng et al. (190), failed to reproduce the findings in our meta-analysis, however, their stratification by ethnicity analyses included subjects from both Japan and China, which might explain the different outcomes. A further meta-analysis conducted by Chen et al. (191) that included 21 case-control studies showed an overall increased risk of GC in individuals harboring *TLR4* Asp299Gly (Allele analysis, OR: 1.84, 95% CI: 1.41–2.39) and *TLR4* Thr399Ile (Allele analysis, OR: 1.97, 95% CI: 1.22–3.18). Consistently, in stratified analyses by ethnicity, these authors only found an association between *TLR4* Asp299Gly (Allele analysis, OR: 1.90, 95% CI: 1.43–2.51) and *TLR4* Thr399Ile (Allele analysis, OR: 2.84, 95% CI: 1.56–5.15) in Caucasian individuals (191). Further, Zhao et al. (192) in an updated version of a meta-analysis that was initially conducted by Zhang et al. (193), on the risk of *TLR4* polymorphisms and risk of cancer in general, found a significant association with GC after stratifying by cancer type (OR: 2.00, 95% CI: 1.53–2.62). In addition, Zou et al. (194), through a meta-analysis that included 10 case-control studies, not only found that *TLR4* Asp299Gly was associated with GC (OR: 1.87, 95% CI: 1.44–2.44), especially non-cardia GC (OR: 2.03, 95% CI: 1.51–2.72), but also gastric precancerous lesions (OR: 2.47, 95% CI: 1.57–3.88), especially in *H. pylori*-infected individuals (OR: 3.43, 95% CI: 1.92–6.13).

Given limited evidence regarding the association between polymorphisms in other molecules of the TLR signaling pathway and the risk of GC, and the fact that 42% of cases of GC worldwide occur in the Chinese population, we conducted a case-control

Table 1 | Genetic polymorphisms in the Toll-like receptor signalling pathway that have been studied in relation to gastric cancer (170).

Gene	Polymorphism	Reference	Population	GC subtype	Total sample size	OR, 95% CI ^a
<i>TLR1</i>	rs5743618 (Ile602Ser)	Yang et al. (154)	German	NS	284 ^b	OR: 0.40, 95% CI: 0.22–0.72
<i>TLR2</i>	-196 to -174del	Castaño-Rodríguez et al. (170) de Oliveira et al. (157) Zeng et al. (158) Hishida et al. (172) Tahara et al. (156)	Chinese Brazilian Chinese Japanese Japanese	Non-cardia Non-cardia NS NS Non-cardia	310 440 744 1680 744	OR: 1.17, 95% CI: 0.81–1.71 OR: 2.32, 95% CI: 1.56–3.46 OR: 0.66, 95% CI: 0.48–0.90 OR: 1.17, 95% CI: 0.79–1.73 ^c OR: 6.06, 95% CI: 1.86–19.72
	rs3804099	de Oliveira et al. (157)	Brazilian	Non-cardia	440	OR: 2.32, 95% CI: 1.56–3.46
	rs3804100	Castaño-Rodríguez et al. (170)	Chinese	Non-cardia	310	OR: 3.16, 95% CI: 1.38–7.24
<i>TLR4</i>	rs4986790 (Asp299Gly)	Qadri et al. (174) de Oliveira et al. (157) Schmidt et al. (175) Santini et al. (160) Trejo de la O (176) Hold et al. (159) Hold et al. (159) Garza-Gonzalez et al. (177)	Indian Brazilian Chinese Italian Mexican Caucasian ^d Caucasian ^e Mexican	NS Non-cardia Non-cardia NS NS Non-cardia Cardia and non-cardia Non-cardia	330 440 222 322 182 731 395 314	OR: 1.15, 95% CI: 0.66–2.03 OR: 2.01, 95% CI: 1.06–3.81 OR: 0.23, 95% CI: 0.03–1.81 OR: 0.97, 95% CI: 0.37–1.14 OR: 2.70, 95% CI: 0.36–10.70 OR: 2.50, 95% CI: 1.60–4.00 OR: 2.10, 95% CI: 1.10–4.20 OR: 1.00, 95% CI: 0.30–2.80
	rs4986791 (Thr399Ile)	Qadri et al. (174) de Oliveira et al. (157) Santini et al. (160) Trejo de la O (176) Garza-Gonzalez et al. (177)	Indian Brazilian Italian Mexican Mexican	NS Non-cardia NS NS Non-cardia	330 440 322 263 314	OR: 1.39, 95% CI: 0.70–2.78 OR: 1.81, 95% CI: 0.64–5.15 OR: 3.62, 95% CI: 1.27–6.01 OR: 1.40, 95% CI: 0.36–5.38 OR: 0.25, 95% CI: 0.01–1.80
	rs10116253	Castaño-Rodríguez et al. (170) Huang et al. (161)	Chinese Chinese	Non-cardia NS	310 511	OR: 0.58, 95% CI: 0.34–1.00 OR: 0.33, 95% CI: 0.18–0.60
	rs10759931	Castaño-Rodríguez et al. (170)	Chinese	Non-cardia	310	OR: 0.56, 95% CI: 0.33–0.97
	rs10759932	Castaño-Rodríguez et al. (170) Huang et al. (178)	Chinese Chinese	Non-cardia Cardia and non-cardia	310 1962	OR: 0.59, 95% CI: 0.34–1.04 OR: 1.03, 95% CI: 0.74–1.45
	rs10983755	Kim et al. (179)	Korean	Non-cardia	974	OR: 1.41, 95% CI: 1.01–1.97
	rs11536889	Castaño-Rodríguez et al. (170) Kupcinskas et al. (180) Hishida et al. (181)	Chinese Caucasian ^f Japanese	Non-cardia NS NS	310 349 1639	OR: 3.58, 95% CI: 1.20–10.65 OR: 1.03, 95% CI: 0.62–1.71 OR: 1.04, 95% CI: 0.66–1.63
	rs1927911	Castaño-Rodríguez et al. (170) Huang et al. (161)	Chinese Chinese	Non-cardia NS	310 511	OR: 0.47, 95% CI: 0.27–0.82 OR: 0.37, 95% CI: 0.21–0.70
	rs2149356	Castaño-Rodríguez et al. (170)	Chinese	Non-cardia	310	OR: 0.59, 95% CI: 0.34–1.02
<i>TLR5</i>	rs5744174	Zeng et al. (158)	Chinese	NS	744	OR: 1.43, 95% CI: 1.03–1.97
<i>TLR9</i>	rs187084 (-1486 T/C)	Wang et al. (163)	Chinese	Cardia and non-cardia	628	OR: 1.63, 95% CI: 1.01–2.64
<i>CD14</i>	rs2569190 (-260 C/T)	Castaño-Rodríguez et al. (170) Companioni et al. (164) Li et al. (133) Kim et al. (179) Hold et al. (182) Hold et al. (182) Tahara et al. (166) Zhao et al. (167) Wu et al. (183)	Chinese Caucasian ^g Tibetan Korean Caucasian ^d Caucasian ^e Japanese Chinese Chinese	Non-cardia Cardia and non-cardia NS Non-cardia Non-cardia Cardia and non-cardia Non-cardia NS Non-cardia	310 1649 462 974 716 395 237 940 414	OR: 0.72, 95% CI: 0.5–1.02 OR: 0.92, 95% CI: 0.77–1.09 OR: 2.16, 95% CI: 1.34–3.47 OR: 0.97, 95% CI: 0.77–1.23 ^h OR: 1.00, 95% CI: 0.70–1.40 OR: 0.80, 95% CI: 0.50–1.30 OR: 0.31, 95% CI: 0.12–0.78 OR: 1.95, 95% CI: 1.20–3.16 OR: 0.98, 95% CI: 0.75–1.29

(Continued)

Table 1 | Continued

Gene	Polymorphism	Reference	Population	GC subtype	Total sample size	OR, 95% CI ^a
MD-2	rs11465996	Castaño-Rodríguez et al. (170)	Chinese	Non-cardia	310	OR: 4.83, 95% CI: 2.02–11.57
	rs16938755	Castaño-Rodríguez et al. (170)	Chinese	Non-cardia	310	OR: 3.80, 95% CI: 1.48–9.77
LBP	rs2232578	Castaño-Rodríguez et al. (170)	Chinese	Non-cardia	310	OR: 3.07, 95% CI: 1.24–7.59
TIRAP	rs7932766	Castaño-Rodríguez et al. (170)	Chinese	Non-cardia	310	OR: 6.04, 95% CI: 1.89–19.36

GC, gastric cancer; OR, odds ratio; CI, confidence intervals; NS, not specified.

^aOR and 95% CI correspond to allele or genotype analysis, depending on available information in the article.

^bThe control group included individuals with high risk gastritis (pangastritis, corpus-predominant gastritis with or without the presence of gastric atrophy, and intestinal metaplasia in either antrum or corpus).

^cCompared to gastric atrophy controls.

^dThe study population is from Poland.

^eThe study population is from the United States. No significant association was found with cardia GC.

^fSubjects from Germany, Lithuania and Latvia.

^gSubjects from France, Italy, Spain, United Kingdom, The Netherlands, Greece, Germany, Sweden, Denmark and Norway.

^hEffect size for intestinal-type GC, diffuse type: OR: 0.99, 95% CI: 0.78–1.26.

study comprising 310 ethnic Chinese individuals (87 non-cardia GC cases and 223 controls with functional dyspepsia), in which 25 polymorphisms involved in the TLR signaling pathway were investigated (170). Seven polymorphisms showed significant associations with GC (*TLR4* rs11536889, *TLR4* rs10759931, *TLR4* rs1927911, *TLR4* rs10116253, *TLR4* rs10759932, *TLR4* rs2149356, and *CD14* –260 C/T). In multivariate analyses, *TLR4* rs11536889 remained a risk factor for GC even after adjustment (OR: 3.58, 95% CI: 1.20–10.65). Further, *TLR4* rs10759932 decreased the risk of *H. pylori* infection (OR: 0.59, 95% CI: 0.41–0.86) (170). Strikingly, statistical analyses assessing the joint effect of *H. pylori* and the selected polymorphisms revealed that *H. pylori*-infected individuals harboring *TLR2* rs3804100, *TLR2* –196 to –174del, *TLR4* rs11536889, *MD-2* rs11465996, *MD-2* rs16938755, *LBP* rs2232578, and *TIRAP* rs7932766 were at most risk of developing GC (Table 1) (170).

The functional relevance of a number of these polymorphisms has already been established. For example, two polymorphisms in *TLR4*, Asp299Gly, and Thr399Ile, have been shown to disrupt the normal structure of the extracellular domain of *TLR4*, and thus, as a result, may reduce responsiveness to *H. pylori* by diminishing the binding affinity of the bacterial ligands (195). In addition, the *TLR4* rs11536889 polymorphism, which is located in the center of the 2818-bp *TLR4* 3' untranslated region (UTR), has recently been shown by Sato et al. (196) to contribute to the translational regulation of *TLR4*, possibly by binding to microRNAs. Further, these authors elegantly demonstrated that subjects harboring *TLR4* rs11536889 exhibited higher levels of *TLR4* receptors on monocytes and secreted higher levels of IL-8 in response to LPS (196). In addition, *TLR4* rs10759932 has been shown to decrease the expression of forkhead box protein P3 (FOXP3), the most specific marker for natural regulatory T (Treg) cells (197). FOXP3+ Treg cells, which suppress the immune response of antigen-specific T cells, have been demonstrated to play a key role in immunologic tolerance (198). Notably, recent studies have not only shown

that *in vivo* depletion of FOXP3+ Treg cells in *H. pylori*-infected mice leads to increased gastric inflammation and reduced bacterial colonization (199), but also recruitment of FOXP3+ Treg cells is increased in *H. pylori*-related human disorders including gastritis (200, 201), duodenal ulcer (202), and GC (200, 203, 204), suggesting that FOXP3+ Treg cells might contribute to lifelong persistence of *H. pylori* infection. Also, *TLR1* rs5743618 appears to impair the surface expression of *TLR1* of NK cells and NK cells-derived IFN-γ production (154). Further, *TLR2* –196 to –174 has been associated with decreased transcriptional activity of *TLR2* (205, 206). Similarly, it has been demonstrated that *TLR9* rs187084 down-regulates *TLR9* expression (207).

Further, *CD14* has been shown to activate macrophages/monocytes to release Th1-type cytokines including IL-12, thus, establishing the chronic inflammation stimulated by *H. pylori* infection (208–210). A Th1 predominant response has been extensively associated with the pathogenesis of *H. pylori*-related gastric disease (211–213). Currently, however, controversy exists regarding the influence of *CD14* –260 on expression of soluble *CD14* (sCD14). According to a number of studies, the *CD14* –260 T allele is believed to increase sCD14 production and therefore, serum sCD14 levels (214–217). In contrast, it has been reported that elevated sCD14 levels are associated with *H. pylori* infection, especially in subjects with the *CD14* –260 CC genotype (167). Alternatively, others have argued that this polymorphism has no effect on transcription (218). Since the evidence to date is conflicting, more functional studies are required to clarify this issue.

Overall, it is clear that genetic variability in genes of the TLR signaling pathway plays an important role in GC pathogenesis. Investigations of polymorphisms in different molecules of this pathway among different populations could provide novel insights into targeted treatment in genetically susceptible individuals, and thus, improve primary and secondary prevention of *H. pylori*-related GC in high risk populations.

Table 2 | Genetic polymorphisms in the Toll-like receptor signalling pathway that have been studied in relation to gastric precancerous lesions.

Reference	Journal	Population	Precancerous lesion	Cases	Controls	Total	Polymorphism	OR (95% CI) ^a
Fan et al. (186)	Human Immunology	Chinese	IM	193	312	505	<i>TLR4</i> Asp299Gly	0.89 (0.46–1.72)
							<i>TLR4</i> Thr399Ile	1.01 (0.33–3.14)
			Dysplasia	140	312	452	<i>TLR4</i> rs10759932	0.42 (0.29–0.62)
							<i>TLR4</i> Asp299Gly	0.81 (0.38–1.73)
de Oliveira et al. (157)	Digestive Diseases and Science	Brazilian	CG	229	240	469	<i>TLR4</i> Thr399Ile	1.08 (0.35–3.39)
							<i>TLR2</i> –196–174 del	1.52 (1.01–2.29)
							<i>TLR4</i> Asp299Gly	1.60 (0.84–3.06)
Kucinskas et al. (180)	BMC Medical Genetics	Caucasian	CG, AG and IM	222	238	460	<i>TLR4</i> rs11536889	0.94 (0.62–1.44)
Zeng et al. (158)	Cancer Epidemiology, Biomarkers and Prevention	Chinese	IM	496	496	992	<i>TLR2</i> –196–174 del	0.99 (0.65–1.52)
							<i>TLR5</i> rs5744174	1.55 (0.78–3.11)
			Dysplasia	350	496	846	<i>TLR2</i> –196–174 del	0.99 (0.73–1.35)
Rigoli et al. (184)	Anti-Cancer Research	Caucasian	CG	60 ^b	87	147	<i>TLR4</i> Thr399Ile	3.73 (1.36–10.14)
							<i>TLR4</i> Asp299Gly	4.80 (1.93–12.35)
Hishida et al. (172)	Gastric Cancer	Japanese	AG ^c	494	443	937	<i>TLR2</i> –196–174 del	1.08 (0.70–1.67)
Hishida et al. (181)	Helicobacter	Japanese	AG ^c	536	1056	1592	<i>TLR4</i> rs11536889	1.20 (0.76–1.89)
Murphy et al. (188)	European Journal of Gastroenterology and Hepatology	Caucasian	CG	91	96	187	<i>TLR4</i> Asp299Gly	1.12 (0.49–2.52)
				90	91	181	<i>TLR4</i> Thr399Ile	0.97 (0.44–2.11)
			IM	63	96	159	<i>TLR4</i> Asp299Gly	1.33 (0.49–3.59)
				62	91	153	<i>TLR4</i> Thr399Ile	0.99 (0.38–2.63)
Hofner et al. (189)	Helicobacter	Caucasian	CG	136 ^d	75	211	<i>TLR4</i> Thr399Ile	0.94 (0.39–2.24)
							<i>TLR4</i> Asp299Gly	1.25 (0.53–2.95)
Achyut et al. (185)	Human Immunology	Indian	AG	68	200	268	<i>TLR4</i> Thr399Ile	4.2 (1.13–15.73)
							<i>TLR4</i> Asp299Gly	1.50 (0.55–3.82)
			IM	50	200	250	<i>TLR4</i> Asp299Gly	1.10 (0.32–3.50)
Hold et al. (159)	Gastroenterology	Caucasian	AG	45 ^e	100	145	<i>TLR4</i> Thr399Ile	4.7 (1.52–14.63)
							<i>TLR4</i> Asp299Gly	11.0 (2.50–48.0)
Kato et al. (187)	Digestive Diseases and Science	Venezuelan	AG	289	1033	1322	<i>CD14</i> –260 C/T	1.17 (0.81–1.70)
				543	1033	1575	<i>CD14</i> –260 C/T	1.45 (1.06–1.99)
			Dysplasia	118	1033	1151	<i>CD14</i> –260 C/T	1.44 (0.82–2.55)

CG, chronic gastritis; AG, atrophic gastritis; IM, intestinal metaplasia; OR, odds ratio; CI, confidence intervals.

^aOR and 95% CI correspond to allele or genotype analysis, depending on available information in the article.

^bOnly individuals with corpus-predominant chronic gastritis were included in the meta-analysis (individual presenting antrum-predominant gastritis were excluded).

^cAnalyses including only *H. pylori* seropositive individuals.

^dOnly patients with chronic gastritis were included in the meta-analysis (patients presenting duodenal ulcer were excluded).

^eCases were GC patients' relatives with gastric atrophy and infected with *H. pylori* from a Scotland population.

NOD-LIKE RECEPTORS AND *HELICOBACTER PYLORI*-RELATED GASTRIC CANCER

NOD-LIKE RECEPTORS RECOGNITION OF *HELICOBACTER PYLORI*

The NLR family not only recognizes PAMPs but also DAMPs in the cytoplasm (93). The NLRs characteristic structure includes a central nucleotide-binding and oligomerization (NACHT) domain

that is present in all NLR family members, a C-terminal LRRs and an N-terminal caspase recruitment (CARD) or pyrin (PYD) domain.

Based on phylogenetic analysis of NACHT domains, the NLR family has been shown to comprise three subfamilies: (1) the NOD family which includes NOD1-2, NOD 3 (NLRC3), NOD4

(NLRC5), NOD5 (NLRX1), and CIITA, (2) the NLRPs including NLRP1-14 (also known as NALPs), and (3) the IPAF subfamily, which consists of IPAF (NLRC4) and NAIP (93).

The NACHT domain belongs to a family of P-loop NTPases known as the signal transduction ATPases with numerous domains (STAND) (219). This domain permits activation of the signaling complex via adenosine ATP-dependent oligomerization (94). NACHT domain oligomerization is essential for the activation of NLRs, forming high molecular weight complexes, probably hexamers or heptamers that characterize inflammasomes (molecular complexes involved in the activation of inflammatory caspases for the maturation and secretion of IL-1 β , IL-18, and possibly IL-33) and NOD signalosomes (complexes that are assembled upon oligomerization of NOD1 or NOD2 and lead to NF- κ B activation through the receptor-interacting protein-2) (94). CARD and PYD are death domains that mediate homotypic protein–protein interactions for down-stream signaling (93, 94). These domains are characterized by six α helices that form trimers or dimers with other members of the same subfamily (94). The third domain, the LRR region, has been implicated in ligand sensing and autoregulation of not only NLRs but TLRs (93, 94). The LRR is formed by tandem repeats of a structural unit consisting of a β strand and an α helix and is composed of 20–30 amino acids that form a horse-shoe shaped structure rich in the hydrophobic amino acid leucine (220). The NLRPs LRR gene is made up of tandem repeats of exons of exactly 171 nucleotides, which encode one central LRR and two halves of the neighboring LRRs (221). This particular modular organization possibly allows extensive alternative splicing of the LRR region leading to maximum variability in the ligand-sensing unit (94). However, a recent publication by Tenthorey et al. (222) analyzing a panel of 43 chimeric NAIPs, showed that LRR was unnecessary for NAIP/NLRC4 inflammasome ligand specificity, leading them to propose a model in which NAIP activation is instead triggered by ligand binding to NACHT-associated helical domains. This recent evidence suggests that the ligand-sensing function of the LRR domain in NLRs, which has been supported primarily by analogy to the well-established ligand-sensing function of the LRR region in TLRs, needs to be re-examined.

The most widely studied NLRs during *H. pylori* infection are NOD1 and NOD2, which are expressed in epithelial and antigen-presenting cells, and are known to specifically recognize peptidoglycan-derived peptides (γ -D-glutamyl-meso-diaminopimelic acid and muramyl dipeptide, respectively). An early study, attempting to determine the mechanism whereby *H. pylori* delivers peptidoglycan to cytosolic host NOD1, demonstrated that *H. pylori* peptidoglycan is delivered to the host cell via a type IV secretion system (223). More recently, Hutton et al. (224) showed, for the first time, that cholesterol-rich microdomains called lipid rafts, were important for the type IV secretion system-dependent peptidoglycan delivery and subsequent NF- κ B activation and IL-8 production, mediated by NOD1. Interestingly, Kaparakis et al. (225) reported a novel mechanism in Gram-negative bacteria, including *H. pylori*, for the delivery of peptidoglycan to cytosolic NOD1 in host cells that involves outer membrane vesicles that enter epithelial cells through lipid rafts. In addition, Necchi et al. (226) demonstrated the formation of

a particle-rich cytoplasmic structure (PaCS) in *H. pylori*-infected human gastric epithelium having metaplastic or dysplastic foci, where VacA, CagA, urease, outer membrane proteins, NOD1 receptor, ubiquitin-activating enzyme E1, polyubiquitinated proteins, proteasome components, and potentially oncogenic proteins like SHP-2 and ERKs colocalized, inferring that this structure is likely to modulate inflammatory and proliferative responses during *H. pylori* infection.

The recent finding that NF- κ B and AP-1 complexes can be physically translocated to the nucleus in response to NOD1 activation has led to the view that NOD1 is likely to be essential for the induction of both NF- κ B and AP-1 activation during *H. pylori* infection (227). A number of studies have shown up-regulation of *NOD1* expression in diverse human cell lines challenged with *H. pylori* in a *cag* PAI-dependent manner (228–230). Further, *H. pylori* *cag* PAI-positive strains have recently been shown to activate the NOD1 pathway through two components of the IFN- γ signaling pathway, STAT1 and IRF1 (228). Similarly, expression of *NOD2* was shown to significantly sensitize HEK293 cells to *H. pylori*-induced NF- κ B activation in a *cag* PAI-dependent manner (231). Further, *NOD2*, but not *NOD1*, seems to be required for induction of pro-IL-1 β and NLRP3 in *H. pylori*-infected DCs (232).

A limited number of studies have assessed the interaction between NLRPs and other inflammasome-associated molecules, and *H. pylori*. NLRPs represent the largest NLR subfamily (14 genes have been identified in humans) and are believed to be the scaffolding proteins of inflammasomes (221, 233). NLRPs interact and recruit the adaptor apoptosis-associated speck-like protein (ASC) via PYD-PYD interaction (94). ASC (also known as PYCARD), a key component required for inflammasome formation, is formed by an N-terminal PYD and a C-terminal CARD (234, 235). This interaction leads to the recruitment of caspase-1, an intracellular aspartate specific cysteine protease, which subsequently leads to the maturation and release of pro-inflammatory cytokines (236).

An early study by Tomita et al. (237) demonstrated that in *H. pylori* positive patients antral IL-18 mRNA expression was increased as compared with *H. pylori* negative patients, however, mature IL-18 protein and active caspase-1 were found to be present in both infected and non-infected gastric mucosa. Interestingly, in the following year, Potthoff et al. (238) reported activation of caspase-3, -8, and -9, but not caspase-1, in AGS cells challenged with *H. pylori*. However, this finding is in contrast with subsequent studies, which have demonstrated an important role for NLRPs and inflammasome-related molecules in *H. pylori* infection. For example, Basak et al. (96) demonstrated that *H. pylori* LPS could activate caspase-1 through Rac1/PAK1 signaling, and that activated caspase-1 played a role in LPS-induced IL-1 β maturation (96). Further, ASC-deficient mice challenged with *H. pylori* have been shown to exhibit higher bacterial loads and significantly lower levels of gastritis, when compared with wild-type mice, and were incapable of producing IL-1 β or IL-18 and produced less INF- γ in response to *H. pylori* infection (239). Later, Hitzler et al. (240) showed in both cultured DCs and *in vivo* that *H. pylori* infection activates caspase-1, leading to IL-1 β /IL-18 processing and secretion. Consistently, three studies, using human GC cell lines, gastric tissue, and murine models, confirmed increased

expression of caspase-1, IL-1 β , and IL-18 in *H. pylori*-infected cells (171, 241, 242). Further, Jiang et al. (243), also using a murine model, have reported the expression of NLRP3 inflammasome-related molecules as well as serum IL-1 β , IL-18, and IL-33 levels to be significantly increased in *H. pylori*-infected mice. More recently, a study by Kim et al. (232) has shown that secretion of IL-1 β by DCs infected with *H. pylori* requires TLR2, NOD2, and the NLRP3 inflammasome.

Given that little is known about the role of NLRPs, inflammasomes, or other molecules involved in the NLR signaling pathways in response to *H. pylori* infection, we recently assessed the gene expression of 84 different molecules involved in the NLR signaling pathways, through quantitative real-time PCR, using THP-1-derived macrophages infected with two strains of *H. pylori*, GC026 (GC) and 26695 (gastritis) (173). Our gene expression analyses showed five genes encoding NLRs to be significantly regulated in *H. pylori*-challenged cells (*NLRC4*, *NLRC5*, *NLRP9*, *NLRP12*, and *NLRX1*) (173). Interestingly, *NLRP12* and *NLRX1*, two known NF- κ B negative regulators, were markedly down-regulated, while *NFKB1* and several NF- κ B target genes encoding pro-inflammatory cytokines (*IFNB1*, *IL12A*, *IL-12B*, *IL6*, and *TNF*), chemokines (*CXCL1*, *CXCL2*, and *CCL5*) and molecules involved in carcinogenesis (*PTGS2* and *BIRC3*) were markedly up-regulated, in THP-1 cells infected with a highly virulent *H. pylori* strain isolated from a GC patient. These findings highlight the relevance of the NLR signaling pathways in gastric carcinogenesis and its close interaction with NF- κ B (173).

Overall, current evidence clearly shows that, in response to *H. pylori*, members of the NOD and NLRP subfamilies are critical for generating mature pro-inflammatory cytokines/chemokines that are crucial for Th1 responses and lead to *H. pylori*-related gastric disorders.

NOD-LIKE RECEPTORS AND GASTRIC CARCINOGENESIS

The role of the NLR signaling pathways in the biological continuum that characterizes GC remains relatively unexplored as a very limited number of studies have addressed this issue. For example, Allison et al. (228) have shown that NOD1 expression was significantly increased in human gastric biopsies displaying severe gastritis, when compared with those without gastritis, as well as in gastric tumor tissues, as compared with paired non-tumor tissues. In contrast, Jee et al. (244), who analyzed human GC tissues and GC cell lines, showed that a significant decrease in the expression of caspase-1 was associated with poor survival and was inversely correlated with p53 expression.

Given the reported interaction of *H. pylori* with NLRs and the importance of this in the development of gastric inflammation and subsequent carcinogenesis, as well as the production of DAMPs during tumor formation (245), further comprehensive studies of the functional relevance of NLRs activation during chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and GC are clearly warranted.

GENETIC POLYMORPHISMS INVOLVED IN THE NOD-LIKE RECEPTOR SIGNALING PATHWAY AND GASTRIC CANCER

The majority of studies examining the association between polymorphisms involved in the NLR signaling pathways and the risk

of GC have focused on *NOD1* and *NOD2* polymorphisms. Studies, conducted in a number of populations, have investigated the association between the polymorphisms *NOD1* rs2907749 (246), *NOD1* rs7789045 (246), *NOD1* rs2075820 (E266K) (179, 247), *NOD1* rs5743336 (180), *NOD2* rs7205423 (246), *NOD2* rs7202124 (164), *NOD2* rs2111235 (164), *NOD2* rs5743289 (164), *NOD2* rs2066842 (P268S) (248, 249), *NOD2* rs2066844 (R702W) (250), *NOD2* rs2066845 (G908R) (184), and *NOD2* rs2066847 (L1007insC) (184, 250), and risk of gastric precancerous lesions and GC (Table 3). Further, a recent meta-analysis by Liu et al. (251) that included six case-control studies has shown consistent associations between *NOD2* R702W, G908R, and L1007insC, and risk of GC.

Given the documented relevance of other NLRs in *H. pylori* infection and related GC, and that polymorphisms in genes such as *NLRP3* (252–255) and *CARD8* (255, 256) have been associated with inflammatory gastrointestinal disorders, we addressed, for the first time, the association between 51 polymorphisms in six genes (*NLRP3*, *NLRP12*, *NLRX1*, *CASP1*, *ASC*, and *CARD8*) involved in the NLR signaling pathways and risk of GC in a high risk Chinese population (173). In this study, we found novel associations between *CARD8* rs11672725 and the risk of GC, and *NLRP12* rs2866112 and the risk of *H. pylori* infection (Table 3). Further, we showed that the concomitant presence of polymorphisms involved in the NLR signaling pathways (*CARD8*, *NLRP3*, *CASP1*, and *NLRP12*) and *H. pylori* infection dramatically increased the risk of GC in Chinese (Table 3) (173).

The functional relevance of a number of these polymorphisms has been examined. For example, the introduction of *NOD2* R702W, a polymorphism located in the LRR of *NOD2*, into the HEK293 cell line, resulted in abrogation of *H. pylori*-induced activation of NF- κ B signaling (231). Further, Maeda et al. (257) observed increased NF- κ B activation in response to muramyl dipeptide in mice harboring a *NOD2* mutation that is homologous to *NOD2* rs5743293 (3020insC) in humans. However, it is worth noting that the conclusions described by Maeda et al. (257) must be interpreted with care given that the authors subsequently found a duplication of the 3' end of the wild-type *Nod2* locus, including exon 11, which was targeted by the mutation, and therefore, they are currently working to recreate a mutant strain without such a duplication.

Given that investigation of the role of polymorphisms involved in the NLR signaling pathways in GC is a relatively recent field of research, further studies are required to assess the association between these polymorphisms and GC in a range of human populations, especially those at high risk of GC.

OTHER PATTERN-RECOGNITION RECEPTORS AND *HELICOBACTER PYLORI*-RELATED GASTRIC CANCER

A further two PRR subfamilies, RLRs and CLRs, have been studied in relation to *H. pylori* infection and gastric carcinogenesis. It is well known that RLRs (RIG-I, MDA-5, and LGP2) induce type I IFN in response to different RNA viruses, however, investigation on the role of RIG-I-like receptors in the recognition of RNA derived from intracellular bacteria is very limited. Interestingly, a study by Rad et al. (112), which used mice lacking simultaneously up to four different TLRs, apart from identifying TLR2 and

Table 3 | Genetic polymorphisms in the NOD-like receptor signalling pathway that have been studied in relation to gastric precancerous lesions and gastric cancer.

Reference	Journal	Population	Gastric lesion	Study sample size	Polymorphism	OR (95% CI) ^a
Castaño-Rodríguez et al. (173)	PLoS One	Chinese	GC	310	<i>CARD8</i> rs11672725 <i>CARD8</i> rs10405717 <i>CARD8</i> rs2043211 <i>NLRP3</i> rs12079994 <i>NLRP3</i> rs3806265 <i>NLRP3</i> rs4612666 <i>NLRP12</i> rs2866112 <i>NLRP12</i> rs4419163 <i>NLRX1</i> rs10790286 <i>CASP1</i> rs2282659 <i>CASP1</i> rs530537 <i>CASP1</i> rs61751523	4.80 (1.39–16.58) 2.46 (1.04–5.84) ^b 0.19 (0.058–0.63) ^b 4.15 (1.70–10.12) ^b 3.33 (1.09–10.13) ^b 4.03 (1.15–14.16) ^b 4.73 (2.06–10.88) ^b 2.42 (1.12–5.23) ^b 4.00 (1.66–9.61) ^b 4.65 (1.67–12.95) ^b 4.65 (1.67–12.95) ^b 4.56 (1.57–13.28) ^b
Companioni et al. (164)	International Journal of Cancer	Caucasian	GC	1649	<i>NOD2</i> rs7202124 <i>NOD2</i> rs2111235 <i>NOD2</i> rs5743289	0.74 (0.61–0.89) 0.77 (0.64–0.93) 3.76 (1.33–10.63) ^c
Kim et al. (179)	Helicobacter	Korean	IM	412	<i>NOD1</i> rs2075820 (E266K)	1.0 (0.74–1.34) ^d
Wang et al. (246)	World Journal of Gastroenterology	Chinese	GC	456	<i>NOD1</i> rs2907749 <i>NOD1</i> rs7789045 <i>NOD2</i> rs7205423	0.50 (0.26–0.95) 2.14 (1.20–3.82) 0.82 (0.39–1.72)
Kupcinskas et al. (180)	BMC Medical Genetics	Caucasian	GC CG, AG and IM	574	<i>NOD1</i> rs5743336	1.01 (0.48–2.16) 0.78 (0.40–1.49)
Rigoli et al. (184)	Anti-cancer Research	Caucasian	CG	147	<i>NOD2</i> G908R <i>NOD2</i> L1007insC	5.18 (1.65–16.09) 3.66 (1.13–11.80)
Kara et al. (247)	Clinical and Experimental Medicine	Turkish	AG IM	150	<i>NOD1</i> rs2075820 (E266K)	13.35 (5.12–34.82) 2.71 (1.26–5.80)
Hnatyszyn et al. (248)	Experimental and Molecular Pathology	Caucasian	CG, AG, IM and GC	244	<i>NOD2</i> rs2066842 (P268S)	2.2 (1.40–3.30)
Angeletti et al. (250)	Human Immunology	Caucasian	GC	326	<i>NOD2</i> rs2066844 (R702W) <i>NOD2</i> rs2066845 (G908R) <i>NOD2</i> rs2066847 (L1007insC)	4.1 (1.75–9.42) ^d 0.56 (0.17–1.65) ^d 16.10 (3.83–67.81) ^d
Wex et al. (249)	Anti-cancer Research	Caucasian	GC	324	<i>NOD2</i> rs2066842 (P268S) <i>NOD2</i> rs2066844 (R702W)	1.5 (1.05–2.17) 1.3 (0.66–2.55)
Hofner et al. (189)	Helicobacter	Caucasian	CG	211	<i>NOD1</i> rs2075820 (E266K)	1.06 (0.66–1.73)

GC, gastric cancer; IM, intestinal metaplasia; AG, atrophic gastritis; GC, chronic gastritis; OR, odds ratio; CI, confidence intervals.

^aOR and 95% CI correspond to allele or genotype analysis, depending on available information in the article.

^bOnly in *H. pylori*-infected individuals.

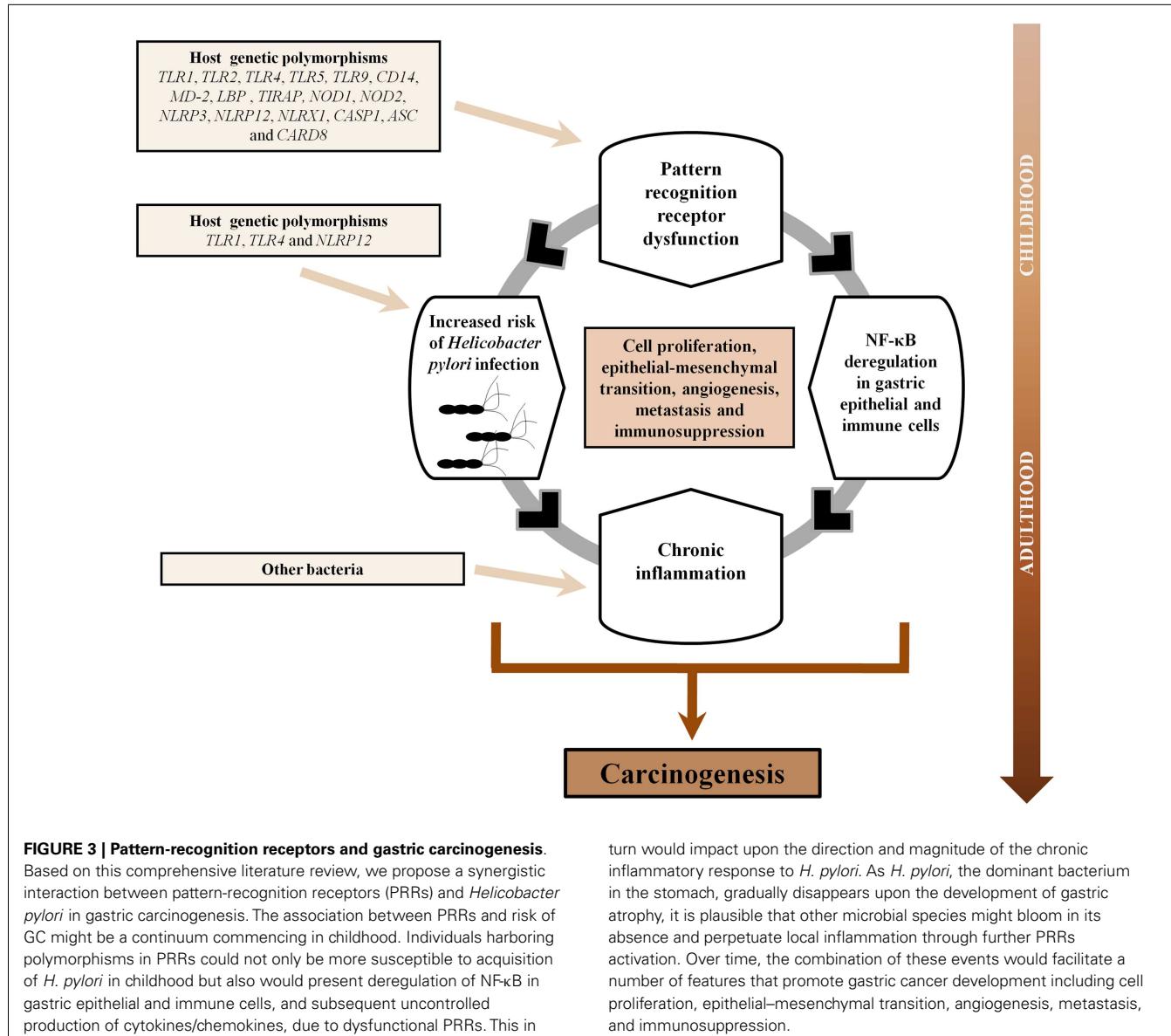
^cSignificant only in non-cardia *H. pylori* CagA negative individuals.

^dResults obtained through a Fisher's exact probability test (two-tailed P-values) conducted in the current review using the information provided in the original article.

TLR9 to be important *H. pylori* recognizing PRRs, also showed that *H. pylori* 5'-triphosphorylated RNA can be sensed by RIG-I and can contribute to the TLR-independent type I IFN response to this bacteria in DCs. Further, Tatsuta et al. (258) have recently shown that MDA-5 expression was significantly increased in the human gastric antral mucosa of *H. pylori*-infected individuals. In

addition, these authors showed that increased MDA-5 levels correlated with atrophy and intestinal metaplasia in the corpus of these individuals (258).

C-type lectin receptors bind to carbohydrates (mannose- or fucose-containing glycans) present on pathogens to tailor immune responses to viruses, bacteria, and fungi. DC-specific intercellular



adhesion molecule-3-grabbing non-integrin (DC-SIGN) is a CLR expressed on the surface of both macrophages and DCs. Interestingly, it has been shown that *H. pylori* harbors fucosylated ligands that can be recognized by DC-SIGN (259). Further, *H. pylori* DC-SIGN ligands appear to actively dissociate the signaling complex down-stream of DC-SIGN (KSR1–CNK–Raf-1) to suppress pro-inflammatory cytokine production (259). In addition, *H. pylori* LPS Lewis blood-group antigens can bind to DC-SIGN in a fucose or galactose-dependent manner (260, 261) and this interaction appears to inhibit a Th1 response in DCs (262). It has also been demonstrated that *H. pylori*-induced IL-10 production in monocyte-derived DCs is significantly suppressed by the addition of anti-DC-SIGN, TLR2, or TLR4 antibodies, either alone or in combination, before *H. pylori* stimulation (263). Further, *in vitro* and *in vivo* experiments have shown that the expression of DC-SIGN is significantly higher

in *H. pylori*-infected individuals as compared with that in their uninfected counterparts (264, 265).

To date, no studies have been conducted to determine the association between genetic polymorphisms involved in the RLR and CLR signaling pathways and GC, however, Kutikhin and Yuzhalin (266) have comprehensively analyzed the oncogenic potential of both RLRs and CLRs, suggesting that future oncogenomic investigations should focus on polymorphisms in *MRC1* (rs1926736, rs2478577, rs2437257, and rs691005), *CD209* (rs2287886, rs735239, rs4804803, and rs735240), *CLEC7A* (rs16910526), and *RIG-I* (rs36055726, rs11795404, and rs10813831).

Given the limited but consistent current evidence suggesting a role of RLRs and CLRs in *H. pylori* infection, and the documented interaction between these signaling pathways and other important PRRs in GC such as TLRs (267, 268) and NLRs (269, 270), further

studies assessing the implications of RLRs and CLRs in *H. pylori*-related inflammation and subsequent carcinogenesis need to be conducted.

PATTERN-RECOGNITION RECEPTORS AS THERAPEUTICS TARGETS IN GASTRIC CANCER

Pattern-recognition receptors are increasingly recognized as important players in immunotherapy as PRRs-specific agonists elicit a potent immune response to cancers, allergic diseases, and chronic viral infections, while reducing the risk of an uncontrolled and detrimental systemic inflammatory response (for comprehensive information on this topic refer to the reviews by Hedayat et al. (271) and Paul-Clark et al. (272)).

In the context of gastric carcinogenesis, Tye et al. (132), using a GC murine model (*gp130^{F/F}*) displaying elevated gastric TLR2 expression levels, have elegantly shown that genetic and antibody-mediated therapeutic targeting of TLR2 leads to a substantial reduction in stomach size and overall tumor burden, including the number of gastric tumors. A further example is presented in the study by Gradiras et al. (273), which suggested that MD-2 is one of the important targets of curcumin (diferuloylmethane), the main component of the spice turmeric (*Curcuma longa*) that is widely used for gastric disorders in the Indian subcontinent, in its suppression of the innate immune response to bacterial infection. Furthermore, curcumin was recently shown to polarize myeloid-derived suppressor cells, extracted from a human GC xenograft mouse model, toward a M1-like phenotype with an increased expression of CCR7 and decreased expression of the CLR dectin 1, being both observed *in vivo* (tumor tissue) and *in vitro* (splenic myeloid-derived suppressor cells from tumor-bearing mice) (274). In addition, a study by Yang et al. (171) demonstrated that the combination of catechins and sialic acid is effective in suppressing the inflammatory responses mediated by the inflammasome/caspase-1 signaling pathway in gastric epithelial cells during *H. pylori* infection. Also, poly(I:C), an agonist of TLR3 and RLRs, has been shown to have a pro-apoptotic effect *in vitro*, and has significantly inhibited xenograft growth of human GC in a mouse model, through up-regulation of RLRs (RIG-I, MDA-5, and LGP2) as well as an increased expression of Bcl-2 family members, suggesting that it may be a promising chemotherapeutic agent against GC (275).

Given that modulation of PRRs has been proven to be relevant in gastric carcinogenesis through diverse mechanisms, including suppression of *H. pylori*-induced inflammation and enhancement of cancer cell apoptosis, this approach should be considered a new and promising angle of immunotherapy in GC.

CONCLUSION

In conclusion, abundant evidence supports the pivotal role of PRRs in gastric carcinogenesis as these receptors of the innate immune system, including TLRs, NLRs, CLRs, and RLRs, have been shown to recognize diverse components of *H. pylori*, the major risk factor of GC. In addition, PRRs are also involved in gastric carcinogenesis *per se* as these receptors are known to exert tumor-promoting functions (cell proliferation, epithelial–mesenchymal transition, angiogenesis, and metastasis) as well as immunosuppression during cancer. Given that host genetic

variability in the TLR and NLR signaling pathways are known to be associated with an increased risk of *H. pylori* infection, the development of gastric precancerous lesions and GC, this knowledge has the potential to allow better prevention of GC through selective treatment and surveillance of individuals harboring high risk genetic profiles. Finally, given that PRRs are increasingly being used as a target for immunotherapy against both cancer and infectious diseases, the established relevance of PRRs in *H. pylori* infection and GC, could suggest that PRR agonists and/or antagonists may potentially improve the outcome of GC. Based on the extensive evidence presented in the current review, we propose a synergistic interaction between PRRs and *H. pylori*, which over time, could facilitate the sequence of events that characterizes GC development including inflammation, atrophy, intestinal metaplasia, dysplasia, and finally, GC (Figure 3).

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REFERENCES

1. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide. *IARC CancerBase No. 11 [Internet]*. Lyon: International Agency for Research on Cancer (2013).
2. Hamilton SR, Altonen LA, editors. *Tumours of the Digestive System*. Lyon: IARC Press (2000).
3. McColl KE. Cancer of the gastric cardia. *Best Pract Res Clin Gastroenterol* (2006) **20**:687–96. doi:10.1016/j.bpg.2006.03.005
4. Ferro A, Peleteiro B, Malvezzi M, Bosetti C, Bertuccio P, Levi F, et al. Worldwide trends in gastric cancer mortality (1980–2011), with predictions to 2015, and incidence by subtype. *Eur J Cancer* (2014) **50**:1330–44. doi:10.1016/j.ejca.2014.01.029
5. Cavaleiro-Pinto M, Peleteiro B, Lunet N, Barros H. *Helicobacter pylori* infection and gastric cardia cancer: systematic review and meta-analysis. *Cancer Causes Control* (2011) **22**:375–87. doi:10.1007/s10552-010-9707-2
6. Hansen S, Vollset SE, Derakhshan MH, Fyfe V, Melby KK, Aase S, et al. Two distinct aetiologies of cardia cancer; evidence from premorbid serological markers of gastric atrophy and *Helicobacter pylori* status. *Gut* (2007) **56**:918–25. doi:10.1136/gut.2006.114504
7. Correa P. A human model of gastric carcinogenesis. *Cancer Res* (1988) **48**:3554–60.
8. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* (2000) **100**:57–70. doi:10.1016/S0092-8674(00)81683-9
9. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) **144**:646–74. doi:10.1016/j.cell.2011.02.013
10. Fuchs CS, Mayer RJ. Gastric carcinoma. *N Engl J Med* (1995) **333**:32–41. doi:10.1056/NEJM199507063330107
11. Correa P. Human gastric carcinogenesis: a multistep and multifactorial process – First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* (1992) **52**:6735–40.
12. Li H, Stoicov C, Cai X, Wang TC, Houghton J. *Helicobacter* and gastric cancer disease mechanisms: host response and disease susceptibility. *Curr Gastroenterol Rep* (2003) **5**:459–67. doi:10.1007/s11894-003-0034-6
13. Oliveira C, Pinheiro H, Figueiredo J, Seruca R, Carneiro F. E-cadherin alterations in hereditary disorders with emphasis on hereditary diffuse gastric cancer. *Prog Mol Biol Transl Sci* (2013) **116**:337–59. doi:10.1016/B978-0-12-394311-8.00015-7
14. Kusters JG, Van Vliet AH, Kuipers EJ. Pathogenesis of *Helicobacter pylori* infection. *Clin Microbiol Rev* (2006) **19**:449–90. doi:10.1128/CMR.00054-05
15. Costa NR, Mendes N, Marcos NT, Reis CA, Caffrey T, Hollingsworth MA, et al. Relevance of MUC1 mucin variable number of tandem repeats polymorphism in *H. pylori* adhesion to gastric epithelial cells. *World J Gastroenterol* (2008) **14**:1411–4. doi:10.3748/wjg.14.1411

16. Atherton JC. The pathogenesis of *Helicobacter pylori*-induced gastro-duodenal diseases. *Annu Rev Pathol* (2006) **1**:63–96. doi:10.1146/annurev.pathol.1.110304.100125
17. Suerbaum S, Michetti P. *Helicobacter pylori* infection. *N Engl J Med* (2002) **347**:1175–86. doi:10.1056/NEJMra020542
18. Wroblewski LE, Peek RM Jr, Wilson KT. *Helicobacter pylori* and gastric cancer: factors that modulate disease risk. *Clin Microbiol Rev* (2010) **23**:713–39. doi:10.1128/CMR.00011-10
19. El-Omar EM, Penman ID, Ardill JE, Chittajallu RS, Howie C, Mccoll KE. *Helicobacter pylori* infection and abnormalities of acid secretion in patients with duodenal ulcer disease. *Gastroenterology* (1995) **109**:681–91. doi:10.1016/0016-5085(95)90374-7
20. Kivi M, Tindberg Y, Sorberg M, Casswall TH, Befrits R, Hellstrom PM, et al. Concordance of *Helicobacter pylori* strains within families. *J Clin Microbiol* (2003) **41**:5604–8. doi:10.1128/JCM.41.12.5604-5608.2003
21. Konno M, Fujii N, Yokota S, Sato K, Takahashi M, Mino E, et al. Five-year follow-up study of mother-to-child transmission of *Helicobacter pylori* infection detected by a random amplified polymorphic DNA fingerprinting method. *J Clin Microbiol* (2005) **43**:2246–50. doi:10.1128/JCM.43.5.2246-2250.2005
22. Rowland M, Daly L, Vaughan M, Higgins A, Bourke B, Drumm B. Age-specific incidence of *Helicobacter pylori*. *Gastroenterology* (2006) **130**:65–72;quiz211. doi:10.1053/j.gastro.2005.11.004
23. Cover TL, Blaser MJ. *Helicobacter pylori* in health and disease. *Gastroenterology* (2009) **136**:1863–73. doi:10.1053/j.gastro.2009.01.073
24. Neiger R, Simpson KW. *Helicobacter* infection in dogs and cats: facts and fiction. *J Vet Intern Med* (2000) **14**:125–33. doi:10.1111/j.1939-1676.2000.tb02225.x
25. Simpson KW, Strauss-Ayali D, Straubinger RK, Scanziani E, Mcdonough PL, Straubinger AF, et al. *Helicobacter pylori* infection in the cat: evaluation of gastric colonization, inflammation and function. *Helicobacter* (2001) **6**:1–14. doi:10.1046/j.1523-5378.2001.00010.x
26. Bik EM, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F, et al. Molecular analysis of the bacterial microbiota in the human stomach. *Proc Natl Acad Sci U S A* (2006) **103**:732–7. doi:10.1073/pnas.0506655103
27. Bauerfeind P, Garner R, Dunn BE, Mobley HL. Synthesis and activity of *Helicobacter pylori* urease and catalase at low pH. *Gut* (1997) **40**:25–30.
28. Marshall BJ, Barrett LJ, Prakash C, Mccallum RW, Guerrant RL. Urea protects *Helicobacter* (*Campylobacter*) *pylori* from the bactericidal effect of acid. *Gastroenterology* (1990) **99**:697–702.
29. Merrell DS, Goodrich ML, Otto G, Tompkins LS, Falkow S. pH-regulated gene expression of the gastric pathogen *Helicobacter pylori*. *Infect Immun* (2003) **71**:3529–39. doi:10.1128/IAI.71.6.3529-3539.2003
30. Ilver D, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, et al. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science* (1998) **279**:373–7. doi:10.1126/science.279.5349.373
31. Mahdavi J, Sonnen B, Hurtig M, Olfat FO, Forsberg L, Roche N, et al. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. *Science* (2002) **297**:573–8. doi:10.1126/science.1069076
32. Putsep K, Branden CI, Boman HG, Normark S. Antibacterial peptide from *H. pylori*. *Nature* (1999) **398**:671–2. doi:10.1038/19439
33. Gebert B, Fischer W, Weiss E, Hoffmann R, Haas R. *Helicobacter pylori* vacuolating cytotoxin inhibits T lymphocyte activation. *Science* (2003) **301**:1099–102. doi:10.1126/science.1086871
34. Sundrud MS, Torres VJ, Unutmaz D, Cover TL. Inhibition of primary human T cell proliferation by *Helicobacter pylori* vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc Natl Acad Sci U S A* (2004) **101**:7727–32. doi:10.1073/pnas.0401528101
35. Supajatura V, Ushio H, Wada A, Yahiro K, Okumura K, Ogawa H, et al. Cutting edge: VacA, a vacuolating cytotoxin of *Helicobacter pylori*, directly activates mast cells for migration and production of proinflammatory cytokines. *J Immunol* (2002) **168**:2603–7. doi:10.4049/jimmunol.168.6.2603
36. Torres VJ, Vancompernolle SE, Sundrud MS, Unutmaz D, Cover TL. *Helicobacter pylori* vacuolating cytotoxin inhibits activation-induced proliferation of human T and B lymphocyte subsets. *J Immunol* (2007) **179**:5433–40. doi:10.4049/jimmunol.179.8.5433
37. Schmee C, Prinz C, Treptau T, Rad R, Hengst L, Voland P, et al. Inhibition of T-cell proliferation by *Helicobacter pylori* gamma-glutamyl transpeptidase. *Gastroenterology* (2007) **132**:1820–33. doi:10.1053/j.gastro.2007.02.031
38. Gobert AP, McGee DJ, Akhtar M, Mendz GL, Newton JC, Cheng Y, et al. *Helicobacter pylori* arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. *Proc Natl Acad Sci U S A* (2001) **98**:13844–9. doi:10.1073/pnas.241443798
39. Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, et al. cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci U S A* (1996) **93**:14648–53. doi:10.1073/pnas.93.25.14648
40. Akopyants NS, Clifton SW, Kersulyte D, Crabtree JE, Youree BE, Reece CA, et al. Analyses of the cag pathogenicity island of *Helicobacter pylori*. *Mol Microbiol* (1998) **28**:37–53. doi:10.1046/j.1365-2958.1998.00770.x
41. Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, et al. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proc Natl Acad Sci U S A* (1993) **90**:5791–5. doi:10.1073/pnas.90.12.5791
42. Tomb JE, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, et al. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* (1997) **388**:539–47. doi:10.1038/41483
43. Odenbreit S, Puls J, Sedlmaier B, Gerland E, Fischer W, Haas R. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* (2000) **287**:1497–500. doi:10.1126/science.287.5457.1497
44. Posselt G, Backert S, Wessler S. The functional interplay of *Helicobacter pylori* factors with gastric epithelial cells induces a multi-step process in pathogenesis. *Cell Commun Signal* (2013) **11**:77. doi:10.1186/1478-811X-11-77
45. Higashi H, Tsutsumi R, Muto S, Sugiyama T, Azuma T, Asaka M, et al. SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. *Science* (2002) **295**:683–6. doi:10.1126/science.1067147
46. Murata-Kamiya N, Kurashima Y, Teishikata Y, Yamahashi Y, Saito Y, Higashi H, et al. *Helicobacter pylori* CagA interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene* (2007) **26**:4617–26. doi:10.1038/sj.onc.1210251
47. Segal ED, Cha J, Lo J, Falkow S, Tompkins LS. Altered states: involvement of phosphorylated CagA in the induction of host cellular growth changes by *Helicobacter pylori*. *Proc Natl Acad Sci U S A* (1999) **96**:14559–64. doi:10.1073/pnas.96.25.14559
48. Blaser MJ, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, et al. Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res* (1995) **55**:2111–5.
49. Figueiredo C, Machado JC, Pharoah P, Seruca R, Sousa S, Carvalho R, et al. *Helicobacter pylori* and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. *J Natl Cancer Inst* (2002) **94**:1680–7. doi:10.1093/jnci/94.22.1680
50. Kuipers EJ, Uterlinde AM, Pena AS, Roosendaal R, Pals G, Nelis GF, et al. Long-term sequelae of *Helicobacter pylori* gastritis. *Lancet* (1995) **345**:1525–8. doi:10.1016/S0140-6736(95)91084-0
51. Plummer M, Van Doorn LJ, Franceschi S, Kleter B, Canzian F, Vivas J, et al. *Helicobacter pylori* cytotoxin-associated genotype and gastric precancerous lesions. *J Natl Cancer Inst* (2007) **99**:1328–34. doi:10.1093/jnci/djm120
52. Nomura A, Stemmermann GN, Chyou PH, Kato I, Perez-Perez GI, Blaser MJ. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. *N Engl J Med* (1991) **325**:1132–6. doi:10.1056/NEJM199110173251604
53. Nomura AM, Lee J, Stemmermann GN, Nomura RY, Perez-Perez GI, Blaser MJ. *Helicobacter pylori* CagA seropositivity and gastric carcinoma risk in a Japanese American population. *J Infect Dis* (2002) **186**:1138–44. doi:10.1086/343808
54. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, et al. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* (1991) **325**:1127–31. doi:10.1056/NEJM199110173251603
55. Pinto-Santini D, Salama NR. The biology of *Helicobacter pylori* infection, a major risk factor for gastric adenocarcinoma. *Cancer Epidemiol Biomarkers Prev* (2005) **14**:1853–8. doi:10.1158/1055-9965.EPI-04-0784
56. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, et al. *Helicobacter pylori* infection and the development of gastric cancer. *N Engl J Med* (2001) **345**:784–9. doi:10.1056/NEJMoa001999
57. Parsonnet J, Friedman GD, Orentreich N, Vogelman H. Risk for gastric cancer in people with CagA positive or CagA negative *Helicobacter pylori* infection. *Gut* (1997) **40**:297–301.

58. Seoane A, Bessa X, Balleste B, O'Callaghan E, Panades A, Alameda F, et al. [Helicobacter pylori and gastric cancer: relationship with histological subtype and tumor location]. *Gastroenterol Hepatol* (2005) **28**:60–4. doi:10.1157/13070701
59. Bornschein J, Selgrad M, Warnecke M, Kuester D, Wex T, Malfertheiner P. *H. pylori* infection is a key risk factor for proximal gastric cancer. *Dig Dis Sci* (2010) **55**:3124–31. doi:10.1007/s10620-010-1351-x
60. Ekstrom AM, Held M, Hansson LE, Engstrand L, Nyren O. *Helicobacter pylori* in gastric cancer established by CagA immunoblot as a marker of past infection. *Gastroenterology* (2001) **121**:784–91. doi:10.1053/gast.2001.27999
61. Siman JH, Engstrand L, Berglund G, Forsgren A, Floren CH. *Helicobacter pylori* and CagA seropositivity and its association with gastric and oesophageal carcinoma. *Scand J Gastroenterol* (2007) **42**:933–40. doi:10.1080/00365520601173863
62. Coffelt SB, Lewis CE, Naldini L, Brown JM, Ferrara N, De Palma M. Elusive identities and overlapping phenotypes of proangiogenic myeloid cells in tumors. *Am J Pathol* (2010) **176**:1564–76. doi:10.2353/ajpath.2010.090786
63. De Palma M, Murdoch C, Venneri MA, Naldini L, Lewis CE. Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. *Trends Immunol* (2007) **28**:519–24. doi:10.1016/j.it.2007.09.004
64. Denardo DG, Andreu P, Coussens LM. Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev* (2010) **29**:309–16. doi:10.1007/s10555-010-9223-6
65. Johansson M, Denardo DG, Coussens LM. Polarized immune responses differentially regulate cancer development. *Immunol Rev* (2008) **222**:145–54. doi:10.1111/j.1600-065X.2008.00600.x
66. Wu MS, Chen CJ, Lin JT. Host-environment interactions: their impact on progression from gastric inflammation to carcinogenesis and on development of new approaches to prevent and treat gastric cancer. *Cancer Epidemiol Biomarkers Prev* (2005) **14**:1878–82. doi:10.1158/1055-9965.EPI-04-0792
67. Coussens LM, Werb Z. Inflammation and cancer. *Nature* (2002) **420**:860–7. doi:10.1038/nature01322
68. Tsuji S, Kawai N, Tsuji M, Kawano S, Hori M. Review article: inflammation-related promotion of gastrointestinal carcinogenesis – a perigenetic pathway. *Aliment Pharmacol Ther* (2003) **18**(Suppl 1):82–9. doi:10.1046/j.1365-2036.18.s1.22.x
69. Correa P, Haenszel W, Cuello C, Zavala D, Fontham E, Zarama G, et al. Gastric precancerous process in a high risk population: cross-sectional studies. *Cancer Res* (1990) **50**:4731–6.
70. Dinarello CA. Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. *J Endotoxin Res* (2004) **10**:201–22. doi:10.1179/096805104225006129
71. Helmby H, Grencis RK. Interleukin 1 plays a major role in the development of Th2-mediated immunity. *Eur J Immunol* (2004) **34**:3674–81. doi:10.1002/eji.200425452
72. Hwang IR, Kodama T, Kikuchi S, Sakai K, Peterson LE, Graham DY, et al. Effect of interleukin 1 polymorphisms on gastric mucosal interleukin 1 β production in *Helicobacter pylori* infection. *Gastroenterology* (2002) **123**:1793–803. doi:10.1053/gast.2002.37043
73. Camargo MC, Mera R, Correa P, Peek RM Jr, Fontham ET, Goodman KJ, et al. Interleukin-1 β and interleukin-1 receptor antagonist gene polymorphisms and gastric cancer: a meta-analysis. *Cancer Epidemiol Biomarkers Prev* (2006) **15**:1674–87. doi:10.1158/1055-9965.EPI-06-0189
74. Xue H, Lin B, Ni P, Xu H, Huang G. Interleukin-1B and interleukin-1RN polymorphisms and gastric carcinoma risk: a meta-analysis. *J Gastroenterol Hepatol* (2010) **25**:1604–17. doi:10.1111/j.1440-1746.2010.06428.x
75. Menu P, Vince JE. The NLRP3 inflammasome in health and disease: the good, the bad and the ugly. *Clin Exp Immunol* (2011) **166**:1–15. doi:10.1111/j.1365-2249.2011.04440.x
76. Hartland S, Newton JL, Griffin SM, Donaldson PT. A functional polymorphism in the interleukin-1 receptor-1 gene is associated with increased risk of *Helicobacter pylori* infection but not with gastric cancer. *Dig Dis Sci* (2004) **49**:1545–50. doi:10.1023/B:DDAS.0000042262.14969.2d
77. Peleteiro B, Lunet N, Carrilho C, Duraes C, Machado JC, La Vecchia C, et al. Association between cytokine gene polymorphisms and gastric precancerous lesions: systematic review and meta-analysis. *Cancer Epidemiol Biomarkers Prev* (2010) **19**:762–76. doi:10.1158/1055-9965.EPI-09-0917
78. Persson C, Canedo P, Machado JC, El-Omar EM, Forman D. Polymorphisms in inflammatory response genes and their association with gastric cancer: a HuGE systematic review and meta-analyses. *Am J Epidemiol* (2011) **173**:259–70. doi:10.1093/aje/kwq370
79. Yuzhalin A. The role of interleukin DNA polymorphisms in gastric cancer. *Hum Immunol* (2011) **72**:1128–36. doi:10.1016/j.humimm.2011.08.003
80. Wang J, He W, Liu J, Nong L, Wei Y, Yang F. Association of IL-6 polymorphisms with gastric cancer risk: evidences from a meta-analysis. *Cytokine* (2012) **59**:176–83. doi:10.1016/j.cyto.2012.03.032
81. Yin YW, Hu AM, Sun QQ, Liu HL, Wang Q, Zeng YH, et al. Association between interleukin-6 gene -174 G/C polymorphism and the risk of coronary heart disease: a meta-analysis of 20 studies including 9619 cases and 10,919 controls. *Gene* (2012) **503**:25–30. doi:10.1016/j.gene.2012.04.075
82. Da Costa DM, Neves-Filho EH, Alves MK, Rabenhorst SH. Interleukin polymorphisms and differential methylation status in gastric cancer: an association with *Helicobacter pylori* infection. *Epigenomics* (2013) **5**:167–75. doi:10.2217/epi.13.7
83. Xue H, Liu J, Lin B, Wang Z, Sun J, Huang G. A meta-analysis of interleukin-8 -251 promoter polymorphism associated with gastric cancer risk. *PLoS One* (2012) **7**:e28083. doi:10.1371/journal.pone.0028083
84. Ni P, Xu H, Xue H, Lin B, Lu Y. A meta-analysis of interleukin-10-1082 promoter polymorphism associated with gastric cancer risk. *DNA Cell Biol* (2012) **31**:582–91. doi:10.1089/dna.2011.1440
85. Yu Z, Liu Q, Huang C, Wu M, Li G. The interleukin 10 –819C/T polymorphism and cancer risk: a HuGE review and meta-analysis of 73 studies including 15,942 cases and 22,336 controls. *OMICS* (2013) **17**:200–14. doi:10.1089/omi.2012.0089
86. Zhuang W, Wu XT, Zhou Y, Liu L, Liu GJ, Wu TX, et al. Interleukin 10 –592 promoter polymorphism associated with gastric cancer among Asians: a meta-analysis of epidemiologic studies. *Dig Dis Sci* (2010) **55**:1525–32. doi:10.1007/s10620-009-0922-1
87. Yuzhalin AE, Kutikhin AG. Interleukin-12: clinical usage and molecular markers of cancer susceptibility. *Growth Factors* (2012) **30**:176–91. doi:10.3109/08977194.2012.678843
88. Haghshenas MR, Hosseini SV, Mahmoudi M, Saberi-Firozi M, Farjadian S, Ghaderi A. IL-18 serum level and IL-18 promoter gene polymorphism in Iranian patients with gastrointestinal cancers. *J Gastroenterol Hepatol* (2009) **24**:1119–22. doi:10.1111/j.1440-1746.2009.05791.x
89. Gorouhi F, Islami F, Bahrami H, Kamangar F. Tumour-necrosis factor- α polymorphisms and gastric cancer risk: a meta-analysis. *Br J Cancer* (2008) **98**:1443–51. doi:10.1038/sj.bjc.6604277
90. Saleh M, Trinchieri G. Innate immune mechanisms of colitis and colitis-associated colorectal cancer. *Nat Rev Immunol* (2011) **11**:9–20. doi:10.1038/nri2891
91. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol* (2011) **29**:707–35. doi:10.1146/annurev-immunol-031210-101405
92. Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, et al. IFI16 is an innate immune sensor for intracellular DNA. *Nat Immunol* (2010) **11**:997–1004. doi:10.1038/ni.1932
93. Schroder K, Tschoch J. The inflammasomes. *Cell* (2010) **140**:821–32. doi:10.1016/j.cell.2010.01.040
94. Martinon F, Mayor A, Tschoch J. The inflammasomes: guardians of the body. *Annu Rev Immunol* (2009) **27**:229–65. doi:10.1146/annurev.immunol.021908.132715
95. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* (2003) **21**:335–76. doi:10.1146/annurev.immunol.21.120601.141126
96. Basak C, Pathak SK, Bhattacharyya A, Mandal D, Pathak S, Kundu M. NF- κ B- and C/EBP β -driven interleukin-1 β gene expression and PAK1-mediated caspase-1 activation play essential roles in interleukin-1 β release from *Helicobacter pylori* lipopolysaccharide-stimulated macrophages. *J Biol Chem* (2005) **280**:4279–88. doi:10.1074/jbc.M412820200
97. Kawahara T, Teshima S, Oka A, Sugiyama T, Kishi K, Rokutan K. Type I *Helicobacter pylori* lipopolysaccharide stimulates toll-like receptor 4 and activates mitogen oxidase 1 in gastric pit cells. *Infect Immun* (2001) **69**:4382–9. doi:10.1128/IAI.69.7.4382-4389.2001
98. Maeda S, Akanuma M, Mitsuno Y, Hirata Y, Ogura K, Yoshida H, et al. Distinct mechanism of *Helicobacter pylori*-mediated NF- κ B activation between gastric cancer cells and monocytic cells. *J Biol Chem* (2001) **276**:44856–64. doi:10.1074/jbc.M105381200

99. Su B, Ceponis PJ, Lebel S, Huynh H, Sherman PM. *Helicobacter pylori* activates Toll-like receptor 4 expression in gastrointestinal epithelial cells. *Infect Immun* (2003) **71**:3496–502. doi:10.1128/IAI.71.6.3496-3502.2003
100. Thomas CJ, Kapoor M, Sharma S, Bausinger H, Zylian U, Lipsker D, et al. Evidence of a trimolecular complex involving LPS, LPS binding protein and soluble CD14 as an effector of LPS response. *FEBS Lett* (2002) **531**:184–8. doi:10.1016/S0014-5793(02)03499-3
101. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* (2004) **4**:499–511. doi:10.1038/nri1391
102. Kawai T, Takeuchi O, Fujita T, Inoue J, Muhradt PF, Sato S, et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol* (2001) **167**:5887–94. doi:10.4049/jimmunol.167.10.5887
103. Basu S, Pathak SK, Chatterjee G, Pathak S, Basu J, Kundu M. *Helicobacter pylori* protein HP0175 transactivates epidermal growth factor receptor through TLR4 in gastric epithelial cells. *J Biol Chem* (2008) **283**:32369–76. doi:10.1074/jbc.M805053200
104. Chochi K, Ichikura T, Kinoshita M, Majima T, Shinomiya N, Tsujimoto H, et al. *Helicobacter pylori* augments growth of gastric cancers via the lipopolysaccharide-toll-like receptor 4 pathway whereas its lipopolysaccharide attenuates antitumor activities of human mononuclear cells. *Clin Cancer Res* (2008) **14**:2909–17. doi:10.1158/1078-0432.CCR-07-4467
105. Ishihara S, Rumi MA, Kadokawa Y, Ortega-Cava CF, Yuki T, Yoshino N, et al. Essential role of MD-2 in TLR4-dependent signaling during *Helicobacter pylori*-associated gastritis. *J Immunol* (2004) **173**:1406–16. doi:10.4049/jimmunol.173.2.1406
106. Cunningham MD, Seachord C, Ratcliffe K, Bainbridge B, Aruffo A, Darveau RP. *Helicobacter pylori* and *Porphyromonas gingivalis* lipopolysaccharides are poorly transferred to recombinant soluble CD14. *Infect Immun* (1996) **64**:3601–8.
107. Smith MF Jr, Mitchell A, Li G, Ding S, Fitzmaurice AM, Ryan K, et al. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF-kappa B activation and chemokine expression by epithelial cells. *J Biol Chem* (2003) **278**:32552–60. doi:10.1074/jbc.M305536200
108. Chang YJ, Wu MS, Lin JT, Sheu BS, Muta T, Inoue H, et al. Induction of cyclooxygenase-2 overexpression in human gastric epithelial cells by *Helicobacter pylori* involves TLR2/TLR9 and c-Src-dependent nuclear factor-kappaB activation. *Mol Pharmacol* (2004) **66**:1465–77. doi:10.1124/mol.104.005199
109. Gobert AP, Bambou JC, Werts C, Balloy V, Chignard M, Moran AP, et al. *Helicobacter pylori* heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism. *J Biol Chem* (2004) **279**:245–50. doi:10.1074/jbc.M307858200
110. Lepper PM, Triantafilou M, Schumann C, Schneider EM, Triantafilou K. Lipopolysaccharides from *Helicobacter pylori* can act as antagonists for Toll-like receptor 4. *Cell Microbiol* (2005) **7**:519–28. doi:10.1111/j.1462-5822.2005.00482.x
111. Mandell L, Moran AP, Cocchiarella A, Houghton J, Taylor N, Fox JG, et al. Intact gram-negative *Helicobacter pylori*, *Helicobacter felis*, and *Helicobacter hepaticus* bacteria activate innate immunity via toll-like receptor 2 but not toll-like receptor 4. *Infect Immun* (2004) **72**:6446–54. doi:10.1128/IAI.72.11.6446-6454.2004
112. Rad R, Ballhorn W, Voland P, Eisenacher K, Mages J, Rad L, et al. Extracellular and intracellular pattern recognition receptors cooperate in the recognition of *Helicobacter pylori*. *Gastroenterology* (2009) **136**:2247–57. doi:10.1053/j.gastro.2009.02.066
113. Chaouche-Drider N, Kaparakis M, Karrar A, Fernandez MI, Carneiro LA, Viala J, et al. A commensal *Helicobacter* sp. of the rodent intestinal flora activates TLR2 and NOD1 responses in epithelial cells. *PLoS One* (2009) **4**:e5396. doi:10.1371/journal.pone.0005396
114. Sayi A, Kohler E, Toller IM, Flavell RA, Muller W, Roers A, et al. TLR-2-activated B cells suppress *Helicobacter*-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells. *J Immunol* (2011) **186**:878–90. doi:10.4049/jimmunol.1002269
115. Smith SM, Moran AP, Duggan SP, Ahmed SE, Mohamed AS, Windle HJ, et al. Tribbles 3: a novel regulator of TLR2-mediated signaling in response to *Helicobacter pylori* lipopolysaccharide. *J Immunol* (2011) **186**:2462–71. doi:10.4049/jimmunol.1000864
116. Obonyo M, Sabet M, Cole SP, Ebmeyer J, Uematsu S, Akira S, et al. Deficiencies of myeloid differentiation factor 88, Toll-like receptor 2 (TLR2), or TLR4 produce specific defects in macrophage cytokine secretion induced by *Helicobacter pylori*. *Infect Immun* (2007) **75**:2408–14. doi:10.1128/IAI.01794-06
117. Takenaka R, Yokota K, Ayada K, Mizuno M, Zhao Y, Fujinami Y, et al. *Helicobacter pylori* heat-shock protein 60 induces inflammatory responses through the Toll-like receptor-triggered pathway in cultured human gastric epithelial cells. *Microbiology* (2004) **150**:3913–22. doi:10.1099/mic.0.27527-0
118. Yokota S, Okabayashi T, Rehli M, Fujii N, Amano K. *Helicobacter pylori* lipopolysaccharides upregulate toll-like receptor 4 expression and proliferation of gastric epithelial cells via the MEK1/2-ERK1/2 mitogen-activated protein kinase pathway. *Infect Immun* (2010) **78**:468–76. doi:10.1128/IAI.00903-09
119. Otani K, Tanigawa T, Watanabe T, Nadatani Y, Sogawa M, Yamagami H, et al. Toll-like receptor 9 signaling has anti-inflammatory effects on the early phase of *Helicobacter pylori*-induced gastritis. *Biochem Biophys Res Commun* (2012) **426**:342–9. doi:10.1016/j.bbrc.2012.08.080
120. Lagunes-Servin H, Torres J, Maldonado-Bernal C, Perez-Rodriguez M, Huerta-Yepez S, Madrazo De La Garza A, et al. Toll-like receptors and cytokines are upregulated during *Helicobacter pylori* infection in children. *Helicobacter* (2013) **18**:423–32. doi:10.1111/hel.12067
121. Gewirtz AT, Yu Y, Krishna US, Israel DA, Lyons SL, Peek RM Jr. *Helicobacter pylori* flagellin evades toll-like receptor 5-mediated innate immunity. *J Infect Dis* (2004) **189**:1914–20. doi:10.1086/386289
122. Kumar Pachathundikandi S, Brandt S, Madassery J, Backert S. Induction of TLR-2 and TLR-5 expression by *Helicobacter pylori* switches cagPAI-dependent signalling leading to the secretion of IL-8 and TNF-alpha. *PLoS One* (2011) **6**:e19614. doi:10.1371/journal.pone.0019614
123. Andersen-Nissen E, Smith KD, Strobe KL, Barrett SL, Cookson BT, Logan SM, et al. Evasion of Toll-like receptor 5 by flagellated bacteria. *Proc Natl Acad Sci U S A* (2005) **102**:9247–52. doi:10.1073/pnas.0502040102
124. Muotiala A, Helander IM, Pyhala L, Kosunen TU, Moran AP. Low biological activity of *Helicobacter pylori* lipopolysaccharide. *Infect Immun* (1992) **60**:1714–6.
125. Moran AP, Lindner B, Walsh EJ. Structural characterization of the lipid A component of *Helicobacter pylori* rough- and smooth-form lipopolysaccharides. *J Bacteriol* (1997) **179**:6453–63.
126. Schmausser B, Andrusis M, Endrich S, Muller-Hermelin HK, Eck M. Toll-like receptors TLR4, TLR5 and TLR9 on gastric carcinoma cells: an implication for interaction with *Helicobacter pylori*. *Int J Med Microbiol* (2005) **295**:179–85. doi:10.1016/j.ijmm.2005.02.009
127. Pimentel-Nunes P, Afonso L, Lopes P, Roncon-Albuquerque R Jr, Goncalves N, Henrique R, et al. Increased expression of toll-like receptors (TLR) 2, 4 and 5 in gastric dysplasia. *Pathol Oncol Res* (2011) **17**:677–83. doi:10.1007/s12253-011-9368-9
128. Pimentel-Nunes P, Goncalves N, Boal-Carvalho I, Afonso L, Lopes P, Roncon-Albuquerque R Jr, et al. *Helicobacter pylori* induces increased expression of Toll-like receptors and decreased Toll-interacting protein in gastric mucosa that persists throughout gastric carcinogenesis. *Helicobacter* (2013) **18**:22–32. doi:10.1111/hel.12008
129. Fernandez-Garcia B, Eiro N, Gonzalez-Reyes S, Gonzalez L, Aguirre A, Gonzalez LO, et al. Clinical significance of toll-like receptor 3, 4, and 9 in gastric cancer. *J Immunother* (2014) **37**:77–83. doi:10.1097/CJI.0000000000000016
130. Yakut M, Ormeci N, Erdal H, Keskin O, Karayel Z, Tutkak H, et al. The association between precancerous gastric lesions and serum pepsinogens, serum gastrin, vascular endothelial growth factor, serum interleukin-1 Beta, serum toll-like receptor-4 levels and *Helicobacter pylori* Cag A status. *Clin Res Hepatol Gastroenterol* (2013) **37**:302–11. doi:10.1016/j.clinre.2012.09.013
131. Song EJ, Kang MJ, Kim YS, Kim SM, Lee SE, Kim CH, et al. Flagellin promotes the proliferation of gastric cancer cells via the Toll-like receptor 5. *Int J Mol Med* (2011) **28**:115–9. doi:10.3892/ijmm.2011.656
132. Tye H, Kennedy CL, Najdovska M, Mcleod L, McCormack W, Hughes N, et al. STAT3-driven upregulation of TLR2 promotes gastric tumorigenesis independent of tumor inflammation. *Cancer Cell* (2012) **22**:466–78. doi:10.1016/j.ccr.2012.08.010
133. Li K, Dan Z, Hu X, Gesang L, Ze Y, Bianba Z. CD14 regulates gastric cancer cell epithelial mesenchymal transition and invasion in vitro. *Oncol Rep* (2013) **30**:2725–32. doi:10.3892/or.2013.2733

134. Wu WK, Sung JJ, Lee CW, Yu J, Cho CH. Cyclooxygenase-2 in tumorigenesis of gastrointestinal cancers: an update on the molecular mechanisms. *Cancer Lett* (2010) **295**:7–16. doi:10.1016/j.canlet.2010.03.015
135. Schumacker PT. Reactive oxygen species in cancer cells: live by the sword, die by the sword. *Cancer Cell* (2006) **10**:175–6. doi:10.1016/j.ccr.2006.08.015
136. Xia C, Meng Q, Liu LZ, Rojanasakul Y, Wang XR, Jiang BH. Reactive oxygen species regulate angiogenesis and tumor growth through vascular endothelial growth factor. *Cancer Res* (2007) **67**:10823–30. doi:10.1158/0008-5472.CAN-07-0783
137. Park JH, Kim TY, Jong HS, Chun YS, Park JW, Lee CT, et al. Gastric epithelial reactive oxygen species prevent normoxic degradation of hypoxia-inducible factor-1alpha in gastric cancer cells. *Clin Cancer Res* (2003) **9**:433–40.
138. Yuan X, Zhou Y, Wang W, Li J, Xie G, Zhao Y, et al. Activation of TLR4 signaling promotes gastric cancer progression by inducing mitochondrial ROS production. *Cell Death Dis* (2013) **4**:e794. doi:10.1038/cddis.2013.334
139. Kutikhin AG. Impact of Toll-like receptor 4 polymorphisms on risk of cancer. *Hum Immunol* (2011) **72**:193–206. doi:10.1016/j.humimm.2010.11.003
140. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, et al. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* (2002) **277**:15028–34. doi:10.1074/jbc.M200497200
141. Guillot L, Balloy V, McCormack FX, Golenbock DT, Chignard M, Si-Tahar M. Cutting edge: the immunostimulatory activity of the lung surfactant protein-A involves Toll-like receptor 4. *J Immunol* (2002) **168**:5989–92. doi:10.4049/jimmunol.168.12.5989
142. Johnson GB, Brunn GJ, Kodaira Y, Platt JL. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. *J Immunol* (2002) **168**:5233–9. doi:10.4049/jimmunol.168.10.5233
143. Smiley ST, King JA, Hancock WW. Fibrinogen stimulates macrophage chemokine secretion through toll-like receptor 4. *J Immunol* (2001) **167**:2887–94. doi:10.4049/jimmunol.167.5.2887
144. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, et al. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med* (2002) **195**:99–111. doi:10.1084/jem.20001858
145. Vabulas RM, Ahmad-Nejad P, Da Costa C, Miethke T, Kirschning CJ, Hacker H, et al. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *J Biol Chem* (2001) **276**:31332–9. doi:10.1074/jbc.M103217200
146. Baniyash M. TCR zeta-chain downregulation: curtailing an excessive inflammatory immune response. *Nat Rev Immunol* (2004) **4**:675–87. doi:10.1038/nri1434
147. Kim CW, Choi SH, Chung EJ, Lee MJ, Byun EK, Ryu MH, et al. Alteration of signal-transducing molecules and phenotypical characteristics in peripheral blood lymphocytes from gastric carcinoma patients. *Pathobiology* (1999) **67**:123–8. doi:10.1159/000028061
148. Takahashi A, Kono K, Amemiya H, Iizuka H, Fujii H, Matsumoto Y. Elevated caspase-3 activity in peripheral blood T cells coexists with increased degree of T-cell apoptosis and down-regulation of TCR zeta molecules in patients with gastric cancer. *Clin Cancer Res* (2001) **7**:74–80.
149. Matsuda M, Petersson M, Lenkei R, Taupin JL, Magnusson I, Mellstedt H, et al. Alterations in the signal-transducing molecules of T cells and NK cells in colorectal tumor-infiltrating, gut mucosal and peripheral lymphocytes: correlation with the stage of the disease. *Int J Cancer* (1995) **61**:765–72. doi:10.1002/ijc.2910610605
150. Healy CG, Simons JW, Carducci MA, Deweese TL, Bartkowiak M, Tong KP, et al. Impaired expression and function of signal-transducing zeta chains in peripheral T cells and natural killer cells in patients with prostate cancer. *Cytometry* (1998) **32**:doi:10.1002/(SICI)1097-0320(19980601)32:2<109::AID-CYTOG>3.0.CO;2-G
151. Kono K, Ressing ME, Brandt RM, Melief CJ, Potkul RK, Andersson B, et al. Decreased expression of signal-transducing zeta chain in peripheral T cells and natural killer cells in patients with cervical cancer. *Clin Cancer Res* (1996) **2**:1825–8.
152. Schmielau J, Nalesnik MA, Finn OJ. Suppressed T-cell receptor zeta chain expression and cytokine production in pancreatic cancer patients. *Clin Cancer Res* (2001) **7**:933s–9s.
153. Wu Y, Zhao Q, Peng C, Sun L, Li XF, Kuang DM. Neutrophils promote motility of cancer cells via a hyaluronan-mediated TLR4/PI3K activation loop. *J Pathol* (2011) **225**:438–47. doi:10.1002/path.2947
154. Yang CA, Scheibenbogen C, Bauer S, Kleinle C, Wex T, Bornschein J, et al. A frequent Toll-like receptor 1 gene polymorphism affects NK- and T-cell IFN-gamma production and is associated with *Helicobacter pylori*-induced gastric disease. *Helicobacter* (2013) **18**:13–21. doi:10.1111/hel.12001
155. Castaño-Rodríguez N, Kaakoush NO, Goh KL, Fock KM, Mitchell HM. The role of TLR2, TLR4 and CD14 genetic polymorphisms in gastric carcinogenesis: a case-control study and meta-analysis. *PLoS One* (2013) **8**:e60327. doi:10.1371/journal.pone.0060327
156. Tahara T, Arisawa T, Wang F, Shibata T, Nakamura M, Sakata M, et al. Toll-like receptor 2 –196 to 174del polymorphism influences the susceptibility of Japanese people to gastric cancer. *Cancer Sci* (2007) **98**:1790–4. doi:10.1111/j.1349-7006.2007.00590.x
157. de Oliveira JG, Rossi AF, Nizato DM, Miyasaki K, Silva AE. Profiles of gene polymorphisms in cytokines and Toll-like receptors with higher risk for gastric cancer. *Dig Dis Sci* (2013) **58**:978–88. doi:10.1007/s10620-012-2460-5
158. Zeng HM, Pan KF, Zhang Y, Zhang L, Ma JL, Zhou T, et al. Genetic variants of toll-like receptor 2 and 5, *helicobacter pylori* infection, and risk of gastric cancer and its precursors in a Chinese population. *Cancer Epidemiol Biomarkers Prev* (2011) **20**:2594–602. doi:10.1158/1055-9965.EPI-11-0702
159. Hold GL, Rabkin CS, Chow WH, Smith MG, Gammon MD, Risch HA, et al. A functional polymorphism of toll-like receptor 4 gene increases risk of gastric carcinoma and its precursors. *Gastroenterology* (2007) **132**:905–12. doi:10.1053/j.gastro.2006.12.026
160. Santini D, Angeletti S, Ruzzo A, Dicuonzo G, Galluzzo S, Vincenzi B, et al. Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms in gastric cancer of intestinal and diffuse histotypes. *Clin Exp Immunol* (2008) **154**:360–4. doi:10.1111/j.1365-2249.2008.03776.x
161. Huang L, Yuan K, Liu J, Ren X, Dong X, Tian W, et al. Polymorphisms of the TLR4 gene and risk of gastric cancer. *Gene* (2014) **537**:46–50. doi:10.1016/j.gene.2013.12.030
162. Kim J, Cho YA, Choi IJ, Lee YS, Kim SY, Hwang JA, et al. Effects of polymorphisms of innate immunity genes and environmental factors on the risk of noncardia gastric cancer. *Cancer Res Treat* (2013) **45**:313–24. doi:10.4143/crt.2013.45.4.313
163. Wang X, Xue L, Yang Y, Xu L, Zhang G. TLR9 promoter polymorphism is associated with both an increased susceptibility to gastric carcinoma and poor prognosis. *PLoS One* (2013) **8**:e65731. doi:10.1371/journal.pone.0065731
164. Companioni O, Bonet C, Munoz X, Weiderpass E, Panico S, Tumino R, et al. Polymorphisms of *Helicobacter pylori* signaling pathway genes and gastric cancer risk in the European Prospective Investigation into Cancer-Eurast cohort. *Int J Cancer* (2014) **134**:92–101. doi:10.1002/ijc.28357
165. Li K, Dan Z, Hu XJ, Gesang LB, Ze YG, Bianba ZX, et al. Association of CD14-260 polymorphism with gastric cancer risk in Highland Tibetans. *World J Gastroenterol* (2014) **20**:2688–94. doi:10.3748/wjg.v20.i10.2688
166. Tahara T, Arisawa T, Shibata T, Hirata I, Nakano H. Association of polymorphism of TLR4 and CD14 genes with gastroduodenal diseases in Japan. *Inflammopharmacology* (2007) **15**:124–8. doi:10.1007/s10787-006-1567-8
167. Zhao D, Sun T, Zhang X, Guo Y, Yu D, Yang M, et al. Role of CD14 promoter polymorphisms in *Helicobacter pylori* infection – related gastric carcinoma. *Clin Cancer Res* (2007) **13**:2362–8. doi:10.1158/1078-0432.CCR-06-2612
168. Ohara T, Kanoh Y, Tani N, Ohdaira H, Suzuki Y, Kameyama J, et al. Single nucleotide polymorphism typing of the human toll-like receptor 4 gene at the 2-kb upstream region of the 5' untranslated region: new enclosure strategy for the risk grouping of poorly-differentiated gastric adenocarcinoma patients. *Mol Med Rep* (2009) **2**:17–21. doi:10.3892/mmr_00000055
169. Ohara T, Morishita T, Suzuki H, Hibi T. Heterozygous Thr 135 Ala polymorphism at leucine-rich repeat (LRR) in genomic DNA of toll-like receptor 4 in patients with poorly-differentiated gastric adenocarcinomas. *Int J Mol Med* (2006) **18**:59–63.
170. Castaño-Rodríguez N, Kaakoush NO, Pardo AL, Goh KL, Fock KM, Mitchell HM. Genetic polymorphisms in the Toll-like receptor signalling pathway in *Helicobacter pylori* infection and related gastric cancer. *Hum Immunol* (2014). doi:10.1016/j.humimm.2014.06.001

171. Yang JC, Yang HC, Shun CT, Wang TH, Chien CT, Kao JY. Catechins and sialic acid attenuate *Helicobacter pylori*-triggered epithelial caspase-1 activity and eradicate *Helicobacter pylori* infection. *Evid Based Complement Alternat Med* (2013) **2013**:248585. doi:10.1155/2013/248585
172. Hishida A, Matsuo K, Goto Y, Naito M, Wakai K, Tajima K, et al. No associations of Toll-like receptor 2 (TLR2) –196 to –174del polymorphism with the risk of *Helicobacter pylori* seropositivity, gastritis, atrophy, and gastric cancer in Japanese. *Gastric Cancer* (2010) **13**:251–7. doi:10.1007/s10120-010-0567-y
173. Castaño-Rodríguez N, Kaakoush NO, Goh KL, Fock KM, Mitchell HM. The NOD-like receptor signalling pathway in *Helicobacter pylori* infection and related gastric cancer: a case-control study and gene expression analyses. *PLoS One* (2014) **9**:e98899. doi:10.1371/journal.pone.0098899
174. Qadri Q, Rasool R, Afroze D, Naqash S, Gulzar GM, Yousuf A, et al. Study of TLR4 and IL-8 gene polymorphisms in *H. pylori*-induced inflammation in gastric cancer in an ethnic Kashmiri population. *Immunol Invest* (2013) **43**:324–36. doi:10.3109/08820139.2013.854378
175. Schmidt HM, Ha DM, Taylor EF, Kovach Z, Goh KL, Fock KM, et al. Variation in human genetic polymorphisms, their association with *Helicobacter pylori* acquisition and gastric cancer in a multi-ethnic country. *J Gastroenterol Hepatol* (2011) **26**:1725–32. doi:10.1111/j.1440-1746.2011.06799.x
176. Trejo-de la O A, Torres J, Perez-Rodríguez M, Camorlinga-Ponce M, Luna LF, Abdo-Francis JM, et al. TLR4 single-nucleotide polymorphisms alter mucosal cytokine and chemokine patterns in Mexican patients with *Helicobacter pylori*-associated gastroduodenal diseases. *Clin Immunol* (2008) **129**:333–40. doi:10.1016/j.clim.2008.07.009
177. Garza-Gonzalez E, Bosques-Padilla FJ, Mendoza-Ibarra SI, Flores-Gutierrez JP, Maldonado-Garza HJ, Perez-Perez GI. Assessment of the toll-like receptor 4 Asp299Gly, Thr399Ile and interleukin-8 –251 polymorphisms in the risk for the development of distal gastric cancer. *BMC Cancer* (2007) **7**:70. doi:10.1186/1471-2407-7-70
178. Huang H, Wu J, Jin G, Zhang H, Ding Y, Hua Z, et al. A 5'-flanking region polymorphism in toll-like receptor 4 is associated with gastric cancer in a Chinese population. *J Biomed Res* (2010) **24**:100–6. doi:10.1016/S1674-8301(10)60017-6
179. Kim EJ, Lee JR, Chung WC, Jung SH, Sung HJ, Lee YW, et al. Association between genetic polymorphisms of NOD 1 and *Helicobacter pylori*-induced gastric mucosal inflammation in healthy Korean population. *Helicobacter* (2013) **18**:143–50. doi:10.1111/hel.12020
180. Kupcinskas J, Wex T, Bornschein J, Selgrad M, Leja M, Juozaityte E, et al. Lack of association between gene polymorphisms of Angiotensin converting enzyme, Nod-like receptor 1, Toll-like receptor 4, FAS/FASL and the presence of *Helicobacter pylori*-induced premalignant gastric lesions and gastric cancer in Caucasians. *BMC Med Genet* (2011) **12**:112. doi:10.1186/1471-2350-12-112
181. Hishida A, Matsuo K, Goto Y, Mitsuda Y, Hiraki A, Naito M, et al. Toll-like receptor 4 +3725 G/C polymorphism, *Helicobacter pylori* seropositivity, and the risk of gastritis, atrophy and gastric cancer in Japanese. *Helicobacter* (2009) **14**:47–53. doi:10.1111/j.1523-5378.2009.00659.x
182. Hold GL, Rabkin CS, Gammon MD, Berry SH, Smith MG, Lissowska J, et al. CD14-159C/T and TLR9-1237T/C polymorphisms are not associated with gastric cancer risk in Caucasian populations. *Eur J Cancer Prev* (2009) **18**:117–9. doi:10.1097/CEJ.0b013e3283101292
183. Wu MS, Cheng TY, Shun CT, Lin MT, Chen LC, Lin JT. Functional polymorphisms of CD14 and toll-like receptor 4 in Taiwanese Chinese with *Helicobacter pylori*-related gastric malignancies. *Hepatogastroenterology* (2006) **53**:807–10.
184. Rigoli L, Di Bella C, Fedele F, Procopio V, Amorini M, Lo Giudice G, et al. TLR4 and NOD2/CARD15 genetic polymorphisms and their possible role in gastric carcinogenesis. *Anticancer Res* (2010) **30**:513–7.
185. Achyut BR, Ghoshal UC, Moorchung N, Mittal B. Association of Toll-like receptor-4 (Asp299Gly and Thr399Ile) gene polymorphisms with gastritis and precancerous lesions. *Hum Immunol* (2007) **68**:901–7. doi:10.1016/j.humimm.2007.10.006
186. Fan YF, Wu YM, Liu H, Yu Y, Jiang YY, Xue YZ, et al. TLR4 polymorphisms associated with developing gastric pre-cancer lesions in a Chinese Han population. *Hum Immunol* (2014) **75**:176–81. doi:10.1016/j.humimm.2013.11.002
187. Kato I, Canzian F, Plummer M, Franceschi S, Van Doorn LJ, Vivas J, et al. Polymorphisms in genes related to bacterial lipopolysaccharide/peptidoglycan signaling and gastric precancerous lesions in a population at high risk for gastric cancer. *Dig Dis Sci* (2007) **52**:254–61. doi:10.1007/s10620-006-9303-1
188. Murphy G, Thornton J, Mcmanus R, Swan N, Ryan B, Hughes DJ, et al. Association of gastric disease with polymorphisms in the inflammatory-related genes IL-1B, IL-1RN, IL-10, TNF and TLR4. *Eur J Gastroenterol Hepatol* (2009) **21**:630–5. doi:10.1097/MEG.0b013e3283140eea
189. Hofner P, Gyulai Z, Kiss ZF, Tiszai A, Tisztavicz L, Toth G, et al. Genetic polymorphisms of NOD1 and IL-8, but not polymorphisms of TLR4 genes, are associated with *Helicobacter pylori*-induced duodenal ulcer and gastritis. *Helicobacter* (2007) **12**:124–31. doi:10.1111/j.1523-5378.2007.00481.x
190. Cheng C, Lingyan W, Yi H, Cheng Z, Huadian Y, Xuting X, et al. Association between TLR2, MTR, MTRR, XPC, TP73, TP53 genetic polymorphisms and gastric cancer: a meta-analysis. *Clin Res Hepatol Gastroenterol* (2014). doi:10.1016/j.clinre.2013.12.009
191. Chen J, Hu S, Liang S, Chen Q, Yang Q, Zheng W, et al. Associations between the four toll-like receptor polymorphisms and the risk of gastric cancer: a meta-analysis. *Cancer Biother Radiopharm* (2013) **28**:674–81. doi:10.1089/cbr.2012.1395
192. Zhao X, Liu L, Kang S, Zhang D. An updated meta-analysis about the association of Asp299Gly in Toll-like receptor 4 gene with risk of cancer. *Eur J Cancer* (2013) **49**:2068–70. doi:10.1016/j.ejca.2013.01.031
193. Zhang K, Zhou B, Wang Y, Rao L, Zhang L. The TLR4 gene polymorphisms and susceptibility to cancer: a systematic review and meta-analysis. *Eur J Cancer* (2013) **49**:946–54. doi:10.1016/j.ejca.2012.09.022
194. Zou TH, Wang ZH, Fang JY. Positive association between Toll-like receptor 4 gene +896A/G polymorphism and susceptibility to gastric carcinogenesis: a meta-analysis. *Tumour Biol* (2013) **34**:2441–50. doi:10.1007/s13277-013-0795-y
195. El-Omar EM, Ng MT, Hold GL. Polymorphisms in Toll-like receptor genes and risk of cancer. *Oncogene* (2008) **27**:244–52. doi:10.1038/sj.onc.1210912
196. Sato K, Yoshimura A, Kaneko T, Ukai T, Ozaki Y, Nakamura H, et al. A single nucleotide polymorphism in 3'-untranslated region contributes to the regulation of Toll-like receptor 4 translation. *J Biol Chem* (2012) **287**:25163–72. doi:10.1074/jbc.M111.338426
197. Liu J, Radler D, Illi S, Klucker E, Turan E, Von Mutius E, et al. TLR2 polymorphisms influence neonatal regulatory T cells depending on maternal atopy. *Allergy* (2011) **66**:1020–9. doi:10.1111/j.1398-9995.2011.02573.x
198. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* (2005) **6**:345–52. doi:10.1038/ni1178
199. Rad R, Brenner L, Bauer S, Schwendy S, Layland L, Da Costa CP, et al. CD25+/Foxp3+ T cells regulate gastric inflammation and *Helicobacter pylori* colonization in vivo. *Gastroenterology* (2006) **131**:525–37. doi:10.1053/j.gastro.2006.05.001
200. Jang TJ. The number of Foxp3-positive regulatory T cells is increased in *Helicobacter pylori* gastritis and gastric cancer. *Pathol Res Pract* (2010) **206**:34–8. doi:10.1016/j.prp.2009.07.019
201. Kandulski A, Wex T, Kuester D, Peitz U, Gebert I, Roessner A, et al. Naturally occurring regulatory T cells (CD4+, CD25high, FOXP3+) in the antrum and cardia are associated with higher *H. pylori* colonization and increased gene expression of TGF-beta1. *Helicobacter* (2008) **13**:295–303. doi:10.1111/j.1523-5378.2008.00612.x
202. Kindlund B, Sjöling A, Hansson M, Edebo A, Hansson LE, Sjovall H, et al. FOXP3-expressing CD4(+) T-cell numbers increase in areas of duodenal gastric metaplasia and are associated to CD4(+) T-cell aggregates in the duodenal of *Helicobacter pylori*-infected duodenal ulcer patients. *Helicobacter* (2009) **14**:192–201. doi:10.1111/j.1523-5378.2009.00673.x
203. Enarsson K, Lundgren A, Kindlund B, Hermansson M, Roncador G, Banham AH, et al. Function and recruitment of mucosal regulatory T cells in human chronic *Helicobacter pylori* infection and gastric adenocarcinoma. *Clin Immunol* (2006) **121**:358–68. doi:10.1016/j.clim.2006.07.002
204. Lundgren A, Stromberg E, Sjöling A, Lindholm C, Enarsson K, Edebo A, et al. Mucosal FOXP3-expressing CD4+ CD25high regulatory T cells in *Helicobacter pylori*-infected patients. *Infect Immun* (2005) **73**:523–31. doi:10.1128/IAI.73.1.523-531.2005
205. Junpee A, Tencomnao T, Sanprasert V, Nuchprayoon S. Association between Toll-like receptor 2 (TLR2) polymorphisms and asymptomatic bancroftian filariasis. *Parasitol Res* (2010) **107**:807–16. doi:10.1007/s00436-010-1932-9
206. Noguchi E, Nishimura F, Fukai H, Kim J, Ichikawa K, Shibasaki M, et al. An association study of asthma and total serum immunoglobulin E levels for Toll-like

- receptor polymorphisms in a Japanese population. *Clin Exp Allergy* (2004) **34**:177–83. doi:10.1111/j.1365-2222.2004.01839.x
207. Tao K, Fujii M, Tsukumo S, Maekawa Y, Kishihara K, Kimoto Y, et al. Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. *Ann Rheum Dis* (2007) **66**:905–9. doi:10.1136/ard.2006.065961
208. Guiney DG, Hasegawa P, Cole SP. *Helicobacter pylori* preferentially induces interleukin 12 (IL-12) rather than IL-6 or IL-10 in human dendritic cells. *Infect Immun* (2003) **71**:4163–6. doi:10.1128/IAI.71.7.4163-4166.2003
209. Mohammadi M, Nedrud J, Redline R, Lycke N, Czinn SJ. Murine CD4 T-cell response to *Helicobacter* infection: TH1 cells enhance gastritis and TH2 cells reduce bacterial load. *Gastroenterology* (1997) **113**:1848–57. doi:10.1016/S0016-5085(97)70004-0
210. Smythies LE, Waites KB, Lindsey JR, Harris PR, Ghiara P, Smith PD. *Helicobacter pylori*-induced mucosal inflammation is Th1 mediated and exacerbated in IL-4, but not IFN-gamma, gene-deficient mice. *J Immunol* (2000) **165**:1022–9. doi:10.4049/jimmunol.165.2.1022
211. Bamford KB, Fan X, Crowe SE, Leary JF, Gourley WK, Luthra GK, et al. Lymphocytes in the human gastric mucosa during *Helicobacter pylori* have a T helper cell 1 phenotype. *Gastroenterology* (1998) **114**:482–92. doi:10.1016/S0016-5085(98)70531-1
212. Eaton KA, Mefford M, Thevenot T. The role of T cell subsets and cytokines in the pathogenesis of *Helicobacter pylori* gastritis in mice. *J Immunol* (2001) **166**:7456–61. doi:10.4049/jimmunol.166.12.7456
213. Hafsi N, Voland P, Schwendy S, Rad R, Reindl W, Gerhard M, et al. Human dendritic cells respond to *Helicobacter pylori*, promoting NK cell and Th1-effector responses in vitro. *J Immunol* (2004) **173**:1249–57. doi:10.4049/jimmunol.173.2.1249
214. Baldini M, Lohman IC, Halonen M, Erickson RP, Holt PG, Martinez FDA. Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. *Am J Respir Cell Mol Biol* (1999) **20**:976–83. doi:10.1165/ajrcmb.20.5.3494
215. Hubacek JA, Rothe G, Pit'ha J, Skodova Z, Stanek V, Poledne R, et al. C(-260)→T polymorphism in the promoter of the CD14 monocyte receptor gene as a risk factor for myocardial infarction. *Circulation* (1999) **99**:3218–20. doi:10.1161/01.CIR.99.25.3218
216. Karhukorpi J, Yan Y, Niemela S, Valtonen J, Koistinen P, Joensuu T, et al. Effect of CD14 promoter polymorphism and *H. pylori* infection and its clinical outcomes on circulating CD14. *Clin Exp Immunol* (2002) **128**:326–32. doi:10.1046/j.1365-2249.2002.01837.x
217. Levan TD, Bloom JW, Bailey TJ, Karp CL, Halonen M, Martinez FD, et al. A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. *J Immunol* (2001) **167**:5838–44. doi:10.4049/jimmunol.167.10.5838
218. Liang XH, Cheung W, Heng CK, Liu JJ, Li CW, Lim B, et al. CD14 promoter polymorphisms have no functional significance and are not associated with atopic phenotypes. *Pharmacogenet Genomics* (2006) **16**:229–36. doi:10.1097/01.fpc.0000197466.14340.0f
219. Leipe DD, Koonin EV, Aravind L. STAND, a class of P-loop NTPases including animal and plant regulators of programmed cell death: multiple, complex domain architectures, unusual phyletic patterns, and evolution by horizontal gene transfer. *J Mol Biol* (2004) **343**:1–28. doi:10.1016/j.jmb.2004.08.023
220. Bella J, Hindle KL, Mcewan PA, Lovell SC. The leucine-rich repeat structure. *Cell Mol Life Sci* (2008) **65**:2307–33. doi:10.1007/s00018-008-8019-0
221. Martinon F, Tschopp J. Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ* (2007) **14**:10–22. doi:10.1038/sj.cdd.4402038
222. Tenthorey JL, Kofoed EM, Daugherty MD, Malik HS, Vance RE. Molecular basis for specific recognition of bacterial ligands by NAIP/NLR4 inflammasomes. *Mol Cell* (2014) **54**:17–29. doi:10.1016/j.molcel.2014.02.018
223. Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, et al. Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat Immunol* (2004) **5**:1166–74. doi:10.1038/ni1131
224. Hutton ML, Kaparakis-Liaskos M, Turner L, Cardona A, Kwok T, Ferrero RL. *Helicobacter pylori* exploits cholesterol-rich microdomains for induction of NF-kappaB-dependent responses and peptidoglycan delivery in epithelial cells. *Infect Immun* (2010) **78**:4523–31. doi:10.1128/IAI.00439-10
225. Kaparakis M, Turnbull L, Carneiro L, Firth S, Coleman HA, Parkington HC, et al. Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. *Cell Microbiol* (2010) **12**:372–85. doi:10.1111/j.1462-5822.2009.01404.x
226. Necchi V, Sommi P, Ricci V, Solcia E. In vivo accumulation of *Helicobacter pylori* products, NOD1, ubiquitinated proteins and proteasome in a novel cytoplasmic structure. *PLoS One* (2010) **5**:e9716. doi:10.1371/journal.pone.0009716
227. Allison CC, Kufer TA, Kremmer E, Kaparakis M, Ferrero RL. *Helicobacter pylori* induces MAPK phosphorylation and AP-1 activation via a NOD1-dependent mechanism. *J Immunol* (2009) **183**:8099–109. doi:10.4049/jimmunol.0900664
228. Allison CC, Ferrand J, Mcleod L, Hassan M, Kaparakis-Liaskos M, Grubman A, et al. Nucleotide oligomerization domain 1 enhances IFN-gamma signaling in gastric epithelial cells during *Helicobacter pylori* infection and exacerbates disease severity. *J Immunol* (2013) **190**:3706–15. doi:10.4049/jimmunol.1200591
229. Boonyanugomol W, Chomvarin C, Hahnvajawong C, Sripa B, Kaparakis-Liaskos M, Ferrero RL. *Helicobacter pylori* cag pathogenicity island (cagPAI) involved in bacterial internalization and IL-8 induced responses via NOD1- and MyD88-dependent mechanisms in human biliary epithelial cells. *PLoS One* (2013) **8**:e77358. doi:10.1371/journal.pone.0077358
230. Grubman A, Kaparakis M, Viala J, Allison C, Badea L, Karrar A, et al. The innate immune molecule, NOD1, regulates direct killing of *Helicobacter pylori* by antimicrobial peptides. *Cell Microbiol* (2010) **12**:626–39. doi:10.1111/j.1462-5822.2009.01421.x
231. Rosenstiel P, Hellwig S, Hampe J, Ott S, Till A, Fischbach W, et al. Influence of polymorphisms in the NOD1/CARD4 and NOD2/CARD15 genes on the clinical outcome of *Helicobacter pylori* infection. *Cell Microbiol* (2006) **8**:1188–98. doi:10.1111/j.1462-5822.2006.00701.x
232. Kim DJ, Park JH, Franchi L, Backert S, Nunez G. The Cag pathogenicity island and interaction between TLR2/NOD2 and NLRP3 regulate IL-1beta production in *Helicobacter pylori* infected dendritic cells. *Eur J Immunol* (2013) **43**:2650–8. doi:10.1002/eji.201243281
233. Tschopp J, Martinon F, Burns K. NALPs: a novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* (2003) **4**:95–104. doi:10.1038/nrm1019
234. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* (2004) **430**:213–8. doi:10.1038/nature02664
235. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* (2002) **10**:417–26. doi:10.1016/S1097-2765(02)00599-3
236. Martinon F, Tschopp J. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* (2004) **117**:561–74. doi:10.1016/j.cell.2004.05.004
237. Tomita T, Jackson AM, Hida N, Hayat M, Dixon MF, Shimoyama T, et al. Expression of Interleukin-18, a Th1 cytokine, in human gastric mucosa is increased in *Helicobacter pylori* infection. *J Infect Dis* (2001) **183**:620–7. doi:10.1086/318541
238. Pothoff A, Ledig S, Martin J, Jandl O, Cornberg M, Obst B, et al. Significance of the caspase family in *Helicobacter pylori* induced gastric epithelial apoptosis. *Helicobacter* (2002) **7**:367–77. doi:10.1046/j.1523-5378.2002.00112.x
239. Benoit BN, Kobayashi M, Kawakubo M, Takeoka M, Sano K, Zou J, et al. Role of ASC in the mouse model of *Helicobacter pylori* infection. *J Histochem Cytochem* (2009) **57**:327–38. doi:10.1369/jhc.2008.952366
240. Hitzler I, Sayi A, Kohler E, Engler DB, Koch KN, Hardt WD, et al. Caspase-1 has both proinflammatory and regulatory properties in *Helicobacter* infections, which are differentially mediated by its substrates IL-1beta and IL-18. *J Immunol* (2012) **188**:3594–602. doi:10.4049/jimmunol.1103212
241. Shimada M, Ando T, Peek RM, Watanabe O, Ishiguro K, Maeda O, et al. *Helicobacter pylori* infection upregulates interleukin-18 production from gastric epithelial cells. *Eur J Gastroenterol Hepatol* (2008) **20**:1144–50. doi:10.1097/MEG.0b013e32830edb15
242. Chen Y, Wang Y, Xu W, Zhang Z. Analysis on the mechanism of *Helicobacter pylori*-induced apoptosis in gastric cancer cell line BGC-823. *Int J Mol Med* (2005) **16**:741–5. doi:10.3892/ijmm.16.4.741
243. Jiang J, Liu S, Luo J, Li X, Tang S, Yu M, et al. [The expressions of NLRP3 inflammasome and its downstream molecules in the mouse model of *Helicobacter pylori* infection]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* (2013) **29**:785–8.
244. Jee CD, Lee HS, Bae SI, Yang HK, Lee YM, Rho MS, et al. Loss of caspase-1 gene expression in human gastric carcinomas and cell lines. *Int J Oncol* (2005) **26**:1265–71. doi:10.3892/ijo.26.5.1265

245. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. *Nat Rev Cancer* (2012) **12**:860–75. doi:10.1038/nrc3380
246. Wang P, Zhang L, Jiang JM, Ma D, Tao HX, Yuan SL, et al. Association of NOD1 and NOD2 genes polymorphisms with *Helicobacter pylori* related gastric cancer in a Chinese population. *World J Gastroenterol* (2012) **18**:2112–20. doi:10.3748/wjg.v18.i17.2112
247. Kara B, Akkiz H, Doran F, Bayram S, Erken E, Gumurdullu Y, et al. The significance of E266K polymorphism in the NOD1 gene on *Helicobacter pylori* infection: an effective force on pathogenesis? *Clin Exp Med* (2010) **10**:107–12. doi:10.1007/s10238-009-0077-6
248. Hnatyszyn A, Szalata M, Stanczyk J, Cichy W, Slomski R. Association of c.802C>T polymorphism of NOD2/CARD15 gene with the chronic gastritis and predisposition to cancer in *H. pylori* infected patients. *Exp Mol Pathol* (2010) **88**:388–93. doi:10.1016/j.yexmp.2010.03.003
249. Wex T, Ebert MP, Kropf S, Dierkes J, Schuttler K, Rocken C, et al. Gene polymorphisms of the NOD-2/CARD-15 gene and the risk of gastric cancer in Germany. *Anticancer Res* (2008) **28**:757–62.
250. Angeletti S, Galluzzo S, Santini D, Ruzzo A, Vincenzi B, Ferraro E, et al. NOD2/CARD15 polymorphisms impair innate immunity and increase susceptibility to gastric cancer in an Italian population. *Hum Immunol* (2009) **70**:729–32. doi:10.1016/j.humimm.2009.04.026
251. Liu J, He C, Xu Q, Xing C, Yuan Y. NOD2 polymorphisms associated with cancer risk: a meta-analysis. *PLoS One* (2014) **9**:e89340. doi:10.1371/journal.pone.0089340
252. Villani AC, Lemire M, Fortin G, Louis E, Silverberg MS, Collette C, et al. Common variants in the NLRP3 region contribute to Crohn's disease susceptibility. *Nat Genet* (2009) **41**:71–6. doi:10.1038/ng.285
253. Pontillo A, Vendramin A, Catamo E, Fabris A, Crovella S. The missense variation Q705K in CIAS1/NALP3/NLRP3 gene and an NLRP1 haplotype are associated with celiac disease. *Am J Gastroenterol* (2011) **106**:539–44. doi:10.1038/ajg.2010.474
254. Pontillo A, Brandao L, Guimaraes R, Segat L, Araujo J, Crovella S. Two SNPs in NLRP3 gene are involved in the predisposition to type-1 diabetes and celiac disease in a pediatric population from northeast Brazil. *Autoimmunity* (2010) **43**:583–9. doi:10.3109/08916930903540432
255. Roberts RL, Topless RK, Phipps-Green AJ, Gearry RB, Barclay ML, Merriman TR. Evidence of interaction of CARD8 rs2043211 with NALP3 rs35829419 in Crohn's disease. *Genes Immun* (2010) **11**:351–6. doi:10.1038/gene.2010.11
256. Yang SK, Kim H, Hong M, Lim J, Choi E, Ye BD, et al. Association of CARD8 with inflammatory bowel disease in Koreans. *J Hum Genet* (2011) **56**:217–23. doi:10.1038/jhg.2010.170
257. Maeda S, Hsu LC, Liu H, Bankston LA, Iimura M, Kagnoff MF, et al. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* (2005) **307**:734–8. doi:10.1126/science.1103685
258. Tatsuta T, Imaizumi T, Shimoyama T, Sawaya M, Kunikazu T, Matsumiya T, et al. Expression of melanoma differentiation associated gene 5 is increased in human gastric mucosa infected with *Helicobacter pylori*. *J Clin Pathol* (2012) **65**:839–43. doi:10.1136/jclinpath-2011-200590
259. Gringhuis SI, Den Dunnen J, Litjens M, Van Der Vlist M, Geijtenbeek TB. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to *Mycobacterium tuberculosis*, HIV-1 and *Helicobacter pylori*. *Nat Immunol* (2009) **10**:1081–8. doi:10.1038/ni.1778
260. Miszczyk E, Rudnicka K, Moran AP, Fol M, Kowalewicz-Kulbat M, Druszczyńska M, et al. Interaction of *Helicobacter pylori* with C-type lectin dendritic cell-specific ICAM grabbing nonintegrin. *J Biomed Biotechnol* (2012) **2012**:206463. doi:10.1155/2012/206463
261. Van Die I, Van Vliet SJ, Nyame AK, Cummings RD, Bank CM, Appelmelk B, et al. The dendritic cell-specific C-type lectin DC-SIGN is a receptor for *Schistosoma mansoni* egg antigens and recognizes the glycan antigen Lewis x. *Glycobiology* (2003) **13**:471–8. doi:10.1093/glycob/cwg052
262. Bergman MP, Engering A, Smits HH, Van Vliet SJ, Van Bodegraven AA, Wirth HP, et al. *Helicobacter pylori* modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN. *J Exp Med* (2004) **200**:979–90. doi:10.1084/jem.20041061
263. Chang LL, Wang SW, Wu IC, Yu FJ, Su YC, Chen YP, et al. Impaired dendritic cell maturation and IL-10 production following *H. pylori* stimulation in gastric cancer patients. *Appl Microbiol Biotechnol* (2012) **96**:211–20. doi:10.1007/s00253-012-4034-z
264. Oertli M, Sundquist M, Hitzler I, Engler DB, Arnold IC, Reuter S, et al. DC-derived IL-18 drives Treg differentiation, murine *Helicobacter pylori*-specific immune tolerance, and asthma protection. *J Clin Invest* (2012) **122**:1082–96. doi:10.1172/JCI61029
265. Wu J, Lin K, Zeng J, Liu W, Yang F, Wang X, et al. Role of DC-SIGN in *Helicobacter pylori* infection of gastrointestinal cells. *Front Biosci (Landmark Ed)* (2014) **19**:825–34. doi:10.2741/4250
266. Kutikhin AG, Yuzhalin AE. C-type lectin receptors and RIG-I-like receptors: new points on the oncogenomics map. *Cancer Manag Res* (2012) **4**:39–53. doi:10.2147/CMAR.S28983
267. Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J Exp Med* (2003) **197**:1107–17. doi:10.1084/jem.20021787
268. Gringhuis SI, Den Dunnen J, Litjens M, Van Het Hof B, Van Kooyk Y, Geijtenbeek TB. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-κappaB. *Immunity* (2007) **26**:605–16. doi:10.1016/j.immuni.2007.03.012
269. Kankkunen P, Teirila L, Rintahaka J, Alenius H, Wolff H, Matikainen S. (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. *J Immunol* (2010) **184**:6335–42. doi:10.4049/jimmunol.0903019
270. Pothlichet J, Meunier I, Davis BK, Ting JP, Skamene E, Von Messling V, et al. Type I IFN triggers RIG-I/TLR3/NLRP3-dependent inflammasome activation in influenza A virus infected cells. *PLoS Pathog* (2013) **9**:e1003256. doi:10.1371/journal.ppat.1003256
271. Hedaya M, Takeda K, Rezaei N. Prophylactic and therapeutic implications of toll-like receptor ligands. *Med Res Rev* (2012) **32**:294–325. doi:10.1002/med.20214
272. Paul-Clark MJ, George PM, Gatheral T, Parzych K, Wright WR, Crawford D, et al. Pharmacology and therapeutic potential of pattern recognition receptors. *Pharmacol Ther* (2012) **135**:200–15. doi:10.1016/j.pharmthera.2012.05.007
273. Gradisar H, Keber MM, Pristovsek P, Jerala R. MD-2 as the target of curcumin in the inhibition of response to LPS. *J Leukoc Biol* (2007) **82**:968–74. doi:10.1189/jlb.1206727
274. Tu SP, Jin H, Shi JD, Zhu LM, Suo Y, Lu G, et al. Curcumin induces the differentiation of myeloid-derived suppressor cells and inhibits their interaction with cancer cells and related tumor growth. *Cancer Prev Res (Phila)* (2012) **5**:205–15. doi:10.1158/1940-6207.CAPR-11-0247
275. Qu J, Hou Z, Han Q, Zhang C, Tian Z, Zhang J. Poly(I:C) exhibits an anti-cancer effect in human gastric adenocarcinoma cells which is dependent on RLRs. *Int Immunopharmacol* (2013) **17**:814–20. doi:10.1016/j.intimp.2013.08.013

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Multiple roles of toll-like receptor 4 in colorectal cancer

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Toll-like receptor (TLR) signaling has been implicated in the inflammatory responses in intestinal epithelial cells (IECs). Such inflammatory signals mediate complex interactions between commensal bacteria and TLRs and are required for IEC proliferation, immune response, repair, and homeostasis. The upregulation of certain TLRs in colorectal cancer (CRC) tissues suggests that TLRs may play an essential role in the prognosis of chronic and inflammatory diseases that ultimately culminate in CRC. Here, we provide a comprehensive review of the literature on the involvement of the TLR pathway in the initiation, progression, and metastasis of CRC, as well as inherited genetic variation and epigenetic regulation. The differential expression of TLRs in epithelial cells has also been discussed. In particular, we emphasize the physiological role of TLR4 in CRC development and pathogenesis, and propose novel and promising approaches for CRC therapeutics with the aid of TLR ligands.

Keywords: colorectal cancer, immune response, inflammation, ligand, toll-like receptor 4

INTRODUCTION

The innate immune system possesses a robust mechanism in the form of evolutionarily conserved toll-like receptors (TLRs) that can detect the signature pattern of invading microorganisms for the protection of the host. TLRs are a class of type I transmembrane glycoproteins. Human and mouse cells comprises of 13 types of TLRs that can detect different kinds of bacterial and viral-associated patterns (1–3). TLR1–9 are highly conserved in both species; while the mouse TLR10 is non-functional due to retroviral insertion, TLR11–13 are undetected in the human genome. Examples of TLR-specific ligands are: lipopeptides for TLR1/2 and 2/6 (4–6), dsRNA for TLR3 (7), lipopolysaccharide (LPS) for TLR4 (8), flagellin for TLR5 (9), ssRNA for TLR7/8 (10, 11), and CpG DNA for TLR9 (12–14). TLRs not only detect invading microbes but also recognize intracellular anomalies and mount an immune response, thereby playing a cardinal role in the homeostasis of the human immune system (15, 16). The abnormal activation of TLRs can jeopardize normal physiological processes and cause several inflammatory diseases, cancers, and autoimmune diseases (17, 18).

Toll-like receptors are ubiquitously expressed, although their expression level may vary according to the circumstances and the tissues. In addition, induced expression of TLRs has been observed when ligands bind to their cognate TLRs (19). Research in the last decade has focused on elucidating various functions, intermediate molecules, and ligands associated with TLRs. There is a well-established link between TLR-induced inflammation and the development and progression of cancer (20, 21). Similarly, TLRs are also known to play a vital role in colorectal cancer (CRC) that affects the large intestine and the rectum. This region is heavily populated by intestinal microbes, highlighting the crucial role of TLRs in CRC pathogenesis (17, 22).

Colorectal cancer is one of the most complex diseases and causes death in many cases in the United States (23). Globally, more than one million new cases of CRC are reported annually (24, 25). The complexity of CRC is primarily attributed to environmental factors, while genetic factors play a minor role. The known risk factors for CRC are food-borne mutagens, pollution, certain commensal bacteria, and chronic intestinal inflammation (25). Commonly, CRC occurs in the right ascending colon with the most common symptom being blood in the stool or rectal bleeding. Genetically, inherited colon polyps also contribute to the development of CRC (26). Since CRC can damage the host immune system during their proliferation period, stimulating it against CRC promises to be an attractive approach for drug discovery (27).

In this review, we discuss the role of TLRs in the maintenance of homeostasis and the development of CRC in intestinal epithelial cells (IECs). Improved techniques to detect dysfunctional TLR signaling in carcinogenesis may stimulate the development of novel therapies to prevent or treat CRC. Recent studies have improved the understanding of TLR-targeted applications such as identifying their differential expression, their role in tumor progression, potential use as immune modulating agents, and development of novel TLR ligands in anti-cancer therapies.

TLR SIGNALING: AN OVERVIEW

The localization of TLRs is heterogeneous and varies from the cell surface (TLR1, 2, 4, 5, 6, 10, and mouse TLR11, 12) to the endosomes (TLR3, 7, 8, and 9) (28), depending on the localization of pathogen-associated molecular patterns (PAMPs). TLRs comprises of the following three domains: ectodomain [contains leucine rich repeats (LRR)] that recognizes PAMPs, a trans-membrane region, and a cytosolic toll/interleukin-1 (IL-1)

receptor (TIR) domain that interacts with adaptor molecules (such as MyD88/MAL and TRIF/TRAM) to propagate downstream signaling. Ligand binding triggers the dimerization of TLRs, facilitating the binding of adaptor molecules, which subsequently activate the IL-1 receptor-associated kinase (IRAK) family (29). Upon IRAK recruitment, IRAK4 phosphorylates IRAK1 at key serine and threonine residues, and enables IRAK1 to eventually activate tumor necrosis factor receptor-associated factor 6 (TRAF6) (30) that subsequently activates transforming growth factor- β -activated protein kinase 1 (TAK1), a member of the mitogen-activated protein (MAP) kinase kinase kinase (MAP3K) family. TAK1 forms a complex with TGF- β -activated kinase 1/MAP3K7 binding protein 1 (TAB1), TAB2, and TAB3 and then activates nuclear factor (NF)- κ B by phosphorylating IKK that in turn phosphorylates I κ B for proteasomal degradation. Following the degradation of I κ B, NF- κ B translocates into the nucleus and induces inflammatory mediators. Moreover, TAK1 activates members of the MAP kinase kinase 3 (MKK3) and MKK6 to activate an alternative closely related pathway that phosphorylates c-Jun N-terminal Kinase (JNK) and p38. TLR signaling can also activate extracellular signal-regulated kinase (ERK) via the activation of MEK1/2. In response to various TLR ligands, reduced activity of NF- κ B, JNK, and p38 was observed in B cells and embryonic fibroblasts derived from TAK1-deficient mice (31). In the TRIF-dependent pathway triggered by TLR3 and TLR4, TRIF recruits TRAF3, TAB1, and IKK and activates the type I IFN. The TRIF-dependent pathway also activates TRAF6 and TAB1, which regulate the delayed activation of NF- κ B and MAP kinases (32) (Figure 1).

TLRs AND THEIR EXPRESSION PATTERNS IN IECs

The human intestinal tract plays a crucial role in maintaining the complex ecosystem of commensal bacteria and also physically isolates the countless resident bacteria from the lamina propria (33). It was originally believed that IECs prevent bacteria from invading the body. However, IECs have a complicated and common beneficial link with the microorganisms in the intestinal gut flora. The commensal bacteria metabolize carbohydrates and the IECs break down the short-chain fatty acids produced as a result of bacterial fermentation of undigested carbohydrates and use them as an energy source (34). IEC membranes express TLRs that detect the commensal PAMPs and mediate signaling to maintain epithelial cell integrity and tight junctions, cell proliferation, immunoglobulin A (IgA) production, and antimicrobial peptide expression (34). In addition, they can also induce a pro-inflammatory response by interacting with the immune cells in the lamina propria (35, 36). Therefore, tight regulation of TLRs is imperative to prevent adverse effects since anomalous or dysregulated TLR signaling can mediate cancer induction and propagation.

Colorectal cancer pathogenesis is governed by TLR expression that is difficult to detect due to the heterogeneous nature of IECs (33). To elucidate the expression profile of TLR2–5 in epithelial cells, small intestinal, and colonic biopsy specimens from patients with inflammatory bowel disease (IBD) were assessed by immunofluorescence histochemistry using polyclonal antibodies against TLR2, 3, 4, and 5. This study showed that TLR3 and TLR5

are ubiquitously expressed while TLR2 and TLR4 are expressed at a very low level in normal cells (37). Conversely, the diseased tissue specimens demonstrated significant overexpression of TLR4 and a decline in TLR3 expression. The expression pattern of TLR2 and TLR5 remained unaltered between the normal and diseased specimens. Furthermore, in normal human IECs, TLR2, and TLR4 were marginally expressed, while TLR3 expression was relatively high. While TLR2 was expressed in the colonic tissue from the epithelium and lamina propria, TLR3 was expressed in the mature epithelial cells of the crypts. Furthermore, TLR5 was moderately overexpressed in a basolateral fashion in the epithelial cells of normal human tissues (38). Tissues from CRC patients demonstrated increased expression of TLR7, 8, 9, and 10 (39); this study also showed that TLR8 expression is an independent marker for CRC.

TLRs AND INTESTINAL HOMEOSTASIS

Toll-like receptor activation is responsible for fighting against microbial infections, while leaving the host cell intact. This is usually accomplished by producing antimicrobial peptides, inflammatory mediators, adenomatous polyposis coli (APCs) maturation, and triggering of cell survival and tissue repairing pathways (40). TLRs are marginally expressed on IECs and are primarily localized on the basolateral surface or in the endosomal vesicles (41). Moreover, regulatory mechanisms such as the expression of TLR inhibitors like single immunoglobulin IL-1-related receptor (SIGIRR), toll-interacting protein (TOLLIP), A20, and IRAK3 are involved in the regulation of TLR signaling (42); these inhibitory molecules prevent TLRs from mounting an immune response even during continuous interaction (34, 43) and nurturing the anti-inflammatory phenotype of homing leukocytes (44). SIGIRR-deficient mice demonstrate defective intestinal homeostasis, and these defects are associated with the microbiota and hyper-expression of inflammatory mediators. Notably, these defects also render the azoxymethane (AOM)-dextran sodium sulfate (DSS)-treated SIGIRR^{−/−} mice prone to colitis and colitis-associated CRC. Interestingly, the rescue of SIGIRR expression in the IECs of SIGIRR^{−/−} mice restored the immune tolerance and abolished the risk of tumor development in these mice (45).

Although elevated TLR activity disrupts the recognition of intestinal microbes by TLR2 and TLR4, TLR signaling is necessary for maintaining homeostasis and regulation of tissue repair in IECs. MyD88-deficient mice, which hamper signaling through IL-1 family members including TLRs, possess profound abnormalities in the mucosa with higher proliferation rates in the crypts (46). Cumulatively, this leads to defects in repair of the intestinal barrier following injury, and increased risk of colitis and CRC (46, 47). Moreover, mice in which normal flora is disrupted by antibiotics display a similar phenotype to mice lacking MyD88, as well as decreased expression of factors [i.e., tumor necrosis factor (TNF), CXC-chemokine ligand 1, IL-6, and heat shock proteins] required for normal intestinal homeostasis (48).

RELATIONSHIP BETWEEN INFLAMMATION AND CRC

The direct link between intestinal inflammation and CRC prognosis is well-established and is also supported by numerous genetic,

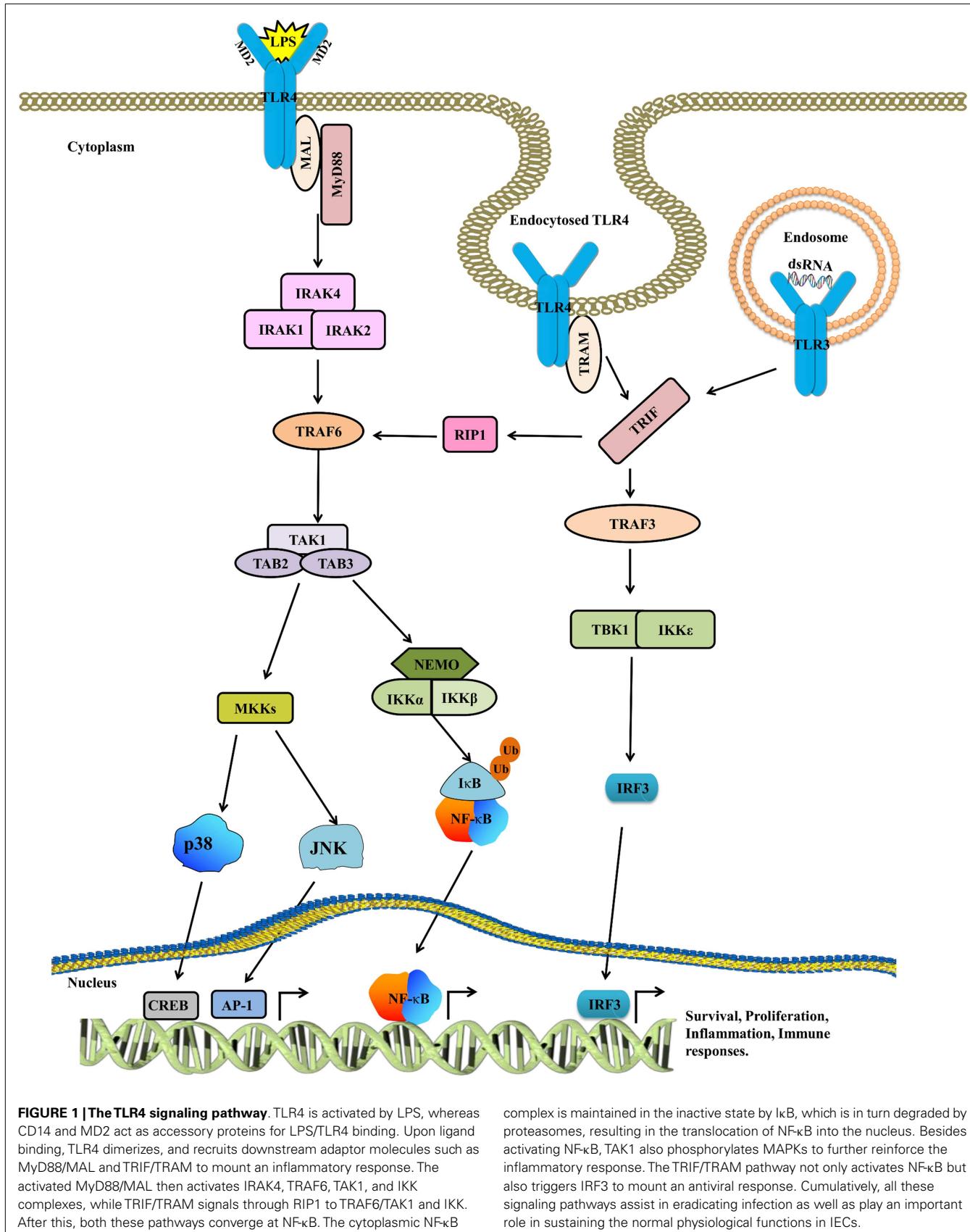


FIGURE 1 | The TLR4 signaling pathway. TLR4 is activated by LPS, whereas CD14 and MD2 act as accessory proteins for LPS/TLR4 binding. Upon ligand binding, TLR4 dimerizes, and recruits downstream adaptor molecules such as MyD88/MAL and TRIF/TRAM to mount an inflammatory response. The activated MyD88/MAL then activates IRAK4, TRAF6, TAK1, and IKK complexes, while TRIF/TRAM signals through RIP1 to TRAF6/TAK1 and IKK. After this, both these pathways converge at NF- κ B. The cytoplasmic NF- κ B

complex is maintained in the inactive state by I κ B, which is in turn degraded by proteasomes, resulting in the translocation of NF- κ B into the nucleus. Besides activating NF- κ B, TAK1 also phosphorylates MAPKs to further reinforce the inflammatory response. The TRIF/TRAM pathway not only activates NF- κ B but also triggers IRF3 to mount an antiviral response. Cumulatively, all these signaling pathways assist in eradicating infection as well as play an important role in sustaining the normal physiological functions in IECs.

pharmacological, and epidemiological studies conducted during the last decade (49). Recent reports demonstrate the complex interplay between distinct immune cells, and also show that pro-inflammatory mediators influence almost all the steps of CRC progression. However, the mechanisms by which inflammation stimulates the development of cancer remain elusive and are expected to vary from colitis-associated CRC to other forms of CRC (25, 50). The relationship between inflammatory responses caused by multiple factors such as the microbiota, IBD, and CRC has been demonstrated by comparative experiments conducted in wild type and *Il10*^{-/-} mice. When treated with AOM, *Il10*^{-/-} mice were found to show an increased risk of colon tumor development, spontaneous colitis, and CRC, while AOM-WT mice were devoid of colitis and rarely progressed to adenomas. In addition, mice with *Bacteroides vulgatus* or dual knockout mice (*Il10*- and *MyD88*-deficient mice) treated with AOM showed reduced transcription of *Il12p40* and TNF- α and remained tumor-free (51).

TLR-induced inflammation is a well-established phenomenon and is perpetuated by several cytokines, ILs, and TNF- α , all of which are known to substantially regulate immune cells and inflammatory responses against cancer (48, 52). Among these, TNF- α is of particular importance and is now recognized as a pro- as well as anti-tumorigenic protein (53). The activation of the TLR4 signaling pathway induces TNF- α and NF- κ B, leading to the promotion of CRC (17, 54–56); TNF- α knockout mice treated with AOM/DSS show significantly less tumor formation, representing the pro-tumorigenic role of TNF- α (57). Immunohistochemistry analyses of mononuclear cells in the lamina propria and colons of patients with advanced stage CRC demonstrate the expression of TNF- α (57). TNF- α also promotes the activation of NF- κ B, which reinforces inflammation by inducing cyclooxygenase-2 (COX-2), IL-6, IL-8, and TNF- α to favor tumorigenesis (55, 58, 59). However, inflammation alone is not sufficient for colon cancer and the contribution of other risk factors is equally essential to the pathogenesis of this complex disease.

CONTRIBUTION OF TLR4 TO CRC DEVELOPMENT

Although IECs are in close proximity to LPS, they do not mount an immune response on the commensal bacteria under normal circumstances. However, in the diseased state, disruption of the coexistence between IECs, and bacteria leads to an inflammatory response. This raises an important question: when and how much inflammation should have to be raised in order to equilibrate the bacterial threat (Figure 2). Numerous studies have been conducted to address this dilemma (60–64). For instance, IFN- α and IFN- γ are known to increase the LPS response in IECs, which is directly linked to the expression of TLR4 and MD2 (63, 65). Moreover, continuous LPS stimulation culminates in reduced TLR4 expression and increased expression of inhibitory proteins (62). However, a conflicting report demonstrated that long-term LPS exposure does not alter TLR4 expression (66). Moreover, hypoxia and numerous endotoxins are known to be prevalent in the inflamed intestinal lining, possibly causing induced TLR4 expression (60). Hung et al. observed an increase in the TLR4 expression from the mucosa of CRC patients of different ages and sexes as well as from a variety of CRC cell lines (HT29, SW480, and KM20) (67). In addition, Maria and colleagues showed that TLR4 expression is required

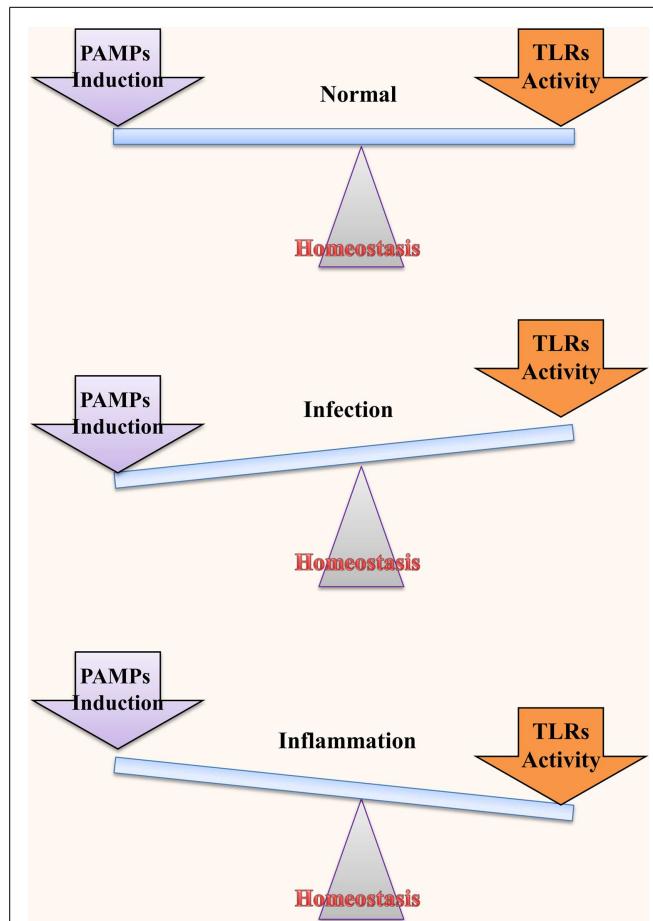


FIGURE 2 | Homeostatic interaction between microbiota and TLRs.

TLRs play an important role in maintaining normal functions of IECs; however, regulation of the activation and induction of TLRs through various mechanisms is necessary for this role. TLRs in the intestine exist in close proximity to and may be stimulated by commensal bacteria. Therefore, it is extremely necessary to regulate their functions. Under normal conditions, homeostasis between bacterial induction and TLR activation is maintained to ensure a disease-free status. On the other hand, if TLRs are inappropriately activated or if they mount an exaggerated immune response to a low level stimulus, they may culminate in bacterial infection and inflammatory disease/cancer, respectively.

for dysplasia and polyp formation. This finding is consistent with results of experiments performed in TLR4 gene knockout mice (56, 68). Collectively, these data present a clear association between TLR4 and CRC development.

In CRC, elevated TLR4 expression is observed in all tumor components such as the epithelial, endothelial, and stromal layers (69). However, the level of this expression varies depending on the type of cancer. Although all TLRs are expressed at the minimal basal mRNA level, IECs can upregulate the TLR expression, based on the inflammatory signals or other stimuli (70). An alternate study demonstrated a low level of TLR4/MD2 expression in normal human colonic epithelial cells and the lamina propria, which is consistent with the level of TLR4/MD2 expression detected in various epithelial cell lines (71). These studies establish the fact

that any alteration in the prevalent inflammatory conditions or the population of luminal bacteria may influence the strength and nature of TLR signaling, paving the way for initiation of inflammatory responses in IECs. Besides this negative role, studies in TLR4- and TLR9-deficient mice have shown that TLR signaling in IECs is essential for protecting the host from inflammation-related damage and for homeostasis (46, 72).

TLR4 CROSSTALK IN CRC PROGRESSION

TLR4 is overexpressed in the liver metastasis of CRC (73). In response to LPS binding, over-stimulation of the TLR4/MD2 complex enhances the phosphorylation of protein kinase B (also known as AKT), which in turn activates the function of β 1 integrin. This complex interplay between multiple pathways promotes the adhesiveness and metastatic behavior of CRC (74). The enhanced AKT phosphorylation can be blocked by eritoran (a TLR4 antagonist), PI 103 [a phosphatidylinositide 3-kinases (PI3K) inhibitor], or anti- β 1 integrin antibodies that are known to ameliorate CRC and its metastatic behavior (75–77), indicating that the PI3K/AKT signaling pathway is induced by TLR4 in response to LPS binding and plays a central role in the growth and progression of CRC. Furthermore, LPS is known to induce the expression of the urokinase plasminogen activator (uPA) system through TLR4 and NF- κ B in human colorectal cell lines. During tumor progression, vital extracellular matrix (ECM) interactions occur, in which uPA and the expression and activity of its receptor facilitate the growth and metastasis of CRC (78). Conversely, inhibition of TLR4, NF- κ B, or the uPA system can attenuate CRC progression. Although NF- κ B is known to impair apoptosis in tumor cells (55, 79, 80), NF- κ B activation through TNFR signaling also protects cells from apoptosis. Studies performed in the Saos-2 cell line reveal that p53-induced cell death is dependent on NF- κ B, and the ablation of NF- κ B leads to the abrogation of p53-dependent cell death (81). Thus, the TNF- α /NF- κ B interaction plays a vital role in CRC and IBD-related diseases and manipulation of this interaction may improve the treatment of CRC.

TLR4 is overexpressed during inflammation-associated colorectal neoplasia in humans and mice. Similarly, mice lacking TLR4 are largely protected from colon carcinogenesis (56). A dissection of this mechanism reveals that TLR4 triggers elevated production of prostaglandin E2, increases Cox-2 induction, and influences epidermal growth factor receptor signaling (EGFR) in chronic colitis. TLR4 can thus manipulate numerous pathways and cause further deterioration of the neoplastic situation. A recent comparative immunohistochemistry analysis between normal mucosa and adenomas showed that TLR4 and MD2 are overexpressed in 20 and 23% of the adenomas, respectively (82), further substantiating the involvement of the TLR4 pathway in CRC. Furthermore, mutations in the *APC* gene cause pre-disposition to CRC. A correlation between the TLR/MyD88 signaling pathway and *APC* mutations was recently proposed (82, 83) since MyD88 signaling was found to facilitate the growth of intestinal polyps while the ablation of MyD88 restricted polyp growth in *Apc*^{min/+}/*Myd88*^{-/-} mice, but not in *Apc*^{min/+} mice (83, 84). In addition, MyD88 induces ERK to block the degradation of the oncoprotein c-Myc, and such cells with continued activation of c-Myc are prone to neoplastic transformation (85).

Similarly, c-Myc is also important for APC-mediated tumorigenesis (86), since knocking out c-Myc in IECs of *Apc*^{min/+} mice impedes tumor growth (84). Furthermore, reduced expression of c-Myc has been reported in *Apc*^{min/+}/*Myd88*^{-/-} of both normal and tumor mice (84, 87). Treatment of *Apc*^{min/+} mice with PD03259012, an inhibitor of MEK1/2, which is the kinase directly upstream of ERK, also inhibits tumor growth. These data indicate that a complex interplay of protein signaling brings about tumor proliferation in the IECs of various transgenic mouse models. Moreover, heritable changes in the *APC* gene frequently lead to familial adenomatous polyposis (FAP). FAP is the most dominant inherited syndrome of CRC (88, 89) and *Apc*^{min/+} mice show increased propensity for the development of adenomatous polyps after the loss of the wild type *APC* allele (88). Up to 80% of sporadic CRCs are known to be initiated by DNA damage of the genes involved in the APC signaling pathway (87).

CORRELATION BETWEEN CRC DEVELOPMENT AND INHERITED GENETIC VARIATIONS OF TLR4

The human *TLR4* gene is located on the long (q) arm of chromosome 9 at position 33.1, and contain four exons. The dominant expression of TLR4 has been observed in lymphocytes, monocytes, leukocytes, and splenocytes (90). Besides CRC, many human pathologies and carcinomas are associated with the polymorphisms of TLR4 (91–93). The *TLR4* gene contains two single-nucleotide polymorphisms (SNPs), namely, Asp299Gly and Thr399Ile that are significantly important in tumor development (94, 95). Both these SNPs are located in the coding sequence for the TLR4 ectodomain and mediate an amino acid substitution. These Asp299Gly and Thr399Ile SNPs in *TLR4* are known to attenuate cytokine expression, leading to an increased propensity for the development of gastric cancer and CRC (94, 96–99). The detection of these two SNPs was carried out using allele-specific polymerase chain reaction and the primer extension method (SNaPshot) for gastric cancer and CRC, respectively. For gastric cancer, only Thr399Ile showed a significant correlation, while both the SNPs were significantly correlated to CRC (94, 100, 101). In addition, the association of the TLR3 (rs3775291) polymorphism and IL-10 promoter variation (rs1800872) to CRC pathogenesis was evaluated in a large cohort of German CRC patients. This study found that the IL-10 promoter variant is significantly associated with an increased risk of lymph node metastasis (for carriers of the TT genotype). Interestingly, a *TLR3* gene polymorphism was found to correlate with patient survival, and the TT genotype was responsible for increased mortality. This TLR3 variation was limited only to stage II patients who were devoid of adjuvant therapy (102, 103).

The LPS-sensing complex is comprises TLR4, MD2, LPS binding protein, and CD14. A positive link between CD14-260 polymorphisms and the occurrence of CRC in the Chinese Han population was demonstrated (104, 105), in which the CD14 polymorphism C/C, but not C/T, was significantly correlated to CRC; no correlation between TLR4 Asp299Gly and CRC was found. However, it is possible that the polymorphism in TLR4 was associated with the population under study (106). A multi-racial study (22 Malays, 20 Chinese, and 18 Indians) conducted in Malaysia showed that there is no correlation between TLR4 polymorphisms (Asp299Gly; Thr399Ile) and the risk of CRC (107). However, a

study on Russian population revealed that IL1B_1473G/C and TLR4_896A/G SNPs are involved in rectal cancer development (108). A conflicting report validated the potential link between TLR4 polymorphisms (Asp299Gly and Thr399Ile) and the digestive tract cancer and CRC (101). This study retrieved and analyzed extensive data from various databases and concluded that Asp299Gly is significantly correlated with an increased risk of gastric cancer, while there was no correlation between this polymorphism and digestive tract cancer and CRC. Moreover, it was also observed that the T allele of Thr399Ile does not influence digestive tract, gastric, or CRC. It is evident that additional studies are necessary to support these findings.

EPIGENETIC REGULATION OF TLR4 IN CRC

Intestinal epithelial cells are stimulated by the commensal bacteria in the intestinal lumen with the help of TLRs for the maintenance of homeostasis. This stimulation from the commensal bacteria is finite, should not trigger an excessive inflammatory response, and is known to influence epigenetic modification in the host cells (109). These epigenetic modifications involve DNA methylation and histone deacetylation that suppress and promote the transcription process, respectively, and in turn regulate gene expression (110).

The *TLR4* gene is methylated in the 5' region; also, the degree of methylation in epithelial cells is higher than that in the splenic cells, caused by the interaction of the commensal bacterial with IECs. Takahashi and colleagues showed that commensal bacteria modulate the epigenetic regulation in IECs by DNA methylation of *TLR4* (111). In their study, the authors compared the methylation levels in the IECs from the small and large intestine obtained from conventional (CV) mice with commensal bacteria and germ-free (GF) mice without commensal bacteria. The methylation level of CpG motifs in the 5' region of *TLR4* from the large intestine was lower in the GF mice compared with CV mice, while in the small intestine, the methylation levels remained unchanged between the GF and CV mice. The frequency of methylation is also found to

depend on the MyD88 adaptor molecule. Results from *in vivo* experiments show that the frequency of CpG methylation is less in the GF mice (MyD88 knockout mice) compared to CV mice (111).

Environmental factors also play a crucial role in regulating epigenetic modifications. In the presence of factors such as myriad food habits and increasing pollution, intestinal commensal bacteria produce short-chain fatty acids known as butyrates that inhibit histone deacetylation (112, 113). Besides *TLR4*, *MD2* can also be downregulated to attenuate the LPS response. IECs are known to poorly express *MD2*, which directly correlates to DNA hypermethylation (114). In IBD, IECs exhibit elevated expression of *MD2* and *TLR4* mRNA, while in normal cells; *TLR4/MD2* transcription is reduced due to DNA methylation. The deacetylation and blocking of methylation enables cells to express higher amounts of *TLR4* and *MD2* mRNA. This study demonstrates how epigenetic regulation of *TLR4* and *MD2* prevents dysregulation of inflammation in IECs and thus provides a novel approach to target CRC.

THERAPEUTIC TARGETING OF TLR4

Synthetic TLR4 ligands are potential targets for therapeutic applications for cancer, allergies, and viral infections (115). By virtue of their cell surface location, quick induction, and the ability to mount a wide array of inflammatory responses, TLRs are one of the most promising targets for therapeutics (91). The clinical trials of various TLR4 ligands are enlisted in Table 1.

TLR4 agonists have immune regulatory applications as adjuvants in vaccines and in the treatment of chronic viral infection and cancer therapy. LPS was the first microbial product identified as a potential TLR4 agonist and implemented for therapeutic applications (116). LPS is very toxic since it induces excessive inflammatory cytokines. However, low-dose LPS combined with non-steroidal anti-inflammatory drug ibuprofene was proved to be safe, with higher levels of TNF- α and IL-1 in all patients (117, 118). Marginal to encouraging results were observed when ibuprofene combined with *Salmonella abortus*

Table 1 | TLR4 agonists in clinical trials.

Compounds	Phase	Note	Indications	Current status	Clinical Trail.gov
LPS	I-II	Combined with KLH-pulsed DCs vaccine	Neuroblastoma and Ewing's sarcoma	Active, not recruiting	NCT00923351
	I-II	Combined with IL-4, KLH, and WT1 peptide-pulsed DC based vaccine	Hematologic malignancies	Completed	NCT00923910
	I	Combined with multipeptide vaccine	Melanoma	Active, not recruiting	NCT01585350
OM-174	I	Injections of OM-174	Solid tumors	Completed	NCT01800812
Stimuvax	II	Combined with chemoradiation therapy	Rectal cancer	Active, not recruiting	NCT01507103
	II	Androgen deprivation and radiation therapy	Prostate cancer	Recruiting	NCT01496131
	II	L-BLP25 vaccination	Colorectal carcinoma	Recruiting	NCT01462513
Picibanil	IV	Intracystic injection	Cystic malformation	Recruiting	NCT01699347
	I-II	Combined with pre-operative intra tumoral DCs	Pancreatic cancer	Unknown	NCT00795977
	I	Combined with cyclophosphamide, docetaxel (chemo-immunotherapy)	Head and neck cancer	Unknown	NCT01149902

equi LPS for non-small cell lung carcinoma (NSCLC) and CRC patients, respectively (119). Currently, a few clinical trials are being conducted for oncological indications involving cell-based vaccination to treat Ewing sarcoma, neuroblastoma, and rhabdomyosarcoma patients (NCT00923351). Besides, peptide-pulsed dendritic cells (DCs) were combined with LPS to treat hematological malignancies (NCT00923910), and to treat melanoma patients (NCT01585350), LPS along with oil-based adjuvant and a peptide vaccine are being investigated (119). A less toxic TLR4 agonist, monophosphoryl lipid A (MPLA), is an immunity modulating agent that activates MyD88-independent pathway in TLR4 signaling, triggers the induction of IFN- γ , and regulation of CD80/86, which forms the crucial aspect of adjuvancy (27, 120). MPLA adjuvant plays a dual role in defending the host from pathogens by stimulating the innate immune system, and induces the long-term adaptive immune system (115, 121, 122). Food and Drug Administration has approved MPLA to use as a vaccine against HPV associated cervical cancer (Cervarix). It also enhances the inflammatory behavior of immune cells, which may be useful in a variety of cancers to overcome the cancer-induced immune suppression. However, this may not be helpful in case of CRC, where TLR should emphasize the tolerance of immune system, not the over-activation. Furthermore, it is established that TLRs can act as double-edge sword that may be exploited in pathologies-dependent circumstances to avoid the undesirable consequences (123). OM-174 is a triacyl lipid A analog that activates TLR4 and culminates in tumor growth regression by increasing the IFN- γ production (124, 125). This is well-tolerated at biological concentrations with strong antitumor effects (NCT01800812) (126). A new anti-cancer vaccine, BLP25 liposome vaccine (Stimuvax), can identify and destroy the cancer antigen MUC1, thereby inducing an immune response against cancer cells (127, 128). However, this could not significantly improve the NSCLC (128). Now, stimuvax is being investigated for the treatment of rectal and prostate cancers (NCT01507103 and NCT01496131). Group A *Streptococcus pyogenes* [in lyophilized form OK-432 (Picibanil)] is shown to stimulate TLR4, which is used to treat gastric, cervical, and oral cancers (119). This compound is currently being examined to treat pancreatic cancer patients in pre-operative settings using intra tumoral injection of DCs (NCT00795977), combined with chemotherapy (cyclophosphamide + docetaxel) for head and neck squamous cell carcinoma (HNSCC) patients (NCT01149902), and via intracystic injection at cystic malformation (NCT01699347). Currently, most of the TLR4 antagonists are being evaluated against cancer-unrelated symptoms.

CONCLUSION AND FUTURE PERSPECTIVES

In this review, we highlight the correlation between CRC and TLRs, in particular, TLR4. We also propose that a beneficial link exists between commensal bacteria and TLRs in order to maintain intestinal homeostasis. In IECs, TLRs are involved in epithelial cell proliferation, IgA production, regulating the permeability of the intestinal barrier, antimicrobial peptide expression, and defense against invading pathogens. Over-stimulation of TLRs in response to minor signals (due to dysregulation) may result in colitis and CRC. Several studies suggested the

relationship of TLR4 signaling with CRC, therefore therapeutic benefit can be achieved by targeting TLR4. However, the development of CRC is highly complex. Experimental studies supported that the gut microbiota contributed to CRC. The studies involving human subjects and considering their microbiota composition revealed the vivid differences in microbial density and population. Therefore, modulating the microbial population, usage of probiotics to favor the growth of certain bacteria, and delineating the interaction of microbiota with the epithelial cells can potentially be used to limit the CRC development.

Furthermore, inflammation is central to the development of cancer, and there are few clinical trials being conducted for anti-inflammatory drugs, but by combining molecular approaches with CV therapies, i.e., chemo- or radiotherapy, anti-inflammatory drugs would increase the efficacy to treat CRC. Additionally, targeting the downstream molecules in TLR4 pathway involved in CRC is also expected to have a tremendous impact on CRC therapeutics. Moreover, differential expression of TLRs leads to tumor development, in which the contribution of TLR4 is considerably higher than in the other TLRs. We hope that extensive studies involving the TLR4 pathway will eventually provide therapeutic targets to treat CRC. Recently developed techniques may also prove helpful in the analyses of differential expression levels of TLRs, their mutations, and epigenetic modifications. These analyses would further aid in the design and development of novel therapeutic approaches for CRC treatment.

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REFERENCES

1. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* (2004) 4(7):499–511. doi:10.1038/nri1391
2. Oldenbourg M, Kruger A, Ferstl R, Kaufmann A, Nees G, Sigmund A, et al. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. *Science* (2012) 337(6098):1111–5. doi:10.1126/science.1220363
3. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. *Nat Immunol* (2010) 11(5):373–84. doi:10.1038/ni.1863
4. Takeuchi O, Kaufmann A, Grote K, Kawai T, Hoshino K, Morr M, et al. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. *J Immunol* (2000) 164(2):554–7. doi:10.4049/jimmunol.164.2.554
5. Takeuchi O, Sato S, Horiochi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of toll-like receptor 1 in mediating immune response to microbial lipoproteins. *J Immunol* (2002) 169(1):10–4. doi:10.4049/jimmunol.169.1.10
6. Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, et al. Discrimination of bacterial lipoproteins by toll-like receptor 6. *Int Immunopharmacol* (2001) 13(7):933–40. doi:10.1093/intimm/13.7.933
7. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by toll-like receptor 3. *Nature* (2001) 413(6857):732–8. doi:10.1038/35099560
8. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/He and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* (1998) 282(5396):2085–8. doi:10.1126/science.282.5396.2085

9. Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by toll-like receptor 5. *Nature* (2001) **410**(6832):1099–103. doi:10.1038/35074106
10. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis E, Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* (2004) **303**(5663):1529–31. doi:10.1126/science.1093616
11. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* (2004) **303**(5663):1526–9. doi:10.1126/science.1093620
12. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A toll-like receptor recognizes bacterial DNA. *Nature* (2000) **408**(6813):740–5. doi:10.1038/35047123
13. Krug A, French AR, Barchet W, Fischer JA, Dzinek A, Pingel JT, et al. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity* (2004) **21**(1):107–19. doi:10.1016/j.jimmuni.2004.06.007
14. Gosu V, Basith S, Kwon OP, Choi S. Therapeutic applications of nucleic acids and their analogues in toll-like receptor signaling. *Molecules* (2012) **17**(11):13503–29. doi:10.3390/molecules17113503
15. Lavelle EC, Murphy C, O'Neill LA, Creagh EM. The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. *Mucosal Immunol* (2010) **3**(1):17–28. doi:10.1038/mi.2009.124
16. Rakoff-Nahoum S, Medzhitov R. Role of toll-like receptors in tissue repair and tumorigenesis. *Biochemistry (Mosc)* (2008) **73**(5):555–61. doi:10.1134/S0006297908050088
17. So EY, Ouchi T. The application of toll like receptors for cancer therapy. *Int J Biol Sci* (2010) **6**(7):675–81. doi:10.7150/ijbs.6.675
18. Keogh B, Parker AE. Toll-like receptors as targets for immune disorders. *Trends Pharmacol Sci* (2011) **32**(7):435–42. doi:10.1016/j.tips.2011.03.008
19. Shi Z, Cai Z, Sanchez A, Zhang T, Wen S, Wang J, et al. A novel toll-like receptor that recognizes vesicular stomatitis virus. *J Biol Chem* (2011) **286**(6):4517–24. doi:10.1074/jbc.M110.159590
20. Karin M. Nuclear factor- κ B in cancer development and progression. *Nature* (2006) **441**(7092):431–6. doi:10.1038/nature04870
21. Karin M, Greten FR. NF- κ B: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* (2005) **5**(10):749–59. doi:10.1038/nri1703
22. Frolova L, Drastich P, Rossmann P, Klimesova K, Tlaskalova-Hogenova H. Expression of toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: upregulated expression of TLR2 in terminal ileum of patients with ulcerative colitis. *J Histochem Cytochem* (2008) **56**(3):267–74. doi:10.1369/jhc.7A7303.2007
23. Smith RA, Brooks D, Cokkinides V, Saslow D, Brawley OW. Cancer screening in the United States, 2013. *CA Cancer J Clin* (2013) **63**(2):87–105. doi:10.3322/caac.21174
24. Tenesa A, Dunlop MG. New insights into the aetiology of colorectal cancer from genome-wide association studies. *Nat Rev Genet* (2009) **10**(6):353–8. doi:10.1038/nrg2574
25. Terzic J, Grivennikov S, Karin E, Karin M. Inflammation and colon cancer. *Gastroenterology* (2010) **138**(6): 2101–14.e5. doi:10.1053/j.gastro.2010.01.058
26. Adelstein BA, Macaskill P, Chan SF, Katelaris PH, Irwig L. Most bowel cancer symptoms do not indicate colorectal cancer and polyps: a systematic review. *BMC Gastroenterol* (2011) **11**:65. doi:10.1186/1471-230X-11-65
27. Evans JT, Cluff CW, Johnson DA, Lacy MJ, Persing DH, Baldridge JR. Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribi.529. *Expert Rev Vaccines* (2003) **2**(2):219–29. doi:10.1586/14760584.2.2.219
28. Blasius AL, Beutler B. Intracellular toll-like receptors. *Immunity* (2010) **32**(3):305–15. doi:10.1016/j.jimmuni.2010.03.012
29. Gosu V, Basith S, Durai P, Choi S. Molecular evolution and structural features of IRAK family members. *PLoS One* (2012) **7**(11):e49771. doi:10.1371/journal.pone.0049771
30. Chen ZJ. Ubiquitin signalling in the NF- κ B pathway. *Nat Cell Biol* (2005) **7**(8):758–65. doi:10.1038/ncb0805-758
31. Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, et al. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat Immunol* (2005) **6**(11):1087–95. doi:10.1038/ni1255
32. Takeda K, Akira S. TLR signaling pathways. *Semin Immunol* (2004) **16**(1):3–9. doi:10.1016/j.smim.2003.10.003
33. Moosavi S, Rezaei N. Toll-like receptor signalling and their therapeutic targeting in colorectal cancer. *Int Immunopharmacol* (2013) **16**(2):199–209. doi:10.1016/j.intimp.2013.03.017
34. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol* (2010) **10**(2):131–44. doi:10.1038/nri2707
35. Khan MA, Ma C, Knodler LA, Valdez Y, Rosenberger CM, Deng W, et al. Toll-like receptor 4 contributes to colitis development but not to host defense during *Citrobacter rodentium* infection in mice. *Infect Immun* (2006) **74**(5):2522–36. doi:10.1128/IAI.74.5.2522-2536.2006
36. Lebeis SL, Bommaraju B, Parkos CA, Sherman MA, Kalman D. TLR signalling mediated by MyD88 is required for a protective innate immune response by neutrophils to *Citrobacter rodentium*. *J Immunol* (2013) **179**:566–77. doi:10.4049/jimmunol.179.1.566
37. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun* (2000) **68**(12):7010–7. doi:10.1128/IAI.68.12.7010-7017.2000
38. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* (2001) **167**(4):1882–5. doi:10.4049/jimmunol.167.4.1882
39. Grimm M, Kim M, Rosenwald A, Heemann U, Germer C-T, Waaga-Gasser AM, et al. Toll-like receptor (TLR) 7 and TLR8 expression on CD133+ cells in colorectal cancer points to a specific role for inflammation-induced TLRs in tumorigenesis and tumour progression. *Eur J Cancer* (2010) **46**(15):2849–57. doi:10.1016/j.ejca.2010.07.017
40. Medzhitov R. Origin and physiological roles of inflammation. *Nature* (2008) **454**(7203):428–35. doi:10.1038/nature07201
41. Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol* (2005) **5**(6):446–58. doi:10.1038/nri1630
42. Anwar MA, Basith S, Choi S. Negative regulatory approaches to the attenuation of toll-like receptor signaling. *Exp Mol Med* (2013) **45**:e11. doi:10.1038/emm.2013.28
43. Lebeer S, Vanderleyden J, De Keersmaecker SC. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nat Rev Microbiol* (2010) **8**(3):171–84. doi:10.1038/nrmicro2297
44. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat Rev Immunol* (2010) **10**(3):159–69. doi:10.1038/nri2710
45. Xiao H, Gulec MF, Qin J, Yao J, Bulek K, Kish D, et al. The toll-interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. *Immunity* (2007) **26**(4):461–75. doi:10.1016/j.immuni.2007.02.012
46. Rakoff NS, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* (2004) **118**(2):229–41. doi:10.1016/j.cell.2004.07.002
47. Salcedo R, Worschach A, Cardone M, Jones Y, Gyulai Z, Dai RM, et al. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. *J Exp Med* (2010) **207**(8):1625–36. doi:10.1084/jem.20100199
48. Clevers H. At the crossroads of inflammation and cancer. *Cell* (2004) **118**(6):671–4. doi:10.1016/j.cell.2004.09.005
49. Elinav E, Nowarski R, Thaiss CA, Hu B, Jin C, Flavell RA. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nat Rev Cancer* (2013) **13**(11):759–71. doi:10.1038/nrc3611
50. Ullman TA, Itzkowitz SH. Intestinal inflammation and cancer. *Gastroenterology* (2011) **140**(6):1807–16. doi:10.1053/j.gastro.2011.01.057
51. Uronis JM, Mühlbauer M, Herfarth HH, Rubinas TC, Jones GS, Jobin C. Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility. *PLoS One* (2009) **4**(6):e6026. doi:10.1371/journal.pone.0006026
52. Swann JB, Vesely MD, Silva A, Sharkey J, Akira S, Schreiber RD, et al. Demonstration of inflammation-induced cancer and cancer immunoediting during primary tumorigenesis. *Proc Natl Acad Sci U S A* (2008) **105**(2):652–6. doi:10.1073/pnas.0708594105

53. Balkwill F. Tumour necrosis factor and cancer. *Nat Rev Cancer* (2009) 9(5):361–71. doi:10.1038/nrc2628
54. Govind S. Control of development and immunity by rel transcription factors in *Drosophila*. *Oncogene* (1999) 18(49):6875–87. doi:10.1038/sj.onc.1203223
55. Perkins ND. NF-kappaB: tumor promoter or suppressor? *Trends Cell Biol* (2004) 14(2):64–9. doi:10.1016/j.tcb.2003.12.004
56. Fukata M, Chen A, Vamadevan AS, Cohen J, Breglio K, Krishnareddy S, et al. Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. *Gastroenterology* (2007) 133(6):1869–81. doi:10.1053/j.gastro.2007.09.008
57. Popivanova BK, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S, et al. Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest* (2008) 118(2):560–70. doi:10.1172/JCI32453
58. Oshima H, Oshima M. The inflammatory network in the gastrointestinal tumor microenvironment: lessons from mouse models. *J Gastroenterol* (2012) 47(2):97–106. doi:10.1007/s00535-011-0523-6
59. Ogino S, Kirkner GJ, Noshio K, Irahara N, Kure S, Shima K, et al. Cyclooxygenase-2 expression is an independent predictor of poor prognosis in colon cancer. *Clin Cancer Res* (2008) 14(24):8221–7. doi:10.1158/1078-0432.CCR-08-1841
60. Leaphart CL, Cavallo J, Gribar SC, Cetin S, Li J, Branca MF, et al. A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *J Immunol* (2007) 179(7):4808–20. doi:10.4049/jimmunol.179.7.4808
61. Jilling T, Simon D, Lu J, Meng FJ, Li D, Schy R, et al. The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis. *J Immunol* (2006) 177(5):3273–82. doi:10.4049/jimmunol.177.5.3273
62. Otte JM, Cario E, Podolsky DK. Mechanisms of cross hyporesponsiveness to toll-like receptor bacterial ligands in intestinal epithelial cells. *Gastroenterology* (2004) 126(4):1054–70. doi:10.1053/j.gastro.2004.01.007
63. Abreu MT, Vora P, Faure E, Thomas LS, Arnold ET, Arditi M. Decreased expression of toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J Immunol* (2001) 167(3):1609–16. doi:10.4049/jimmunol.167.3.1609
64. Suzuki M, Hisamatsu T, Podolsky DK. Gamma interferon augments the intracellular pathway for lipopolysaccharide (LPS) recognition in human intestinal epithelial cells through coordinated up-regulation of LPS uptake and expression of the intracellular toll-like receptor 4-MD-2 complex. *Infect Immun* (2003) 71(6):3503–11. doi:10.1128/IAI.71.6.3503-3511.2003
65. Abreu MT, Arnold ET, Thomas LS, Gonsky R, Zhou Y, Hu B, et al. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. *J Biol Chem* (2002) 277(23):20431–7. doi:10.1074/jbc.M110333200
66. Hornef MW, Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J Exp Med* (2002) 195(5):559–70. doi:10.1084/jem.20011788
67. Doan HQ, Bowen KA, Jackson LA, Evers BM. Toll-like receptor 4 activation increases Akt phosphorylation in colon cancer cells. *Anticancer Res* (2009) 29(7):2473–8.
68. Fukata M, Chen A, Klepper A, Krishnareddy S, Vamadevan AS, Thomas LS, et al. Cox-2 is regulated by toll-like receptor-4 (TLR4) signaling: role in proliferation and apoptosis in the intestine. *Gastroenterology* (2006) 131(3):862–77. doi:10.1053/j.gastro.2006.06.017
69. Cammarota R, Bertolini V, Pennesi G, Bucci EO, Gottardi O, Garlanda C, et al. The tumor microenvironment of colorectal cancer: stromal TLR-4 expression as a potential prognostic marker. *J Transl Med* (2010) 8(1):112. doi:10.1186/1479-5876-8-112
70. Fukata M, Abreu MT. TLR4 signalling in the intestine in health and disease. *Biochem Soc Trans* (2007) 35(Pt 6):1473–8. doi:10.1042/BST0351473
71. Abreu MT, Thomas LS, Arnold ET, Lukasek K, Michelsen KS, Arditi M. TLR signaling at the intestinal epithelial interface. *J Endotoxin Res* (2003) 9(5):322–30. doi:10.1179/096805103225002593
72. Kataoka K, Lee J, Rachmilewitz D, Li G, Eckmann L, Raz E. Toll-like receptor 9 – induced type I IFN protects mice from experimental colitis. *J Clin Invest* (2005) 115(3):695–702. doi:10.1172/JCI200522996C1
73. Laird MH, Rhee SH, Perkins DJ, Medvedev AE, Piao W, Fenton MJ, et al. TLR4/MyD88/PI3K interactions regulate TLR4 signaling. *J Leukoc Biol* (2009) 85(6):966–77. doi:10.1189/jlb.1208763
74. Sheng H, Shao J, Townsend CM Jr, Evers BM. Phosphatidylinositol 3-kinase mediates proliferative signals in intestinal epithelial cells. *Gut* (2003) 52(10):1472–8. doi:10.1136/gut.52.10.1472
75. Somanath PR, Kandel ES, Hay N, Byzova TV. Akt1 signaling regulates integrin activation, matrix recognition, and fibronectin assembly. *J Biol Chem* (2007) 282(31):22964–76. doi:10.1074/jbc.M700241200
76. Hsu RY, Chan CH, Spicer JD, Rousseau MC, Giannias B, Rousseau S, et al. LPS-induced TLR4 signaling in human colorectal cancer cells increases beta1 integrin-mediated cell adhesion and liver metastasis. *Cancer Res* (2011) 71(5):1989–98. doi:10.1158/0008-5472.CAN-10-2833
77. Earl TM, Nicoud IB, Pierce JM, Wright JP, Majoras NE, Rubin JE, et al. Silencing of TLR4 decreases liver tumor burden in a murine model of colorectal metastasis and hepatic steatosis. *Ann Surg Oncol* (2009) 16(4):1043–50. doi:10.1245/s10434-009-0325-8
78. Killeen S, Wang J, Andrews E, Redmond H. Bacterial endotoxin enhances colorectal cancer cell adhesion and invasion through TLR-4 and NF- κ B-dependent activation of the urokinase plasminogen activator system. *Br J Cancer* (2009) 100(10):1589–602. doi:10.1038/sj.bjc.6604942
79. Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. *J Clin Invest* (2001) 107(3):241–6. doi:10.1172/JCI11991
80. Hatta Y, Koeffler HP. Role of tumor suppressor genes in the development of adult T cell leukemia/lymphoma (ATLL). *Leukemia* (2002) 16(6):1069–85. doi:10.1038/sj.leu.2402458
81. Ryan KM, Ernst MK, Rice NR, Vousden KH. Role of NF-kappaB in p53-mediated programmed cell death. *Nature* (2000) 404(6780):892–7. doi:10.1038/35009130
82. Wang EL, Qian ZR, Nakasono M, Tanahashi T, Yoshimoto K, Bando Y, et al. High expression of toll-like receptor 4/myeloid differentiation factor 88 signals correlates with poor prognosis in colorectal cancer. *Br J Cancer* (2010) 102(5):908–15. doi:10.1038/sj.bjc.6605558
83. Rakoff NS, Medzhitov R. Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. *Science* (2007) 317(5834):124–7. doi:10.1126/science.1140488
84. Lee SH, Hu LL, Gonzalez-Navajas J, Seo GS, Shen C, Brick J, et al. ERK activation drives intestinal tumorigenesis in Apc(min+) mice. *Nat Med* (2010) 16(6):665–70. doi:10.1038/nm.2143
85. Gustafson WC, Weiss WA. Myc proteins as therapeutic targets. *Oncogene* (2010) 29(9):1249–59. doi:10.1038/onc.2009.512
86. Wilkins JA, Sansom OJ. C-Myc is a critical mediator of the phenotypes of Apc loss in the intestine. *Cancer Res* (2008) 68(13):4963–6. doi:10.1158/0008-5472.CAN-07-5558
87. Fukata M, Abreu MT. Microflora in colorectal cancer: a friend to fear. *Nat Med* (2010) 16(6):639–41. doi:10.1038/nm0610-639
88. Groden J, Thliveris A, Samowitz W, Carlson M, Gelbert L, Albertsen H, et al. Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* (1991) 66(3):589–600. doi:10.1016/0092-8674(81)90021-0
89. Goss KH, Groden J. Biology of the adenomatous polyposis coli tumor suppressor. *J Clin Oncol* (2000) 18(9):1967–79.
90. Opal SM, Esmon CT. Bench-to-bedside review: functional relationships between coagulation and the innate immune response and their respective roles in the pathogenesis of sepsis. *Crit Care* (2002) 7(1):23. doi:10.1186/cc1854
91. Hennessy EJ, Parker AE, O'Neill LA. Targeting toll-like receptors: emerging therapeutics? *Nat Rev Drug Discov* (2010) 9(4):293–307. doi:10.1038/nrd3203
92. Slattery ML, Herrick JS, Bondurant KL, Wolff RK. Toll-like receptor genes and their association with colon and rectal cancer development and prognosis. *Int J Cancer* (2012) 130(12):2974–80. doi:10.1002/ijc.26314
93. Pimentel-Nunes P, Teixeira AL, Pereira C, Gomes M, Brandao C, Rodrigues C, et al. Functional polymorphisms of toll-like receptors 2 and 4 alter the risk for colorectal carcinoma in Europeans. *Dig Liver Dis* (2013) 45(1):63–9. doi:10.1016/j.dld.2012.08.006
94. Omrane I, Baroudi O, Kourda N, Bignon YJ, Uhrhammer N, Desrichard A, et al. Positive link between variant toll-like receptor 4 (Asp299Gly and Thr399Ile)

- and colorectal cancer patients with advanced stage and lymph node metastasis. *Tumour Biol* (2013) **35**(1):545–51. doi:10.1007/s13277-013-1075-6
95. Mockenhaupt FP, Cramer JP, Hamann L, Stegemann MS, Eckert J, Oh NR, et al. Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria. *Proc Natl Acad Sci U S A* (2006) **103**(1):177–82. doi:10.1073/pnas.0506803102
96. Garza-Gonzalez E, Bosques-Padilla FJ, Mendoza-Ibarra SI, Flores-Gutierrez JP, Maldonado-Garza HJ, Perez-Perez GI. Assessment of the toll-like receptor 4 Asp299Gly, Thr399Ile and interleukin-8-251 polymorphisms in the risk for the development of distal gastric cancer. *BMC Cancer* (2007) **7**(1):70. doi:10.1186/1471-2407-7-70
97. Ferwerda B, McCall MB, Verheijen K, Kullberg BJ, Van Der Ven AJ, Van Der Meer JW, et al. Functional consequences of toll-like receptor 4 polymorphisms. *Mol Med* (2008) **14**(5–6):346–52. doi:10.2119/2007-00135.Ferwerda
98. Boraska Jelavic T, Barisic M, Drmic Hofman I, Boraska V, Vrdoljak E, Peruzovic M, et al. Microsatellite GT polymorphism in the toll-like receptor 2 is associated with colorectal cancer. *Clin Genet* (2006) **70**(2):156–60. doi:10.1111/j.1399-0004.2006.00651.x
99. Li XX, Sun GP, Meng J, Li X, Tang YX, Li Z, et al. Role of toll-like receptor 4 in colorectal carcinogenesis: a meta-analysis. *PLoS One* (2014) **9**(4):e93904. doi:10.1371/journal.pone.0093904
100. Santini D, Angeletti S, Russo A, Dicuonzo G, Galluzzo S, Vincenzi B, et al. Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms in gastric cancer of intestinal and diffuse histotypes. *Clin Exp Immunol* (2008) **154**(3):360–4. doi:10.1111/j.1365-2249.2008.03776.x
101. Zhao X, Kang S, Liu L, Zhang D. Correlation of Asp299Gly and Thr399Ile polymorphisms in toll-like receptor 4 gene with digestive cancer risk: a meta-analysis. *Biomed Rep* (2013) **1**(2):294–302. doi:10.3892/br.2012.32
102. Castro FA, Forstai A, Buch S, Kalthoff H, Krauss C, Bauer M, et al. TLR-3 polymorphism is an independent prognostic marker for stage II colorectal cancer. *Eur J Cancer* (2011) **47**(8):1203–10. doi:10.1016/j.ejca.2010.12.011
103. Tsilidis KK, Helzlsouer KJ, Smith MW, Grinberg V, Hoffman-Bolton J, Clipp SL, et al. Association of common polymorphisms in IL10, and in other genes related to inflammatory response and obesity with colorectal cancer. *Cancer Causes Control* (2009) **20**(9):1739–51. doi:10.1007/s10552-009-9427-7
104. Guo Q, Zhu J, Xia B. Polymorphism of CD14 gene but not the mutation of TLR4 gene is associated with colorectal cancer in Chinese patients. *J Gastroenterol Hepatol* (2006) **21**(1 Pt 1):92–7. doi:10.1111/j.1440-1746.2005.04156.x
105. Chen R, Luo FK, Wang YL, Tang JL, Liu YS. LBP and CD14 polymorphisms correlate with increased colorectal carcinoma risk in Han Chinese. *World J Gastroenterol* (2011) **17**(18):2326–31. doi:10.3748/wjg.v17.i18.2326
106. Landi S, Gemignani F, Bottari F, Gioia-Patriconi L, Guino E, Cambray M, et al. Polymorphisms within inflammatory genes and colorectal cancer. *J Negat Results Biomed* (2006) **5**:15. doi:10.1186/1477-5751-5-15
107. Davoodi H, Seow HF. Variant toll-like receptor4 (Asp299Gly and Thr399Ile alleles) and toll-like receptor2 (Arg753Gln and Arg677Trp alleles) in colorectal cancer. *Iran J Allergy Asthma Immunol* (2011) **10**(2):91–9. doi:0.02/ijaa.9199
108. Kutikhin AG, Yuzhalin AE, Volkov AN, Zhivotovskiy AS, Brusina EB. Correlation between genetic polymorphisms within IL-1B and TLR4 genes and cancer risk in a Russian population: a case-control study. *Tumour Biol* (2014) **35**(5):4821–30. doi:10.1007/s13277-014-1633-6
109. Takahashi K, Sugi Y, Hosono A, Kaminogawa S. Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis. *J Immunol* (2009) **183**(10):6522–9. doi:10.4049/jimmunol.0901271
110. Govind CK, Ginsburg D, Hinnebusch AG. Measuring dynamic changes in histone modifications and nucleosome density during activated transcription in budding yeast. *Methods Mol Biol* (2012) **833**:15–27. doi:10.1007/978-1-61779-477-3_2
111. Takahashi K, Sugi Y, Nakano K, Tsuda M, Kurihara K, Hosono A, et al. Epigenetic control of the host gene by commensal bacteria in large intestinal epithelial cells. *J Biol Chem* (2011) **286**(41):35755–62. doi:10.1074/jbc.M111.271007
112. Davie JR. Inhibition of histone deacetylase activity by butyrate. *J Nutr* (2003) **133**(7 Suppl):2485S–93S.
113. Waldecker M, Kautenburger T, Daumann H, Busch C, Schrenk D. Inhibition of histone-deacetylase activity by short-chain fatty acids and some polyphenol metabolites formed in the colon. *J Nutr Biochem* (2008) **19**(9):587–93. doi:10.1016/j.jnutbio.2007.08.002
114. Vamadevan AS, Fukata M, Arnold ET, Thomas LS, Hsu D, Abreu MT. Regulation of toll-like receptor 4-associated MD-2 in intestinal epithelial cells: a comprehensive analysis. *Innate Immun* (2010) **16**(2):93–103. doi:10.1177/1753425909339231
115. Kanzler H, Barrat PJ, Hessel EM, Coffman RL. Therapeutic targeting of innate immunity with toll-like receptor agonists and antagonists. *Nat Med* (2007) **13**(5):552–9. doi:10.1038/nm1589
116. Akira S. Mammalian toll-like receptors. *Curr Opin Immunol* (2003) **15**(1):5–11. doi:10.1016/S0952-7915(03)00005-0
117. Mackensen A, Galanos C, Engelhardt R. Treatment of cancer patients with endotoxin induces release of endogenous cytokines. *Pathobiology* (1991) **59**(4):264–7. doi:10.1159/000163659
118. Engelhardt R, Mackensen A, Galanos C. Phase I trial of intravenously administered endotoxin (*Salmonella abortus equi*) in cancer patients. *Cancer Res* (1991) **51**(10):2524–30.
119. Galluzzi L, Vacchelli E, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, et al. Trial watch: experimental toll-like receptor agonists for cancer therapy. *Oncotarget* (2012) **1**(5):699–716. doi:10.4161/onci.20696
120. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science* (2007) **316**(5831):1628–32. doi:10.1126/science.1138963
121. Persing DH, Coler RN, Lacy MJ, Johnson DA, Baldridge JR, Hershberg RM, et al. Taking toll: lipid A mimetics as adjuvants and immunomodulators. *Trends Microbiol* (2002) **10**(10):s32–7. doi:10.1016/S0966-842X(02)02426-5
122. Krieg AM. Toll-free vaccines? *Nat Biotechnol* (2007) **25**(3):303–5. doi:10.1038/nbt0307-303
123. Basith S, Manavalan B, Yoo TH, Kim SG, Choi S. Roles of toll-like receptors in cancer: a double-edged sword for defense and offense. *Arch Pharm Res* (2012) **35**(8):1297–316. doi:10.1007/s12272-012-0802-7
124. D’Agostini C, Pica F, Febbraro G, Grelli S, Chiavaroli C, Garaci E. Antitumour effect of OM-174 and cyclophosphamide on murine B16 melanoma in different experimental conditions. *Int Immunopharmacol* (2005) **5**(7–8):1205–12. doi:10.1016/j.intimp.2005.02.013
125. De Ridder M, Verovski VN, Chiavaroli C, Van Den Berghe DL, Monsaert C, Law K, et al. The radiosensitizing effect of immunoadjuvant OM-174 requires cooperation between immune and tumor cells through interferon-gamma and inducible nitric oxide synthase. *Int J Radiat Oncol Biol Phys* (2006) **66**(5):1473–80. doi:10.1016/j.ijrobp.2006.07.1381
126. Isambert N, Fumoleau P, Paul C, Ferrand C, Zanetta S, Bauer J, et al. Phase I study of OM-174, a lipid A analogue, with assessment of immunological response, in patients with refractory solid tumors. *BMC Cancer* (2013) **13**:172. doi:10.1186/1471-2407-13-172
127. Butts C, Murray N, Maksymiuk A, Goss G, Marshall E, Soulieres D, et al. Randomized phase IIB trial of BLP25 liposome vaccine in stage IIIB and IV non-small-cell lung cancer. *J Clin Oncol* (2005) **23**(27):6674–81. doi:10.1200/JCO.2005.13.011
128. Kroemer G, Zitvogel L, Galluzzi L. Victories and deceptions in tumor immunology: Stimuvax. *Oncotarget* (2013) **2**(1):e23687. doi:10.4161/onci.23687
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The role of toll-like receptors in colorectal cancer progression: evidence for epithelial to leucocytic transition

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Toll-like receptors (TLRs) are expressed by immune cells, intestinal epithelium, and tumor cells. In the homeostatic setting, they help to regulate control over invading pathogens and maintain the epithelial lining of the large and small intestines. Aberrant expression of certain TLRs by tumor cells can induce growth inhibition while others contribute to tumorigenesis and progression. Activation of these TLRs can induce inflammation, tumor cell proliferation, immune evasion, local invasion, and distant metastasis. These TLR-influenced behaviors have similarities with properties observed in leukocytes, suggesting that tumors may be hijacking immune programs to become more aggressive. The concept of epithelial to leucocytic-transition (ELT) is proposed, akin to epithelial to mesenchymal transition, in which tumors develop the ability to activate leucocytic traits otherwise inaccessible to epithelial cells. Understanding the mechanisms of ELT could lead to novel therapeutic strategies for inhibiting tumor metastasis.

Keywords: colorectal cancer, toll-like receptors, epithelial to leucocytic transition, ELT, metastasis, immune evasion, cell plasticity, inflammation

INTRODUCTION

Toll-like receptors (TLRs) are a diverse family of pattern recognition receptors expressed by immune cells from both the innate and adaptive arms of the immune system (1–4). Hence, TLRs stimulate both innate and adaptive immune responses to invading pathogen-associated molecular patterns (PAMPs) as well as danger-associated molecular patterns (DAMPs) from damaged epithelial cells. Ligand specificity, downstream signaling, and subsequent immune stimulation vary greatly among the TLR subtypes (5, 6). The complex expression patterns of TLRs differ among cell types, physiological location, and are controlled by the microenvironment (7–9). In the healthy gastrointestinal (GI) tract, TLRs are expressed by intestinal epithelial cells (IEC) and play a role in immune modulation and tissue homeostasis by stimulating the immune response to bacterial pathogens, attenuating the immune response against favorable microbes, sensing breakdown of the protective intestinal barriers, and triggering proliferative signaling (6, 10, 11). The lumen of the gut is subjected to continual interactions with the microbiome and would be in a constant state of inflammation were it not for the controlled expression and normal function of these TLRs (2, 6).

Inflammation has been implicated as an underlying factor in tumorigenesis and cancer progression, leading transformed cells to develop the “hallmarks of cancer” (12, 13). Some of the same TLRs (TLR 2–4) that normally regulate inflammation in the gut are also found to be aberrantly expressed in colorectal cancers (CRCs) (14, 15). Overexpression of TLR4 in CRC is associated with poor survival (16). Deletion of TLR4, its signaling partner Myd88, or absence of its ligand LPS in the colon

can lead to increased or reduced inflammation, depending on the cancer subtype, which can then lead to either increased or decreased tumorigenesis and tumor progression (17, 18). It is notable that the role of TLRs in CRC reflects a “natural history” of selection events that lead from normal TLR function in unaffected colon tissue and throughout all stages of CRC progression. More specifically, the normal role of TLR immune modulation in the gut involves the controlled release of cytokines and danger signals that stimulate the immune response to bacterial pathogens and attenuate the immune response against favorable microbes (6, 10). Microbial imbalance and/or dysregulation of these responses leads to chronic inflammation of the bowel (15). Chronic inflammation is correlated with initiation of cancer development and in the progression of cancer into more aggressive forms of malignancies (2). Aberrant TLR signaling and the resulting cytokine imbalance leads to increased epithelial proliferation and decreased cell death (19). Additionally, an active immune environment creates selection pressures for initiating cancer cells resulting in the evolution of an immune-evasive tumor phenotype (14). Furthermore, TLR dysregulation is implicated in cancer invasion and metastasis (2, 19–21). Understanding the role of TLRs in the natural evolution of metastatic disease is crucial for developing new therapies and optimizing current treatments.

ROLE OF TLRs IN INFLAMMATION-MEDIATED TUMORIGENESIS

The intestines house approximately 70% of the body’s immune cells under normal conditions (10). Signaling between these

immune cells, commensal bacteria, and IECs is critical for normal digestion and protection against invading pathogens (6). TLRs are key modulators of the immune system of the GI tract. In order to maintain homeostasis and suppress immune responses to commensal bacteria (11), TLR expression and signaling are tightly controlled in this environment (6, 20). However, these controls are disrupted in diseases such as Crohn's disease and ulcerative colitis, resulting in chronic inflammation (11, 15). Inflammation is linked to cancer through two pathways: extrinsic inflammation induced by non-transformed cells (e.g., invading pathogens or autoimmune disease), and intrinsic inflammation induced by transformed cells (22). In CRC, TLRs are involved in both. Autoimmune diseases cause chronic, smoldering levels of inflammation that predispose individuals into developing CRC (22). Once initiated, tumors can intrinsically activate inflammation through TLR binding by cancer-related DAMPs. Intrinsically and extrinsically induced TLR activation results in tumor-promoting inflammation through NF- κ B signaling, leading to expression of the inflammatory cytokines IL-1 β , TNF α , and IL-6 (17). This aberrant expression by tumor cells in early carcinogenesis can recruit tumor-promoting immune cells, leading to inflammation and protection from cytotoxic immune cells. Additional data from Kim et al. links mutations in p53 and PTEN to SOCS-mediated activation of IL-6 signaling, leading to intrinsic inflammation (23). Since p53 mutation is a very common event in the natural history of CRC, this is likely a major mechanism of tumor-induced inflammation. Additionally, inflammation can drive genetic and epigenetic changes in cells as well as possible alterations in lineage differentiation programs leading to increased plasticity. This process is also thought to involve NF- κ B signaling; however, further studies are needed (22, 24).

ROLE OF TLRs IN INFLAMMATION-MEDIATED PROLIFERATION AND SURVIVAL

Inflammatory pathways are tightly linked to aberrant proliferation and resistance to cell death, which are key cancer hallmarks that can be mediated through TLR activation (14). IECs are the barrier layer that protects the interstitial layers from the changing exterior environment of the GI tract. Infiltrating bacteria and the resulting immune response can cause tissue damage. To prepare for this, IECs utilize TLR4 signaling as an early warning system to initiate proliferation, maintain tissue integrity, and protect the interstitial compartments (6). Tumors can co-opt this system, allowing cells to proliferate unchecked (25).

Tumor growth is further fueled through an overabundance of growth factors (e.g., TGF- β , IL-8, CXCR4, and VEGF) (15), a decline in immune surveillance, and the evolution of mobile and invasive phenotypes. TLR expression on tumor cells stimulates the release of cytokines that recruit favorable immune cells further driving proliferation. Additionally, the release of cytokines and chemokines due to TLR signaling generates an autocrine loop that further stimulates tumor cell growth. The cumulative result is tumor control over its own environment.

ROLE OF TLRs IN IMMUNE EVASION

An active immune environment selects for the natural evolution of cancer cells with decreased immunogenic phenotypes. TLR expression in tumors can confer the advantages of both immune evasion and immunosuppression (26). Often pro-inflammatory signals reduce elements of the adaptive immune response. TLR signaling causes a shift in this response from anti-tumor to pro-tumor by affecting the balance toward inflammation and suppression of anti-tumor immunity. Direct TLR activation results in production of immunosuppressive cytokines IL-10 and TGF- β (14, 27), as well as increased expression of immune modulating surface markers PD-L1 and HLA-G (19, 20, 28). These secreted and surface proteins have a tolerizing effect on immune cells. TLR-activated IECs induce the transformation of dendritic cells (DC) into an antigen-specific CD103+ phenotype. These DC promote contact-dependent antigen-specific regulatory T cells (Tregs) that express gut-homing integrins, which further attenuates the anti-tumor immune response (10). Each of these mechanisms are used in the healthy gut to avoid food hypersensitivity or auto-immune diseases. However, dysregulation through abnormal TLR expression can lead to malignant progression.

ROLE OF TLRs IN INVASION AND METASTASIS

The most dangerous effect of tumoral TLR signaling is the acquisition of invasive and metastatic tumor phenotypes (29). Ninety percent of patients who succumb to their disease have metastatic lesions (30). TLR expression in tumors is linked to increased grade and distant metastasis (2, 18, 21, 31). The ability of a tumor cell to detach from its epithelial neighbors, break through the basement membrane, and invade nearby tissues is, in part, the result of a long history of aberrant TLR signaling. In CRC, TLR-mediated alterations of the immune system components in the tumor microenvironment can change intracellular signaling (NF- κ B), integrin expression (B1 integrin), and motility (29, 32). Activation of TLR4 by LPS *in vitro* and *in vivo* induces epithelial to mesenchymal transition (EMT) and invasive phenotypes in certain cell lines (29, 33).

Immune cells are educated by tumor-secreted factors and then actively migrate through the lymphatic vessels and secondary lymphoid organs. These tightly gated organs allow entry and passage to soluble antigens and select immune cell phenotypes, and yet lymph nodes are often the first site of metastasis (34). While it was once thought that tumors cells passively filter into draining lymph nodes, it has recently been shown that tumor cells require chemokine-mediated (CCR7 and CCR8) active transport through the subcapsular sinus epithelium (35, 36). Furthermore, it has been shown that tumor-mediated lymphatic remodeling of peritumoral lymph vessels and draining lymph nodes facilitates metastasis (37–40). TLRs may play a role in this metastatic process, since TLR activation leads to increased expression of CCR7 and CCR8 (41), which are key molecules expressed by leukocytes to access lymphatics (35, 42). This suggests that the tumor cells can harness existing leucocytic mechanisms to begin the metastatic cascade through the lymph nodes.

Lymphocytes typically traffic throughout the body to sites of inflammation, using chemokines, selectins, and integrins as

homing signals (43). Many metastatic tumors have been shown to use the expression of these same molecules to colonize distal sites (44, 45). As an example, CXCR4 is a well-characterized bone marrow homing receptor expressed by T cells (46); research has found that both prostate cancers (47) and breast cancers (48) that metastasize to the bone commonly express CXCR4. CRC typically metastasizes to the liver or lung. Aberrant expression of CXCR3, CXCR4/CXCR7, and CCR6 are commonly found in liver and lung metastasis of colon cancer (49–55). Ligands for these receptors (CXCL19, SDF-1, and CCL20, respectively) are highly expressed in the liver and lungs of metastatic CRC patients (53, 56–58). Local inflammation in these organs induces ligand expression and preferential organ metastasis is determined by their expression (59, 60).

Alteration in integrin signaling is another metastatic mechanism induced by TLR signaling (26). Integrin signaling is used in healthy systems to aid immune cell trafficking (61). Aberrant expression of these integrins via TLR signaling allows circulating tumor cells to respond to the same trafficking mechanisms that an immune cell uses to migrate to distal sites (2, 32, 62, 63). Similar examples have been shown with integrins in colon cancer (64), breast cancer (65), and melanoma (66). These expressed surface markers are a natural part of the lymphocytic trafficking system, and their expression on tumor cells could be evidence that tumor cells use leucocytic trafficking mechanisms to metastasize.

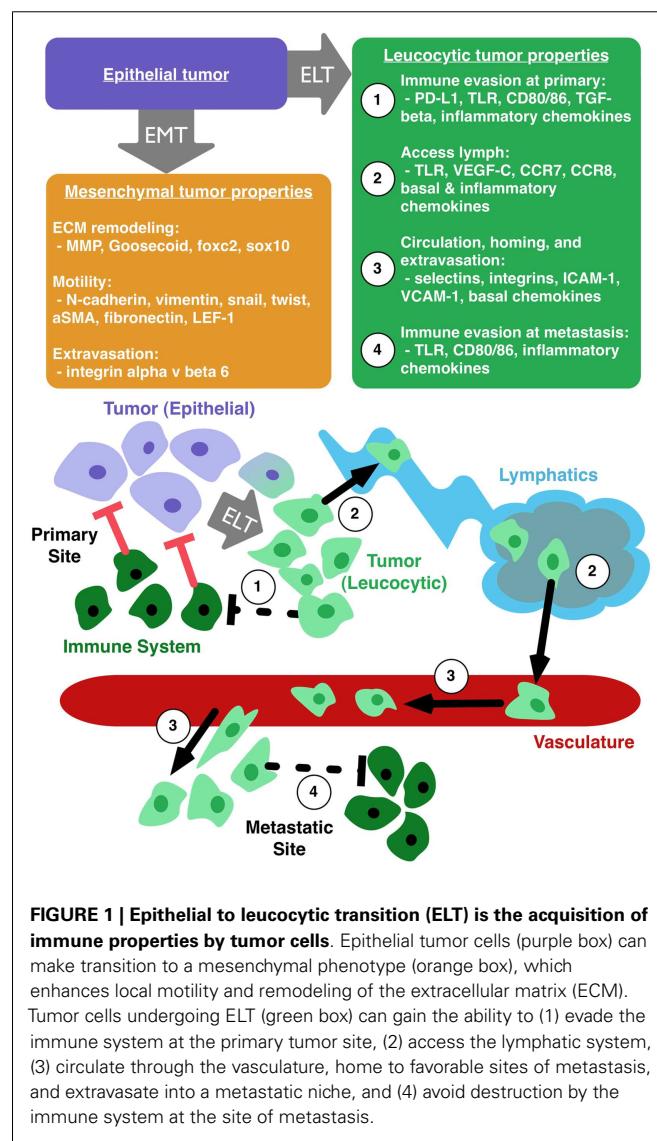
EPITHELIAL TO LEUCOCYTIC TRANSITION

The co-opting of immune cell signaling and migration mechanisms by tumor cells is well documented, with many citing the plasticity of tumor cells and inappropriate gene expression as the underlying cause of treatment resistance and metastatic growth (13, 67–70). Pressures from cytotoxic immune cells, abundant inflammation, cytotoxic drugs, and targeted therapies push tumor cells into plastic states where they may begin to access programmed mechanisms outside of their usual function (68). The survivors of these selection pressures are adaptive and dynamic cells, many of which express patterns of proteins found in other normal cell types (70, 71). These protein expression patterns have been used to define and detect EMT, for example. An increasing number of publications suggest that although EMT is important in locally invasive disease, it is not enough to allow tumor cells access to lymph nodes, lymphatic and vascular systems, as well as entrance and settlement into distant tissues (35, 69, 72, 73). Others hypothesize a myeloid lineage expression pattern gained from horizontal gene transfer and Lamarckian inheritance, tumor cell myeloid cell fusion, or a possible myeloid cell origin (69, 74–76). Here, we build on these observations and propose a new concept, the transition from epithelial phenotype to leucocytic phenotype.

Immune cells of myeloid and lymphoid origins house a diverse set of mechanisms that make them perfect trafficking cells. They can shift their metabolism easily, survive in low oxygenated areas, roll along the endothelium in the presence of high shear forces, read integrin codes, and facilitate tissue specific extravasation (77–79). As described above, the aberrant expression of TLRs by CRC cells results in the acquisition of a number of tumor-promoting mechanisms. At the same time, these mechanisms are key properties of the immune system, as is TLR expression. In a broad

sense, immunosuppression, migration through tissue, intra- and extravasation through lymph and blood vessels, rapid proliferation, altered metabolism, and homing to specific tissues are key hallmarks of both cancer and the immune system.

Pathogenic EMT has its roots in normal embryogenesis. In cancer, this transition results in epithelial cells with a range of mesenchymal protein expression. These alterations increase motility and invasive capability of tumor cells, but do not necessarily explain immuno-evasion, lymphatic access, and metastatic spread (35, 69, 72). We therefore propose the parallel concept of epithelial to leucocytic transition (ELT) as a framework, akin to EMT, with which to understand the metastatic properties of cancer cells. Figure 1 illustrates the primary properties gained by tumor cells that undergo ELT. We consider ELT to be a partial transition in which epithelial cells retain their epithelial origin while at the same time acquiring a set of leucocytic traits. Tumor cells co-opt many mechanisms of the immune system for their own transport and these mechanisms are activated by proteins typically reserved for



the immune response. A leucocytic tumor cell expresses proteins that allow for regulation and co-opting of the immune system such as PD-L1, CD80/86, TLR, TGF- β , CCL4, and CCL5 (80) (**Figure 1**, properties 1 and 4). Additional leucocytic proteins (CXCR4, CCR7, CCR8) facilitate invasion and proliferation within lymph nodes (**Figure 1**, property 2) (35, 42, 81). Processes critical to survival in circulation, homing to tissue specific sites, and successful extravasation are mediated by E/P-selectins, L-selectin ligands, α 4 β 1, ICAM-1, and VCAM-1 (61, 73, 82–86) (**Figure 1**, property 3). By harnessing mechanisms usually reserved for immune cells, tumor cells gain the ability to become more aggressive. In the case of TLRs, a cycle of overexpression and resulting inflammation promotes plasticity of the epithelial phenotype. This plasticity permits tumor cells to undergo ELT, accessing immune programs that facilitate invasion and metastasis of the cancer. ELT, as with other plastic states, is likely transient, making the evaluation of these phenotypes a significant challenge. TLR-mediated evolution of CRC may be a good model to study how ELT occurs, since TLRs are primarily seen in immune cells and the overexpression of TLRs appears to promote an immune-like phenotype in CRC.

Understanding the acquisition of the leucocytic phenotype could reveal key targets that would prevent CRC cells from accessing dangerous invasion and trafficking mechanisms through a plastic transition. Simply antagonizing TLRs and associated molecules may not be enough, since resistance is likely to develop. However, if the mechanisms of plasticity induced by TLRs are understood, new targets may be developed to inhibit ELT.

It is important to note that the key functional activities of immune cells, specifically the CD8 cytotoxic T-cell phenotype and the antibody producing activated B-cell phenotypes, have not yet been described in tumor cells. However, other cytotoxic mechanisms utilized by immune cells have been seen in normal and neoplastic epithelial cells. Tumor cell cannibalism, resembling phagocytosis, of neighboring apoptotic cells as well as infiltrating immune cells has been seen during times of metabolic stress (87). During mammary involution, epithelial-derived FAS plays a role in FASL-mediated cell death (88). Tumor cells can secrete FAS, TNF α , and TGF β , proteins capable of promoting and inhibiting epithelial cell death (89–91). Additionally, PD-L1 proteins on tumor cells result in T-cell anergy and apoptosis (92, 93). Although, none of these represent the antigen-specific killing of the adaptive immune system, it is our opinion that further exploration is needed to determine how far epithelial cells can evolve to obtain immune-like processes and that cell killing can not yet be included or excluded from that hypothesis.

CONCLUSION

Originally, lymphatic dissemination into draining lymph nodes was considered a clear indicator of prognosis and was attributed to tumor chronology based on the correlation of tumor volumes and lymph node metastasis. However, later larger studies often showed conflicting results. Jatoi et al. (94, 95) and others attributed these differences to the tumor phenotype as opposed to a simple passage of time. This means that tumor phenotypes can exist on a continuum from slow growing with late lymph node metastasis to aggressive early disseminators much more capable of exiting the lymph node and establishment at distant sites (94, 95).

While lymph node positivity is a useful tool for treatment decisions understanding the complexities of these aggressive phenotypes is key to halting the lymphatic dissemination of cancer.

Many host parameters contribute to natural progression of tumor metastasis and the extent of tumor cell plasticity is not yet fully appreciated. In an opinion article on tumor and immune cell plasticity, Holzel et al. (68) recognize the similarity between cancer cells and immune cells by linking inflammation and evolutionary pressures to the creation of plastic phenotypes. We think that this idea needs to be taken further to include a plastic transition to an immune-like phenotype, i.e., ELT, in the context tumor development, invasion, metastasis, and resistance to therapies. Specifically in CRC, the tumor cells acquire many hallmarks of the immune system, and this transition is intimately tied to aberrant TLR expression. By considering TLR expression in the context of ELT, the transition to a migratory immune-like and therefore metastatic phenotype might be better understood, and therefore, lead to better therapeutic strategies.

REFERENCES

- Rahman AH, Taylor DK, Turka LA. The contribution of direct TLR signaling to T cell responses. *Immunol Res* (2009) **45**(1):25–36. doi:10.1007/s12026-009-8113-x
- Lu Q, Ding H, Li W. Role of toll-like receptors in microbiota-associated gastrointestinal cancer metastasis. *J Cancer Res Ther* (2013) **9**(Suppl):S142–9. doi:10.4103/0973-1482.122509
- Du T, Zhou ZG, You S, Huang G, Lin J, Yang L, et al. Modulation of monocyte hyperresponsiveness to TLR ligands by 1,25-dihydroxy-vitamin D3 from LADA and T2DM. *Diabetes Res Clin Pract* (2009) **83**(2):208–14. doi:10.1016/j.diabres.2008.09.046
- Hua Z, Hou B. TLR signaling in B-cell development and activation. *Cell Mol Immunol* (2013) **10**(2):103–6. doi:10.1038/cmi.2012.61
- Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* (2004) **4**(7):499–511. doi:10.1038/nri1391
- Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nat Rev Immunol* (2010) **10**(2):131–44. doi:10.1038/nri2707
- Zaremba KA, Godowski PJ. Tissue expression of human toll-like receptors and differential regulation of toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* (2002) **168**(2):554–61. doi:10.4049/jimmunol.168.2.554
- Applequist SE, Wallin RP, Ljunggren HG. Variable expression of toll-like receptor in murine innate and adaptive immune cell lines. *Int Immunopharmacol* (2002) **14**(9):1065–74. doi:10.1093/intimm/dxf069
- Takenaka S, McCormick S, Safronova E, Xing Z, Gauldie J. Influence of the tissue microenvironment on toll-like receptor expression by CD11c+ antigen-presenting cells isolated from mucosal tissues. *Clin Vaccine Immunol* (2009) **16**(11):1615–23. doi:10.1128/CVI.00216-09
- de Kvit S, Tobin MC, Forsyth CB, Keshavarzian A, Landay AL. Regulation of intestinal immune responses through TLR activation: implications for pro- and prebiotics. *Front Immunol* (2014) **5**:60. doi:10.3389/fimmu.2014.00060
- Cario E. Toll-like receptors in inflammatory bowel diseases: a decade later. *Inflamm Bowel Dis* (2010) **16**(9):1583–97. doi:10.1002/ibd.21282
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) **144**(5):646–74. doi:10.1016/j.cell.2011.02.013
- Coussens LM, Werb Z. Inflammation and cancer. *Nature* (2002) **420**(6917):860–7. doi:10.1038/nature01322
- Ridnour LA, Cheng RY, Switzer CH, Heinecke JL, Ambs S, Glynn S, et al. Molecular pathways: toll-like receptors in the tumor microenvironment – poor prognosis or new therapeutic opportunity. *Clin Cancer Res* (2013) **19**(6):1340–6. doi:10.1158/1078-0432.CCR-12-0408
- Sato Y, Goto Y, Narita N, Hoon DS. Cancer cells expressing toll-like receptors and the tumor microenvironment. *Cancer Microenviron* (2009) **2**(Suppl 1):205–14. doi:10.1007/s12307-009-0022-y

16. Eiro N, Gonzalez L, Gonzalez LO, Fernandez-Garcia B, Andicocchea A, Barbon E, et al. Toll-like receptor-4 expression by stromal fibroblasts is associated with poor prognosis in colorectal cancer. *J Immunother* (2013) **36**(6):342–9. doi:10.1097/CJI.0b013e31829d85e6
17. Pradere JP, Dapito DH, Schwabe RF. The Yin and Yang of toll-like receptors in cancer. *Oncogene* (2013) **33**(27):3485–95. doi:10.1038/onc.2013.302
18. Salcedo R, Worschach A, Cardone M, Jones Y, Gyulai Z, Dai RM, et al. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. *J Exp Med* (2010) **207**(8):1625–36. doi:10.1084/jem.20100199
19. Yu L, Wang L, Chen S. Dual character of toll-like receptor signaling: pro-tumorigenic effects and anti-tumor functions. *Biochim Biophys Acta* (2013) **1835**(2):144–54. doi:10.1016/j.bbcan.2012.10.006
20. Huang B, Zhao J, Unkeless JC, Feng ZH, Xiong H. TLR signaling by tumor and immune cells: a double-edged sword. *Oncogene* (2008) **27**(2):218–24. doi:10.1038/sj.onc.121904
21. O'Leary DP, Bhatt L, Woolley JE, Gough DR, Wang JH, Cotter TG, et al. TLR-4 signalling accelerates colon cancer cell adhesion via NF-κappaB mediated transcriptional up-regulation of Nox-1. *PLoS One* (2012) **7**(10):e44176. doi:10.1371/journal.pone.0044176
22. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* (2008) **454**(7203):436–44. doi:10.1038/nature07205
23. Kim G, Ouzounova M, Quraishi AA, Davis A, Tawakkol N, Clouthier SG, et al. SOCS3-mediated regulation of inflammatory cytokines in PTEN and p53 inactivated triple negative breast cancer model. *Oncogene* (2014). doi:10.1038/onc.2014.4
24. Kwon OJ, Zhang L, Ittmann MM, Xin L. Prostatic inflammation enhances basal-to-luminal differentiation and accelerates initiation of prostate cancer with a basal cell origin. *Proc Natl Acad Sci U S A* (2014) **111**(5):E592–600. doi:10.1073/pnas.1318157111
25. Yang H, Zhou H, Feng P, Zhou X, Wen H, Xie X, et al. Reduced expression of toll-like receptor 4 inhibits human breast cancer cells proliferation and inflammatory cytokines secretion. *J Exp Clin Cancer Res* (2010) **29**:92. doi:10.1186/1756-9966-29-92
26. Montero Vega MT, de Andres Martin A. The significance of toll-like receptors in human diseases. *Allergol Immunopathol (Madr)* (2009) **37**(5):252–63. doi:10.1016/j.aller.2009.04.004
27. Lu H. TLR agonists for cancer immunotherapy: tipping the balance between the immune stimulatory and inhibitory effects. *Front Immunol* (2014) **5**:83. doi:10.3389/fimmu.2014.00083
28. Huang B, Zhao J, Li H, He KL, Chen Y, Chen SH, et al. Toll-like receptors on tumor cells facilitate evasion of immune surveillance. *Cancer Res* (2005) **65**(12):5009–14. doi:10.1158/0008-5472.CAN-05-0784
29. Killeen SD, Wang JH, Andrews EJ, Redmond HP. Bacterial endotoxin enhances colorectal cancer cell adhesion and invasion through TLR-4 and NF-κappaB-dependent activation of the urokinase plasminogen activator system. *Br J Cancer* (2009) **100**(10):1589–602. doi:10.1038/sj.bjc.6604942
30. Weigelt B, Peters JL, van't Veer LJ. Breast cancer metastasis: markers and models. *Nat Rev Cancer* (2005) **5**(8):591–602. doi:10.1038/nrc1670
31. Gonzalez-Reyes S, Marin L, Gonzalez L, Gonzalez LO, del Casar JM, Lamelas ML, et al. Study of TLR3, TLR4 and TLR9 in breast carcinomas and their association with metastasis. *BMC Cancer* (2010) **10**:665. doi:10.1186/1471-2407-10-665
32. Wang JH, Manning BJ, Wu QD, Blankson S, Boucher-Hayes D, Redmond HP. Endotoxin/lipopolysaccharide activates NF-κappa B and enhances tumor cell adhesion and invasion through a beta 1 integrin-dependent mechanism. *J Immunol* (2003) **170**(2):795–804. doi:10.4049/jimmunol.170.2.795
33. Jing YY, Han ZP, Sun K, Zhang SS, Hou J, Liu Y, et al. Toll-like receptor 4 signaling promotes epithelial-mesenchymal transition in human hepatocellular carcinoma induced by lipopolysaccharide. *BMC Med* (2012) **10**:98. doi:10.1186/1741-7015-10-98
34. Alitalo K. The lymphatic vasculature in disease. *Nat Med* (2011) **17**(11):1371–80. doi:10.1038/nm.2545
35. Das S, Sarrou E, Podgrabska S, Cassella M, Mungamuri SK, Feirt N, et al. Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses. *J Exp Med* (2013) **210**(8):1509–28. doi:10.1084/jem.20111627
36. Ben-Baruch A. Organ selectivity in metastasis: regulation by chemokines and their receptors. *Clin Exp Metastasis* (2008) **25**(4):345–56. doi:10.1007/s10585-007-9097-3
37. Fukumura D, Duda DG, Munn LL, Jain RK. Tumor microvasculature and microenvironment: novel insights through intravital imaging in pre-clinical models. *Microcirculation* (2010) **17**(3):206–25. doi:10.1111/j.1549-8719.2010.00029.x
38. Ji RC. Lymph node lymphangiogenesis: a new concept for modulating tumor metastasis and inflammatory process. *Histo Histopathol* (2009) **24**(3):377–84.
39. Achen MG, Stacker SA. Molecular control of lymphatic metastasis. *Ann N Y Acad Sci* (2008) **1131**:225–34. doi:10.1196/annals.1413.020
40. He Y, Kozaki K, Karpanen T, Koshikawa K, Yla-Herttula S, Takahashi T, et al. Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. *J Natl Cancer Inst* (2002) **94**(11):819–25. doi:10.1093/jnci/94.11.819
41. Monteleone I, Platt AM, Jaenson E, Agace WW, Mowat AM. IL-10-dependent partial refractoriness to toll-like receptor stimulation modulates gut mucosal dendritic cell function. *Eur J Immunol* (2008) **38**(6):1533–47. doi:10.1002/eji.200737909
42. Fusi A, Liu Z, Kummerlen V, Nonnenmacher A, Jeske J, Keilholz U. Expression of chemokine receptors on circulating tumor cells in patients with solid tumors. *J Transl Med* (2012) **10**:52. doi:10.1186/1479-5876-10-52
43. Huber-Lang M, Sarma VJ, Lu KT, McGuire SR, Padgaonkar VA, Guo RF, et al. Role of C5a in multiorgan failure during sepsis. *J Immunol* (2001) **166**(2):1193–9. doi:10.4049/jimmunol.166.2.1193
44. Paschos KA, Canovas D, Bird NC. The role of cell adhesion molecules in the progression of colorectal cancer and the development of liver metastasis. *Cell Signal* (2009) **21**(5):665–74. doi:10.1016/j.cellsig.2009.01.006
45. Laubli H, Borsig L. Selectins promote tumor metastasis. *Semin Cancer Biol* (2010) **20**(3):169–77. doi:10.1016/j.semcan.2010.04.005
46. Alberda WJ, Dassen HP, Dwarkasing RS, Willemse FF, van der Pool AE, de Wilt JH, et al. Prediction of tumor stage and lymph node involvement with dynamic contrast-enhanced MRI after chemoradiotherapy for locally advanced rectal cancer. *Int J Colorectal Dis* (2013) **28**(4):573–80. doi:10.1007/s00384-012-1576-6
47. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res* (2002) **62**(6):1832–7.
48. Jain S, Sharma P, Mukherjee A, Bal C, Kumar R. “Witch's milk” and 99mTc-pertechnetate uptake in neonatal breast tissue: an uncommon but not unexpected finding. *Clin Nucl Med* (2013) **38**(7):586–7. doi:10.1097/RLU.0b013e318292aa
49. Murakami T, Kawada K, Iwamoto M, Akagami M, Hida K, Nakanishi Y, et al. The role of CXCR3 and CXCR4 in colorectal cancer metastasis. *Int J Cancer* (2013) **132**(2):276–87. doi:10.1002/ijc.27670
50. Kawada K, Hosogi H, Sonoshita M, Sakashita H, Manabe T, Shimahara Y, et al. Chemokine receptor CXCR3 promotes colon cancer metastasis to lymph nodes. *Oncogene* (2007) **26**(32):4679–88. doi:10.1038/sj.onc.1210267
51. Kim J, Takeuchi H, Lam ST, Turner RR, Wang HJ, Kuo C, et al. Chemokine receptor CXCR4 expression in colorectal cancer patients increases the risk for recurrence and for poor survival. *J Clin Oncol* (2005) **23**(12):2744–53. doi:10.1200/JCO.2005.07.078
52. Ghadjari P, Coupland SE, Na IK, Noutsias M, Letsch A, Stroux A, et al. Chemokine receptor CCR6 expression level and liver metastases in colorectal cancer. *J Clin Oncol* (2006) **24**(12):1910–6. doi:10.1200/JCO.2005.04.1822
53. Rubie C, Oliveira V, Kempf K, Wagner M, Tilton B, Rau B, et al. Involvement of chemokine receptor CCR6 in colorectal cancer metastasis. *Tumour Biol* (2006) **27**(3):166–74. doi:10.1159/000092777
54. Rubie C, Oliveira-Frick V, Rau B, Schilling M, Wagner M. Chemokine receptor CCR6 expression in colorectal liver metastasis. *J Clin Oncol* (2006) **24**(32):5173–4; author reply 4. doi:10.1200/JCO.2006.07.9095
55. Rubie C, Kollmar O, Frick VO, Wagner M, Brittnar B, Gruber S, et al. Differential CXCR receptor expression in colorectal carcinomas. *Scand J Immunol* (2008) **68**(6):635–44. doi:10.1111/j.1365-3083.2008.02163.x
56. Iwasa S, Yanagawa T, Fan J, Katoh R. Expression of CXCR4 and its ligand SDF-1 in intestinal-type gastric cancer is associated with lymph node and liver metastasis. *Anticancer Res* (2009) **29**(11):4751–8.
57. Matsusue R, Kubo H, Hisamori S, Okoshi K, Takagi H, Hida K, et al. Hepatic stellate cells promote liver metastasis of colon cancer cells by the action of SDF-1/CXCR4 axis. *Ann Surg Oncol* (2009) **16**(9):2645–53. doi:10.1245/s10434-009-0599-x

58. Ghadjar P, Rubie C, Aebersold DM, Keilholz U. The chemokine CCL20 and its receptor CCR6 in human malignancy with focus on colorectal cancer. *Int J Cancer* (2009) **125**(4):741–5. doi:10.1002/ijc.24468
59. Cambien B, Karimjee BF, Richard-Fiardo P, Bziouech H, Barthel R, Millet MA, et al. Organ-specific inhibition of metastatic colon carcinoma by CXCR3 antagonism. *Br J Cancer* (2009) **100**(11):1755–64. doi:10.1038/sj.bjc.6605078
60. Guillemot E, Karimjee-Soilihi B, Pradelli E, Benchettit M, Goguet-Surmenian E, Millet MA, et al. CXCR7 receptors facilitate the progression of colon carcinoma within lung not within liver. *Br J Cancer* (2012) **107**(12):1944–9. doi:10.1038/bjc.2012.503
61. Strell C, Entschladen F. Extravasation of leukocytes in comparison to tumor cells. *Cell Commun Signal* (2008) **6**:10. doi:10.1186/1478-811X-6-10
62. Vega MT, Martin AD. The significance of toll-like receptors in human diseases. *Allergol Immunopathol (Madr)* (2009) **37**(5):252–63. doi:10.1016/j.aller.2009.04.004
63. Harmey JH, Bucana CD, Lu W, Byrne AM, McDonnell S, Lynch C, et al. Lipopolysaccharide-induced metastatic growth is associated with increased angiogenesis, vascular permeability and tumor cell invasion. *Int J Cancer* (2002) **101**(5):415–22. doi:10.1002/ijc.10632
64. Koretz K, Schlag P, Boumstell L, Moller P. Expression of VLA-alpha 2, VLA-alpha 6, and VLA-beta 1 chains in normal mucosa and adenomas of the colon, and in colon carcinomas and their liver metastases. *Am J Pathol* (1991) **138**(3):741–50.
65. Liapis H, Flath A, Kitazawa S. Integrin alpha V beta 3 expression by bone-residing breast cancer metastases. *Diagn Mol Pathol* (1996) **5**(2):127–35. doi:10.1097/00019606-199606000-00008
66. Seftor RE, Seftor EA, Gehlsen KR, Stetler-Stevenson WG, Brown PD, Ruoslahti E, et al. Role of the alpha v beta 3 integrin in human melanoma cell invasion. *Proc Natl Acad Sci U S A* (1992) **89**(5):1557–61. doi:10.1073/pnas.89.5.1557
67. Man YG, Stojadinovic A, Mason J, Avital I, Bilchik A, Bruecher B, et al. Tumor-infiltrating immune cells promoting tumor invasion and metastasis: existing theories. *J Cancer* (2013) **4**(1):84–95. doi:10.7150/jca.5482
68. Holzel M, Bovier A, Tuting T. Plasticity of tumour and immune cells: a source of heterogeneity and a cause for therapy resistance? *Nat Rev Cancer* (2013) **13**(5):365–76. doi:10.1038/nrc3498
69. Seyfried TN, Huysement LC. On the origin of cancer metastasis. *Crit Rev Oncog* (2013) **18**(1–2):43–73. doi:10.1615/CritRevOncog.v18.i1-2.40
70. Tarin D. Inappropriate gene expression in human cancer and its far-reaching biological and clinical significance. *Cancer Metastasis Rev* (2012) **31**(1–2):21–39. doi:10.1007/s10555-011-9326-8
71. Seftor RE, Hess AR, Seftor EA, Kirschmann DA, Hardy KM, Margaryan NV, et al. Tumor cell vasculogenic mimicry: from controversy to therapeutic promise. *Am J Pathol* (2012) **181**(4):1115–25. doi:10.1016/j.ajpath.2012.07.013
72. Tarin D, Thompson EW, Newgreen DF. The fallacy of epithelial mesenchymal transition in neoplasia. *Cancer Res* (2005) **65**(14):5996–6000; discussion 6000–1. doi:10.1158/0008-5472.CAN-05-0699
73. Wirtz D, Konstantopoulos K, Searson PC. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nat Rev Cancer* (2011) **11**(7):512–22. doi:10.1038/nrc3080
74. Schramm HM. Should EMT of cancer cells be understood as epithelial-myeloid transition? *J Cancer* (2014) **5**(2):125–32. doi:10.7150/jca.8242
75. Pawelek JM. Tumour-cell fusion as a source of myeloid traits in cancer. *Lancet Oncol* (2005) **6**(12):988–93. doi:10.1016/S1470-2045(05)70466-6
76. Pawelek JM, Chakraborty AK. The cancer cell – leukocyte fusion theory of metastasis. *Adv Cancer Res* (2008) **101**:397–444. doi:10.1016/S0065-230X(08)00410-7
77. Masopust D, Schenkel JM. The integration of T cell migration, differentiation and function. *Nat Rev Immunol* (2013) **13**(5):309–20. doi:10.1038/nri3442
78. Brinkman CC, Peske JD, Engelhard VH. Peripheral tissue homing receptor control of naive, effector, and memory CD8 T cell localization in lymphoid and non-lymphoid tissues. *Front Immunol* (2013) **4**:241. doi:10.3389/fimmu.2013.00241
79. Tufail S, Badrealam KF, Sherwani A, Gupta UD, Owais M. Tissue specific heterogeneity in effector immune cell response. *Front Immunol* (2013) **4**:254. doi:10.3389/fimmu.2013.00254
80. Erreni M, Bianchi P, Laghi L, Mirolo M, Fabbri M, Locati M, et al. Expression of chemokines and chemokine receptors in human colon cancer. *Methods Enzymol* (2009) **460**:105–21. doi:10.1016/S0076-6879(09)05205-7
81. Hwang TL, Lee LY, Wang CC, Liang Y, Huang SF, Wu CM. CCL7 and CCL21 over-expression in gastric cancer is associated with lymph node metastasis and poor prognosis. *World J Gastroenterol* (2012) **18**(11):1249–56. doi:10.3748/wjg.v18.i11.1249
82. Kitayama J, Nagawa H, Tsuno N, Osada T, Hatano K, Sunami E, et al. Laminin mediates tethering and spreading of colon cancer cells in physiological shear flow. *Br J Cancer* (1999) **80**(12):1927–34. doi:10.1038/sj.bjc.6690622
83. Roy J, Audette M, Tremblay MJ. Intercellular adhesion molecule-1 (ICAM-1) gene expression in human T cells is regulated by phosphotyrosyl phosphatase activity. Involvement of NF-kappaB, Ets, and palindromic interferon-gamma-responsive element-binding sites. *J Biol Chem* (2001) **276**(18):14553–61. doi:10.1074/jbc.M005067200
84. Ding YB, Chen GY, Xia JG, Zang XW, Yang HY, Yang L. Association of VCAM-1 overexpression with oncogenesis, tumor angiogenesis and metastasis of gastric carcinoma. *World J Gastroenterol* (2003) **9**(7):1409–14.
85. Burdick MM, Chu JT, Godar S, Sackstein R. HCELL is the major E- and L-selectin ligand expressed on LS174T colon carcinoma cells. *J Biol Chem* (2006) **281**(20):13899–905. doi:10.1074/jbc.M513617200
86. Zetter BR. Adhesion molecules in tumor metastasis. *Semin Cancer Biol* (1993) **4**(4):219–29.
87. Caruso RA, Fedele F, Finocchiaro G, Arena G, Venuti A. Neutrophil-tumor cell phagocytosis (cannibalism) in human tumors: an update and literature review. *Exp Oncol* (2012) **34**(3):306–11.
88. Song J, Sapi E, Brown W, Nilsen J, Tartaro K, Kacinski BM, et al. Roles of Fas and Fas ligand during mammary gland remodeling. *J Clin Invest* (2000) **106**(10):1209–20. doi:10.1172/JCI10411
89. Mullauer L, Mosberger J, Grusch M, Rudas M, Chott A. Fas ligand is expressed in normal breast epithelial cells and is frequently up-regulated in breast cancer. *J Pathol* (2000) **190**(1):20–30. doi:10.1002/(SICI)1096-9896(200001)190:1<20::AID-PATH497>3.0.CO;2-S
90. Spriggs DR, Imamura K, Rodriguez C, Sariban E, Kufe DW. Tumor necrosis factor expression in human epithelial tumor cell lines. *J Clin Invest* (1988) **81**(2):455–60. doi:10.1172/JCI113341
91. Humbert L, Lebrun JJ. TGF-beta inhibits human cutaneous melanoma cell migration and invasion through regulation of the plasminogen activator system. *Cell Signal* (2013) **25**(2):490–500. doi:10.1016/j.cellsig.2012.10.011
92. Shi F, Shi M, Zeng Z, Qi RZ, Liu ZW, Zhang JY, et al. PD-1 and PD-L1 upregulation promotes CD8(+) T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients. *Int J Cancer* (2011) **128**(4):887–96. doi:10.1002/ijc.25397
93. Shi SJ, Wang LJ, Wang GD, Guo ZY, Wei M, Meng YL, et al. B7-H1 expression is associated with poor prognosis in colorectal carcinoma and regulates the proliferation and invasion of HCT116 colorectal cancer cells. *PLoS One* (2013) **8**(10):e76012. doi:10.1371/journal.pone.0076012
94. Koscielny S, Le MG, Tubiana M. The natural history of human breast cancer. The relationship between involvement of axillary lymph nodes and the initiation of distant metastases. *Br J Cancer* (1989) **59**(5):775–82. doi:10.1038/bjc.1989.162
95. Jatoi I, Hilsenbeck SG, Clark GM, Osborne CK. Significance of axillary lymph node metastasis in primary breast cancer. *J Clin Oncol* (1999) **17**(8):2334–40.

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Toll-like receptors and prostate cancer

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Prostate cancer is the second leading cause of cancer-related death in men after lung cancer. Immune responses clearly play a critical role in the tumorigenesis and in the efficacy of radiation therapy and chemotherapy in prostate cancer; however, the underlying molecular mechanisms are still poorly understood. Toll-like receptors (TLRs) are a well-known family of pattern recognition receptors that play a key role in host immune system. Recent studies demonstrate that there are links between TLRs and cancer; however, the function and biological importance of TLRs in prostate cancer seems complex. To elucidate the role of TLRs and innate immunity in prostate cancer might provide us with a better understanding of the molecular mechanisms of this disease. Moreover, utilizing the agonists or antagonists of TLRs might represent a promising new strategy against prostate cancer. In this review, we summarize recent advances on the studies of association between TLR signaling and prostate cancer, TLR polymorphisms and prostate cancer risk, and provide some insights about TLRs as potential targets for prostate cancer immunotherapy.

Keywords: toll-like receptor, TLR signaling, prostate cancer, innate immunity, immunotherapy

INTRODUCTION

Based on the latest cancer statistics, prostate cancer predictably ranks first among all the cancers in men and second in cancer-related deaths in the United States in 2014 (1). Treatments against prostate cancer, including chemotherapy and radiotherapy, could improve survival; however, many patients will endure relapse and metastasis, which eventually leads to death. These treatments also destroy cancer cells and normal cells alike. Therefore, a more effective and less toxic therapy needs discovery. A promising strategy for dramatically preventing cancer development and improving cancer treatment might rely on immunotherapy. Immune evasion is a hallmark of cancer pathogenesis. Cancer cells escape from immune attack through a variety of mechanisms. A compromised immune system and chronic inflammation increase the incidence of cancer development. Inflammation has been proposed as the seventh hallmark of cancer (2) and an excellent review has elegantly summarized the role of inflammation in prostate cancer development and potential underlying mechanisms (3). Immunotherapy, which utilizes host immune system to fight cancer, has been recently highlighted with several advantages including specificity, less side effects, and less likely to develop resistance. It could be achieved in two ways: stimulating immune system to attack cancer cells or taking away the inhibitory machinery to the immune system in cancer. One potential approach to modulate immune system is targeting pattern recognition receptors (PRRs) in innate immune system, among which toll-like receptors are most well studied.

TOLL-LIKE RECEPTOR: A WELL-KNOWN FAMILY OF PATTERN RECOGNITION RECEPTORS IN INNATE IMMUNITY

Toll-like receptors are a family of transmembrane receptors that play a key role in the innate immunity. TLRs prevent invading pathogens by recognizing pathogen-associated molecular patterns (PAMPs), which are highly conserved components derived from

bacteria, viruses, fungi, and parasites (4, 5). It can also recognize endogenous damage-associated molecular patterns (DAMPs) in different disorders and diseases such as cancer (4, 5). At present, 10 TLRs have been identified in human. TLR1s, TLR2, TLR4, TLR5, and TLR6 are expressed on cell surface; however, TLR3, TLR7, TLR8, and TLR9 are found exclusively within endosomes (Figure 1). Different TLRs exhibit specificity for ligand recognition. TLR2 recognizes bacterial lipoproteins, TLR3 recognizes double-stranded RNA/polyinosinic–polycytidyl acid [poly (I:C)], TLR4 recognizes lipopolysaccharides (LPS), TLR5 recognizes flagellin, TLR7 recognizes single-stranded RNA, and TLR9 recognizes CpG-containing DNA (CpG-ODN) (6–11). TLR10 is so far an orphan receptor and highly expressed in the human spleen (12) and B cells (13). Upon activation, TLRs transmit signals through one or more of four adaptor proteins: myeloid differentiation factor 88 (MyD88), TICAM1 (also known as TRIF), TIRAP (also known as MAL), and TICAM2 (also known as TRAM and TIRP). All TLRs (except for TLR3) and IL-1 receptor family members signal through MyD88. TLR3 signals through TRIF pathway; TLR4 signals through both the MyD88 and the TRIF pathways (4). Stimulation of TLRs leads to activation of NF-κB, MAPKs, Jun N-terminal kinases (JNKs), p38, and ERKs, as well as interferon regulatory factor (IRF3, IRF5, and IRF7) signaling pathways, which results in the production of inflammatory cytokines (14). Activation of TLRs in antigen-presenting cells (APC) also triggers adaptive immunity. TLRs have also been shown to regulate cell death and increase expression of the anti-apoptotic proteins Bcl-2-related protein A1 (BCL2A1), inhibitor of apoptosis 1 (cIAP1), cIAP2, XIAP, and Bcl-2 family members (15).

TLR EXPRESSION AND FUNCTION IN PROSTATE CANCER

Toll-like receptors are predominantly expressed in innate immune cells such as dendritic cells, macrophages, and natural killing

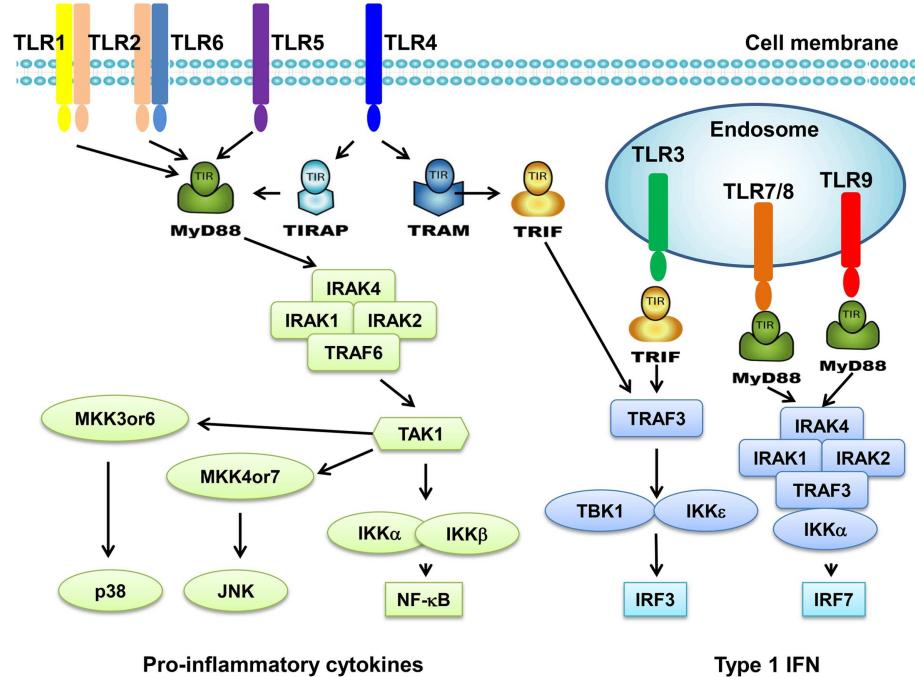


FIGURE 1 | Toll-like receptors and TLR-mediated signaling pathway. TLR1 and TLR6 recognize their ligands as heterodimers with TLR2. For TLR4, MD2, and CD14 are required for LPS recognition and signaling. TLR3, TLR4, TLR5, TLR7, and TLR9 are currently thought to deliver their signal by forming homodimers after interacting with

their ligands. TLR3, TLR7/8, and TLR9 are intracellular TLRs and are involved in the recognition of nucleic acids. Most TLRs, except for TLR3, signal through MyD88 pathway to activate NF- κ B and AP1. TLR3 and TLR4 can signal through MyD88-independent pathway (TRIF pathway) to activate INF- β .

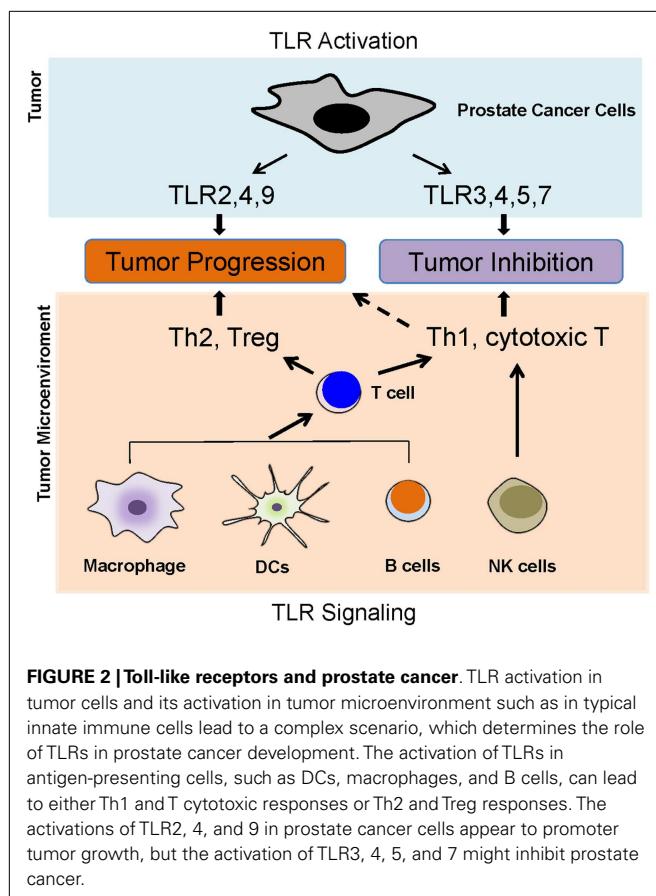
(NK) cells. Activation of TLRs in these cells leads to the activation of innate immunity and results in the production of pro-inflammatory cytokines, chemokines, as well as adhesion molecules, and then facilitates the activation of adaptive immunity (16). Intriguingly, growing evidence has demonstrated that TLRs are also expressed in tumor cells. TLR activation in tumor cells and its activation in tumor microenvironment such as in typical innate immune cells lead to a complex scenario (Figure 2); therefore, the activation of TLRs might play a “double-edged sword” role in the influence of tumor progression.

In most cases, it is difficult to figure out a specific pathogen to activate TLR signaling in prostate cancer. An endogenous TLR ligand, DAMPs released from damaged and/or necrotic tissues, might play a pivotal role. In term of endogenous TLR ligands in cancer, HMGB1 can activate TLR2 and TLR4 (17), and versican acts as a TLR2 agonist (18). Peroxiredoxin 1 (Prx1) appears to be an agonist of TLR4 in prostate cancer development (19). Perhaps, there are more endogenous TLR ligands that need to be further identified and verified.

The activation of some TLRs might prevent the tumor growth of prostate cancer (Figure 2). It has been shown that TLR3 is expressed in prostate cancer cells (20–25). TLR3 mRNA is detected in three prostate cancer cell lines including LNCaP, PC3, and DU-145. TLR3 mRNA level was clearly enhanced in prostate cancer cells by stimulating with poly (I:C), which suggests a functional role of TLR3 in prostate cancer (20). TLR3

protein was also expressed at similar levels in LNCaP and DU-145 cells, with a slightly lower expression in PC3 cells. Treatment with poly (I:C) rapidly triggered NF- κ B-dependent expression of inflammatory molecules. Condition medium from poly (I:C)-treated LNCaP and DU145 cells recruited leukocyte subpopulation, indicating that TLR3 activation might influence early immune responses in tumor microenvironment (25). Stimulation with poly (I:C) strongly suppressed prostate tumor growth *in vivo*, perhaps due to increased infiltration of T lymphocytes and NK cells in a type I IFN-dependent manner (24). In human prostate cancer patients, 85 in 112 prostate carcinomas samples showed positive expression of TLR3. High TLR3 expression level was significantly associated with high probability of the recurrence of prostate cancer (23). Paone and colleagues found that TLR3 could regulate the process of angiogenesis and apoptosis in prostate cancer cells through hypoxia-inducible factor 1 α (HIF-1 α) and PKC-dependent mechanism (21, 22). TLR5 is expressed in LNCaP and DU-145 by which stimulation triggers the production of chemokines that recruit immune cells, including NK cells and cytotoxic CD8 cells, which most likely contribute to tumor inhibition (25).

The activation of other TLRs might play a different role in the tumor growth of prostate cancer (Figure 2). The expression of TLR4 in prostate cancer has been demonstrated in several animal models. Studies revealed a constitutive expression of TLR4 in the epithelial cells of rat ventral prostate as well as in a rat



adenocarcinoma cell line and in prostate primary culture cells (26, 27). TLR4 is also expressed in DU-145, PC3, and normal prostate gland in both stroma and epithelium (28, 29). In addition, TLR4 has also been shown to be expressed in clinical samples of prostate cancer. Initially, TLR9 expression was thought to be restricted to immune cells, but recent studies have shown that a variety of tumor cell types including prostate cancer also express functional TLR9 (23, 30, 31). A clinical study demonstrated that TLR9 is expressed in prostate cancer specimens (23). Joanna et al. found that TLR9 is expressed in human prostate cancer cell lines LnCaP, C4-2B, Du-145, PC3, and in clinical samples of prostate cancer through immunohistochemistry and western blotting, but not in MDA Pca2b and stromal cells of the clinical adenocarcinoma samples (32). TLR9 expression was also statistically significantly increased in prostate cancer epithelium and stroma, compared with the same cellular compartments in benign hyperplasia, especially in the most poorly differentiated forms (30).

The function and biological importance of TLRs in prostate cancer seems complex (Figure 2). Perhaps the distinct and unidentified TLR signaling pathways are activated in cancer cells or innate immune cells during tumor progression; or, the first activation of TLR in cancer cells or innate immune cells markedly affect the subsequently activation and induced effectors. The mystery will be further investigated and will affect the potential of TLR agonists or antagonists as anti-tumor therapeutic agents.

MicroRNA REGULATE TLRs IN PROSTATE CANCER

MicroRNAs (miRNAs) are a class of small non-coding RNAs (~22 nt in length), which negatively regulate gene expression at the post-transcriptional level (33). By binding to target sequences within the 3' UTR of mRNA, miRNAs induce gene silencing by either inhibiting translation or leading to degradation of mRNA. MiRNA alterations are shown to be involved in both initiation and progression of human cancer (34–39). Deregulation of miRNAs is implicated as an important mechanism in tumorigenesis and several miRNAs have been proposed as oncogenes or tumor suppressors (40–42).

MicroRNAs are emerging as a fundamental mechanism in the regulation of TLR signaling (43–47). Recent works have linked miRNAs and TLRs in prostate cancer. MiR-29a has been shown as a potential tumor suppressor miRNA to regulate TRAF-4 expression in metastatic prostate cancer (48). TLR3 activation by poly (I:C) induces upregulation of miRNAs including miR-29b, -29c, -148b, and -152, which target DNA methyltransferases and leads to reexpression of oncosuppressor RAR β in prostate cancer cells (49). TLRs activation facilitates either prostate cancer inhibition or progression. MiRNAs are likely to act as important regulators to control TLRs expression and signaling, thus contribute to prostate cancer development.

TLR SIGNALING IN PROSTATE CANCER

Toll-like receptor signaling pathway has been well defined in innate immune cells. TLR ligation recruits one or more adaptor proteins such as MyD88, TRIF, Mal, and TRAM through TIR domain interactions. Most TLRs except TLR3 go through a MyD88-dependent signaling pathway. MyD88 engagement activates IL-1 receptor associated kinase (IRAK), which interacts with tumor necrosis factor receptor associated factor 6 (TRAF6), resulting in the activation of MAPK and NF- κ B signaling. TLR3 and TLR4 activate a MyD88-independent signaling pathway. TRIF is recruited upon stimulation and leads to the activation of NF- κ B and type I IFN signaling.

Although TLR3 can be activated in prostate cancer cells, the molecular signaling pathway has not been fully elucidated. A recent study in human prostate cancer cells suggests that TLR3 signaling triggers apoptosis and growth arrest of LNCaP cells partially through inactivation of the PI3K/Akt pathway. CyclinD1, c-Myc, p53, and NOXA are indicated to play a role in poly (I:C)-treated LNCaP cells (20). In other studies, HIF-1 α facilitates apoptosis through a PKC-dependent mechanism in poly (I:C)-treated prostate cancer cells. TLR3 activation by poly (I:C) activates JNK and p38 through PKC- α and triggers apoptosis in a caspase-8 dependent manner (21, 22). In LNCap cells, poly (I:C) treatment upregulates a pattern of chemokines, including CCL3, CCL4, CCL5, CCL8, CXCL9, and CXCL10, which could induce massive NK cell and CD8 T cell chemotaxis. Moreover, poly (I:C) induced the expression of inflammatory molecules such as IL-6 and IL-12, which are NF- κ B signaling dependent (25). In TRAMP tumor model, poly (I:C) treatment recruits NK cells and T lymphocytes through a type I IFN dependent mechanism, resulting in suppression of tumor growth (24). TLR5 agonist flagellin can activate NF- κ B signaling in LNCaP and DU145 cells, and lead to the production of pro-inflammatory molecules (25).

Stimulation of TLR4 in DU145 by LPS activates NF- κ B signaling pathway, which leads to production of pro-inflammatory cytokines such as IL-6 and IL-1 β through MyD88-dependent pathway (29). In addition, TLR4 activation increases expression of VEGF and TGF- β 1 in PC3 cells, which promote tumor development (28). Also, knockdown of TLR4 using siRNA in PC3 cells reduces tumor cell migration and invasion (50). TLR9 stimulation by CpG-ODN plays an important role in prostate cancer invasion. This effect is mediated by activating NF- κ B and upregulation of COX-2 (31). TLR9 expression in prostate cancer cells has similarly been found to enhance invasiveness via induction of MMP-13 *in vitro* (32). In both studies, CpG-ODN stimulation did not affect cellular proliferation, which suggests TLR9 signaling plays a role in cancer progression and metastasis.

These defined TLR signaling pathways seem difficult to help understand why the activation of some TLRs such as TLR3 inhibits tumor growth but the activation of other TLRs such as TLR2 promotes tumor growth (Figure 2). Some distinct TLR signal pathways must exist to determine the specific effectors in the different TLR activations leading opposite consequences.

TLR GENE POLYMORPHISMS AND PROSTATE CANCER RISK

Polymorphisms in TLR genes are reportedly related to susceptibility of a large spectrum of infectious and inflammatory diseases. Growing evidence suggest that chronic intra-prostatic inflammation contribute to prostate cancer progression. It was suggested that TLR gene polymorphisms might alter TLR signaling, thus affecting inflammation and prostate cancer risk. A number of studies have been done to investigate whether there is a connection between TLR gene polymorphisms and prostate cancer risk, and the results are controversial (51, 52).

Single nucleotide polymorphisms (SNPs) in TLR4 were reported to be associated with prostate cancer risk in several studies (53–58). Sequence variants in TLR gene cluster (TLR6-TLR1-TLR10) were also reported to be associated with prostate cancer risk (51, 52). However, controversial results were also obtained. Shui and colleagues investigated 10 SNPs in TLR4 and found no significant correlation between TLR4 genetic variation and prostate cancer risks (59). Chen et al. reported that sequence variants of gene cluster TLR6-TLR1-TLR10 were not associated with the risk of prostate cancer (60). A meta-analysis by Lindström et al. did not show clear correlation between TLR gene polymorphisms and prostate cancer risks.

The discrepancies among these results might be due to multiple factors including detection method, the race of population, and sample size. It is important to clarify this issue because it will determine not only whether the TLR polymorphisms can be used as a diagnosis/prognosis marker but also whether we can develop a novel strategy to treat prostate cancer by targeting TLRs and their signaling pathway. A more comprehensive study including a sufficient sample size should be performed to investigate the association between TLR gene polymorphisms and prostate cancer risk.

TARGETING TLRs FOR PROSTATE CANCER IMMUNOTHERAPY

The ability of TLRs to manipulate prostate cancer development has raised the interests in developing immunotherapy against prostate

cancer with the TLR agonists or antagonists. Actually, three drugs targeting TLRs have been approved by FDA for use in cancer patients: the bacillus Calmette–Guérin (BCG), monophosphoryl lipid A (MPL), and imiquimod (61). BCG is prepared from an attenuated strain of *Mycobacterium bovis* and activates TLR2/4. BCG is used as a vaccine in prevention of tuberculosis, but also for treatment of *in situ* bladder carcinoma. Derived from LPS as a potent TLR4 agonist, MPL is an active component of Cervarix, which is used against cancer-causing human papillomavirus (HPV) (62, 63). Imiquimod, one of the most successful drugs targeting TLRs, is a synthetic imidazoquinoline that signals through TLR7 and is commonly used in the treatment of skin cancer such as basal cell carcinoma and Bowen's disease (64–66). Imiquimod induces the proinflammatory cytokines including IFN α , IL-6, and TNF- α (67). The activation of TLR7/8 leads to a Th1 response and an anti-tumor activity, which depends on IFN γ (68). In prostate cancer, to support this concept, Han et al., reported that Imiquimod can inhibit both human and mouse prostate cancer growth by inducing apoptosis (69, 70).

A number of preclinical and clinical studies are ongoing to investigate the immunotherapeutic potency utilizing TLRs against prostate cancer. TLR3 activation directly triggers apoptosis of human prostate cancer cells (21); therefore, TLR3 agonists have potential to be developed as anti-tumor therapeutic agents. Indeed, Ampligen, composed of poly (I:C) (a TLR3 agonist), has been shown to inhibit a variety of tumor growth in early clinical trials (71, 72). Hiltonol, a particular formulation of poly (I:C), is currently in Phase I/II clinical trial to evaluate its safety and efficacy (71). Meanwhile, a phase 2 clinical study (NCT00514072) utilizing a BCG vaccine to treat prostate cancer is ongoing. A multi-peptide, dual-adjuvant telomerase vaccine (GX301) in which Imiquimod is an active component showed less toxic and highly immunogenic in prostate cancer patients, but requires future studies to determine its clinical efficacy (73). Furthermore, TLR4 stimulation by LPS is shown to contribute to chemoresistance to docetaxel in prostate cancer cells (74).

CONCLUDING REMARKS

Toll-like receptors play a critical role in innate immunity. TLRs are expressed not only in innate immune cells, but also in non-immune cells including cancer cells. Functional expression of TLRs has been linked to prostate cancer development. TLRs may serve as a double-edged sword in prostate cancer tumorigenesis by promoting malignant transformation of epithelial cells and tumor growth, or on the contrary, inducing apoptosis, and inhibiting tumor progression. The consequences might be dependent on complex signaling networks triggered by TLRs activation and tumor microenvironment. Genetic variations and polymorphisms of TLRs have been associated with prostate cancer; however, the results are inconclusive and need further validation (75, 76). The ability of boosting immune responses but with less serious side effect makes TLRs a good target to treat cancers. A wave of pre-clinical and clinical studies showed the potential of developing treatment targeting TLRs against prostate cancer. Based on these researches, one of the most probable approaches is to use agents targeting TLRs as adjuvants along with other treatments (67, 68, 71, 77, 78). Above all, elucidation of the mechanisms of cancer cell

TLR signaling and crosstalk with other signaling pathways as well as the mechanisms of cancer progression will definitely provide a promising novel strategy for cancer treatment.

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REFERENCES

- Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin* (2014) **64**(1):9–29. doi:10.3322/caac.21208
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* (2011) **144**(5):646–74. doi:10.1016/j.cell.2011.02.013
- De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, Drake CG, et al. Inflammation in prostate carcinogenesis. *Nat Rev Cancer* (2007) **7**(4):256–69. doi:10.1038/nrc2090
- Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* (2003) **21**:335–76. doi:10.1146/annurev.immunol.21.120601.141126
- Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* (1989) **54**(Pt 1):1–13. doi:10.1101/SQB.1989.054.01.003
- Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* (1998) **282**(5396):2085–8. doi:10.1126/science.282.5396.2085
- Aliprantis AO, Yang RB, Mark MR, Suggett S, Devaux B, Radolf JD, et al. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science* (1999) **285**(5428):736–9. doi:10.1126/science.285.5428.736
- Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A toll-like receptor recognizes bacterial DNA. *Nature* (2000) **408**(6813):740–5. doi:10.1038/35047123
- Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by toll-like receptor 3. *Nature* (2001) **413**(6857):732–8. doi:10.1038/35099560
- Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by toll-like receptor 5. *Nature* (2001) **410**(6832):1099–103. doi:10.1038/35074106
- Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* (2004) **303**(5663):1526–9. doi:10.1126/science.1093620
- Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol* (2005) **174**(5):2942–50. doi:10.4049/jimmunol.174.5.2942
- Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, et al. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* (2002) **168**(9):4531–7. doi:10.4049/jimmunol.168.9.4531
- Lee MS, Kim YJ. Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annu Rev Biochem* (2007) **76**:447–80. doi:10.1146/annurev.biochem.76.060605.122847
- Salaun B, Romero P, Lebecque S. Toll-like receptors' two-edged sword: when immunity meets apoptosis. *Eur J Immunol* (2007) **37**(12):3311–8. doi:10.1002/eji.200737744
- Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* (2004) **5**(10):987–95. doi:10.1038/ni1112
- Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* (2007) **13**(9):1050–9. doi:10.1038/nm1622
- Kim S, Takahashi H, Lin WW, Descargues P, Grivennikov S, Kim Y, et al. Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis. *Nature* (2009) **457**(7225):102–6. doi:10.1038/nature07623
- Riddell JR, Bshara W, Moser MT, Sperryk JA, Foster BA, Gollnick SO. Peroxiredoxin 1 controls prostate cancer growth through toll-like receptor 4-dependent regulation of tumor vasculature. *Cancer Res* (2011) **71**(5):1637–46. doi:10.1158/0008-5472.CAN-10-3674
- Harashima N, Inao T, Imamura R, Okano S, Suda T, Harada M. Roles of the PI3K/Akt pathway and autophagy in TLR3 signaling-induced apoptosis and growth arrest of human prostate cancer cells. *Cancer Immunol Immunother* (2012) **61**(5):667–76. doi:10.1007/s00262-011-1132-1
- Paone A, Starace D, Galli R, Padula F, De Cesaris P, Filippini A, et al. Toll-like receptor 3 triggers apoptosis of human prostate cancer cells through a PKC-alpha-dependent mechanism. *Carcinogenesis* (2008) **29**(7):1334–42. doi:10.1093/carcin/bgn149
- Paone A, Galli R, Gabellini C, Lukashev D, Starace D, Gorlach A, et al. Toll-like receptor 3 regulates angiogenesis and apoptosis in prostate cancer cell lines through hypoxia-inducible factor 1 alpha. *Neoplasia* (2010) **12**(7):539–49. doi:10.1593/neo.92106
- Gonzalez-Reyes S, Fernandez JM, Gonzalez LO, Aguirre A, Suarez A, Gonzalez JM, et al. Study of TLR3, TLR4, and TLR9 in prostate carcinomas and their association with biochemical recurrence. *Cancer Immunol Immunother* (2011) **60**(2):217–26. doi:10.1007/s00262-010-0931-0
- Chin AI, Miyahira AK, Covarrubias A, Teague J, Guo B, Dempsey PW, et al. Toll-like receptor 3-mediated suppression of TRAMP prostate cancer shows the critical role of type I interferons in tumor immune surveillance. *Cancer Res* (2010) **70**(7):2595–603. doi:10.1158/0008-5472.CAN-09-1162
- Galli R, Starace D, Busa R, Angelini DF, Paone A, De Cesaris P, et al. TLR stimulation of prostate tumor cells induces chemokine-mediated recruitment of specific immune cell types. *J Immunol* (2010) **184**(12):6658–69. doi:10.4049/jimmunol.0902401
- Quintar AA, Roth FD, De Paul AL, Aoki A, Maldonado CA. Toll-like receptor 4 in rat prostate: modulation by testosterone and acute bacterial infection in epithelial and stromal cells. *Biol Reprod* (2006) **75**(5):664–72. doi:10.1095/biolreprod.106.053967
- Gatti G, Rivero V, Motrich RD, Maccioni M. Prostate epithelial cells can act as early sensors of infection by up-regulating TLR4 expression and proinflammatory mediators upon LPS stimulation. *J Leukoc Biol* (2006) **79**(5):989–98. doi:10.1189/jlb.1005597
- Pei Z, Lin D, Song X, Li H, Yao H. TLR4 signaling promotes the expression of VEGF and TGFbeta1 in human prostate epithelial PC3 cells induced by lipopolysaccharide. *Cell Immunol* (2008) **254**(1):20–7. doi:10.1016/j.cellimm.2008.06.007
- Gatti G, Quintar AA, Andreani V, Nicola JP, Maldonado CA, Masini-Repiso AM, et al. Expression of toll-like receptor 4 in the prostate gland and its association with the severity of prostate cancer. *Prostate* (2009) **69**(13):1387–97. doi:10.1002/pros.20984
- Vaisanen MR, Vaisanen T, Jukkola-Vuorinen A, Vuopala KS, Desmond R, Selander KS, et al. Expression of toll-like receptor-9 is increased in poorly differentiated prostate tumors. *Prostate* (2010) **70**(8):817–24. doi:10.1002/pros.21115
- Di JM, Pang J, Sun QP, Zhang Y, Fang YQ, Liu XP, et al. Toll-like receptor 9 agonists up-regulates the expression of cyclooxygenase-2 via activation of NF-kappaB in prostate cancer cells. *Mol Biol Rep* (2010) **37**(4):1849–55. doi:10.1007/s11033-009-9620-5
- Ilvesaro JM, Merrell MA, Swain TM, Davidson J, Zayzafoon M, Harris KW, et al. Toll like receptor-9 agonists stimulate prostate cancer invasion in vitro. *Prostate* (2007) **67**(7):774–81. doi:10.1002/pros.20562
- He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* (2004) **5**(7):522–31. doi:10.1038/nrg1415
- Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. *Nature* (2005) **435**(7043):834–8. doi:10.1038/nature03702
- Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* (2006) **6**(11):857–66. doi:10.1038/nrc1997
- Calin GA, Croce CM. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res* (2006) **66**(15):7390–4. doi:10.1158/0008-5472.CAN-06-0800
- Esquela-Kerscher A, Slack FJ. Oncomirs – microRNAs with a role in cancer. *Nat Rev Cancer* (2006) **6**(4):259–69. doi:10.1038/nrc1840
- Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* (2009) **10**(10):704–14. doi:10.1038/nrg2634
- Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. *Annu Rev Med* (2009) **60**:167–79. doi:10.1146/annurev.med.59.053006.104707
- Zhang B, Pan X, Cobb GP, Anderson TA. MicroRNAs as oncogenes and tumor suppressors. *Dev Biol* (2007) **302**(1):1–12. doi:10.1016/j.ydbio.2006.08.028
- Shenouda SK, Alahari SK. MicroRNA function in cancer: oncogene or a tumor suppressor? *Cancer Metastasis Rev* (2009) **28**(3–4):369–78. doi:10.1007/s10555-009-9188-5

42. Ventura A, Jacks T. MicroRNAs and cancer: short RNAs go a long way. *Cell* (2009) **136**(4):586–91. doi:10.1016/j.cell.2009.02.005
43. Nahid MA, Satoh M, Chan EK. MicroRNA in TLR signaling and endotoxin tolerance. *Cell Mol Immunol* (2011) **8**(5):388–403. doi:10.1038/cmi.2011.26
44. O’Neill LA, Sheedy FJ, McCoy CE. MicroRNAs: the fine-tuners of toll-like receptor signalling. *Nat Rev Immunol* (2011) **11**(3):163–75. doi:10.1038/nri2957
45. Quinn SR, O’Neill LA. A trio of microRNAs that control toll-like receptor signalling. *Int Immunol* (2011) **23**(7):421–5. doi:10.1093/intimm/dxr034
46. Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, et al. MicroRNAs bind to toll-like receptors to induce prometastatic inflammatory response. *Proc Natl Acad Sci U S A* (2012) **109**(31):E2110–6. doi:10.1073/pnas.1209414109
47. He X, Jing Z, Cheng G. MicroRNAs: new regulators of toll-like receptor signalling pathways. *Biomed Res Int* (2014) **2014**:945169. doi:10.1155/2014/945169
48. Ahmed F, Shiraishi T, Vessella RL, Kulkarni P. Tumor necrosis factor receptor associated factor-4: an adapter protein overexpressed in metastatic prostate cancer is regulated by microRNA-29a. *Oncol Rep* (2013) **30**(6):2963–8. doi:10.3892/or.2013.2789
49. Galli R, Paone A, Fabbri M, Zanesi N, Calore F, Cascione L, et al. Toll-like receptor 3 (TLR3) activation induces microRNA-dependent reexpression of functional RAR β and tumor regression. *Proc Natl Acad Sci U S A* (2013) **110**(24):9812–7. doi:10.1073/pnas.1304610110
50. Hua D, Liu MY, Cheng ZD, Qin XJ, Zhang HM, Chen Y, et al. Small interfering RNA-directed targeting of toll-like receptor 4 inhibits human prostate cancer cell invasion, survival, and tumorigenicity. *Mol Immunol* (2009) **46**(15):2876–84. doi:10.1016/j.molimm.2009.06.016
51. Sun J, Wiklund F, Zheng SL, Chang B, Balter K, Li L, et al. Sequence variants in toll-like receptor gene cluster (TLR6-TLR1-TLR10) and prostate cancer risk. *J Natl Cancer Inst* (2005) **97**(7):525–32. doi:10.1093/jnci/dji070
52. Stevens VL, Hsing AW, Talbot JT, Zheng SL, Sun J, Chen J, et al. Genetic variation in the toll-like receptor gene cluster (TLR10-TLR1-TLR6) and prostate cancer risk. *Int J Cancer* (2008) **123**(11):2644–50. doi:10.1002/ijc.23826
53. Zheng SL, Augustsson-Balter K, Chang B, Hedelin M, Li L, Adami HO, et al. Sequence variants of toll-like receptor 4 are associated with prostate cancer risk: results from the Cancer Prostate in Sweden Study. *Cancer Res* (2004) **64**(8):2918–22. doi:10.1158/0008-5472.CAN-03-3280
54. Chen YC, Giovannucci E, Lazarus R, Kraft P, Ketkar S, Hunter DJ. Sequence variants of toll-like receptor 4 and susceptibility to prostate cancer. *Cancer Res* (2005) **65**(24):11771–8. doi:10.1158/0008-5472.CAN-05-2078
55. Cheng I, Plummer SJ, Casey G, Witte JS. Toll-like receptor 4 genetic variation and advanced prostate cancer risk. *Cancer Epidemiol Biomarkers Prev* (2007) **16**(2):352–5. doi:10.1158/1055-9965.EPI-06-0429
56. Song J, Kim DY, Kim CS, Kim HJ, Lee DH, Lee HM, et al. The association between toll-like receptor 4 (TLR4) polymorphisms and the risk of prostate cancer in Korean men. *Cancer Genet Cytogenet* (2009) **190**(2):88–92. doi:10.1016/j.cancergenryo.2008.12.011
57. Wang MH, Helzlsouer KJ, Smith MW, Hoffman-Bolton JA, Clipp SL, Grinberg V, et al. Association of IL10 and other immune response- and obesity-related genes with prostate cancer in CLUE II. *Prostate* (2009) **69**(8):874–85. doi:10.1002/pros.20933
58. Kim HJ, Bae JS, Chang IH, Kim KD, Lee J, Shin HD, et al. Sequence variants of Toll-like receptor 4 (TLR4) and the risk of prostate cancer in Korean men. *World J Urol* (2012) **30**(2):225–32. doi:10.1007/s00345-011-0690-3
59. Shui IM, Stark JR, Penney KL, Schumacher FR, Epstein MM, Pitt MJ, et al. Genetic variation in the toll-like receptor 4 and prostate cancer incidence and mortality. *Prostate* (2012) **72**(2):209–16. doi:10.1002/pros.21423
60. Chen YC, Giovannucci E, Kraft P, Lazarus R, Hunter DJ. Association between Toll-like receptor gene cluster (TLR6, TLR1, and TLR10) and prostate cancer. *Cancer Epidemiol Biomarkers Prev* (2007) **16**(10):1982–9. doi:10.1158/1055-9965.EPI-07-0325
61. Vacchelli E, Galluzzi L, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, et al. Trial watch: FDA-approved toll-like receptor agonists for cancer therapy. *Oncoimmunology* (2012) **1**(6):894–907. doi:10.4161/onci.20931
62. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science* (2007) **316**(5831):1628–32. doi:10.1126/science.1138963
63. Schiffman M, Wacholder S. Success of HPV vaccination is now a matter of coverage. *Lancet Oncol* (2012) **13**(1):10–2. doi:10.1016/S1470-2045(11)70324-2
64. van Egmond S, Hoedemaker C, Sinclair R. Successful treatment of perianal Bowen’s disease with imiquimod. *Int J Dermatol* (2007) **46**(3):318–9. doi:10.1111/j.1365-4632.2007.03200.x
65. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol* (2002) **3**(2):196–200. doi:10.1038/ni758
66. Holcmann M, Drobis B, Sibilia M. How imiquimod licenses plasmacytoid dendritic cells to kill tumors. *Oncoimmunology* (2012) **1**(9):1661–3. doi:10.4161/onci.22033
67. Hennessy EJ, Parker AE, O’Neill LA. Targeting toll-like receptors: emerging therapeutics? *Nat Rev Drug Discov* (2010) **9**(4):293–307. doi:10.1038/nrd3203
68. O’Neill LA, Bryant CE, Doyle SL. Therapeutic targeting of toll-like receptors for infectious and inflammatory diseases and cancer. *Pharmacol Rev* (2009) **61**(2):177–97. doi:10.1124/pr.109.001073
69. Han JH, Park SY, Kim JB, Cho SD, Kim B, Kim BY, et al. TLR7 expression is decreased during tumour progression in transgenic adenocarcinoma of mouse prostate mice and its activation inhibits growth of prostate cancer cells. *Am J Reprod Immunol* (2013) **70**(4):317–26. doi:10.1111/aji.12146
70. Han JH, Lee J, Jeon SJ, Choi ES, Cho SD, Kim BY, et al. In vitro and in vivo growth inhibition of prostate cancer by the small molecule imiquimod. *Int J Oncol* (2013) **42**(6):2087–93. doi:10.3892/ijo.2013.1898
71. Galluzzi L, Vacchelli E, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, et al. Trial watch: experimental toll-like receptor agonists for cancer therapy. *Oncoimmunology* (2012) **1**(5):699–716. doi:10.4161/onci.20696
72. Brodsky I, Strayer DR, Krueger LJ, Carter WA. Clinical studies with ampligen (mismatched double-stranded RNA). *J Biol Response Mod* (1985) **4**(6):669–75.
73. Fenoglio D, Traverso P, Parodi A, Tomasello L, Negrini S, Kalli F, et al. A multi-peptide, dual-adjuvant telomerase vaccine (GX301) is highly immunogenic in patients with prostate and renal cancer. *Cancer Immunol Immunother* (2013) **62**(6):1041–52. doi:10.1007/s00262-013-1415-9
74. Zhang Y, Wang Y, Yuan J, Qin W, Liu F, Wang F, et al. Toll-like receptor 4 ligation confers chemoresistance to docetaxel on PC-3 human prostate cancer cells. *Cell Biol Toxicol* (2012) **28**(4):269–77. doi:10.1007/s10565-012-9221-2
75. Kutikhin AG, Yuzhalin AE. Are toll-like receptor gene polymorphisms associated with prostate cancer? *Cancer Manag Res* (2012) **4**:23–9. doi:10.2147/CMAR.S28683
76. Lindstrom S, Schumacher F, Siddiq A, Travis RC, Campa D, Berndt SI, et al. Characterizing associations and SNP-environment interactions for GWAS-identified prostate cancer risk markers – results from BPC3. *PLoS One* (2011) **6**(2):e17142. doi:10.1371/journal.pone.0017142
77. Malara AE, Fedele C, Aloj L, Arra C, Laccetti P, D’Alessio G, et al. Effects of a human compact anti-ErbB2 antibody on prostate cancer. *Oncol Rep* (2012) **28**(1):297–302. doi:10.3892/or.2012.1760
78. Gora J, Hopfgartner J, Kuess P, Paskeviciute B, Georg D. Is there room for combined modality treatments? Dosimetric comparison of boost strategies for advanced head and neck and prostate cancer. *J Radiat Res* (2013) **54**(Suppl 1):i97–112. doi:10.1093/jrr/rrt067

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Toll-like receptors in ovarian cancer as targets for immunotherapies

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In the last decade, it has become apparent that toll-like receptor (TLR) signaling can play an important role in ovarian cancer (OC) progression. Interestingly, TLR activation in immune cells can help activate an anti-tumor response, while TLR signaling in tumor cells themselves is often associated with cancer-promoting inflammation. For example, it has been shown that TLR activation in dendritic cells can result in more effective antigen presentation to T cells, thereby favoring tumor eradication. However, aberrant TLR expression in OC cells is associated with more aggressive disease (likely due to recruitment of pro-tumoral leukocytes to the tumor site) and has also been implicated in resistance to mainstream chemotherapy. The delicate balance of TLR activation in the tumor microenvironment in different cell types altogether help shape the inflammatory profile and outcome of tumor growth or regression. With further studies, specific activation or repression of TLRs may be harnessed to offer novel immunotherapies or adjuvants to traditional chemotherapy for some OC patients. Herewith, we review recent literature on basic and translational research concerning therapeutic targeting of TLR pathways for the treatment of OC.

Keywords: ovarian cancer, toll-like receptors, tumor microenvironment, immunotherapy, pattern-recognition receptors

INTRODUCTION

Ovarian cancer (OC) has the most devastating death rate of gynecological cancers with only 44% of women surviving 5 years after diagnosis (1–4). The low long-term survival statistics are in part due to lack of efficient screening technology; by the time symptoms occur, most patients exhibit advanced-stage disease (over 60% of OC is diagnosed after distant metastasis). The survival rates decrease with each later stage of diagnosis with only a 27% 5-year relative survival rate for distantly metastasized tumors, highlighting the need for more efficacious treatments for advanced OC (4). Today, the standard of therapy includes surgery (hysterectomy and bilateral salpingoophorectomy) and several rounds of platinum- or taxane-based chemotherapy (1–3). Chemotherapy typically produces significant side effects, such as nausea, weight loss, fatigue, and alopecia, largely a result of toxicity of the treatment to healthy cells (5). Moreover, many cancers become resistant to treatment, further warranting the development of additional and more tumor-specific therapies. Thus, although chemotherapy remains the gold-standard of OC management, replacement as well as adjuvant treatments are in process of intensive investigation. As it is well-established that the immune system (if properly functioning) can fight tumor growth, tumor immunology research and immunotherapy clinical trials are taking center-stage in the quest for better clinical outcomes for late-staged OC (6–9).

As aggressive OC often correlates with an immunosuppressive leukocyte population in the tumor environment, efforts to modulate these cells to potentiate an anti-tumor immune response are ongoing (10–12). Of particular interest to the processes of tumor infiltration by immune cells and their activation are the toll-like

receptors (TLRs), pattern-recognition receptors (PRRs) that ligate conserved pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS) or viral dsRNA (13–20). TLR expression is well-established in immune cells, such as macrophages and dendritic cells (DCs), where upon PAMP recognition, an inflammatory response occurs, activating numerous transcription factors, such as NF-κB and IRF 3/7 (21–23). Cytokine and chemokine secretion subsequently ensues, further activating inflammation and stimulating the adaptive immune response. In fact, TLR activation in leukocytes (e.g., DCs) can trigger a shift in the inflammatory profile of the tumor site by decreasing immuno-suppression and activating immune cells that can actively fight tumors (11, 24, 25). However, in addition to their expression in leukocytes, TLRs are found in multiple tumor types, including in OC, where their activation can have tumor-promoting effects (26–29). In fact, high levels of different TLRs in cancer cells have been associated with disease aggressiveness, treatment resistance, and poor clinical outcome. Most likely, this is a result of cytokine and chemokine-induced (e.g., as a result of NF-κB activation) recruitment of immunosuppressive and pro-angiogenic leukocytes to the tumor site (13, 30–32). In this mini review, we summarize recent studies and clinical trials aimed at exploiting TLR signaling pathways for OC immunotherapy.

TOLL-LIKE RECEPTOR SIGNALING IN LEUKOCYTES

Toll-like receptors expressed in leukocytes (e.g., macrophages) serve a crucial function at the start of the immune response, activating numerous pro-inflammatory pathways resulting in cytokine secretion, and further activation of immune cells,

including the adaptive immune response (21–23). It is known that the white blood cell population infiltrating the tumor environment differs between cancers and it has been established that the specific leukocyte profile at the site has a profound effect on tumor progression or regression (8, 30, 33). As the microenvironment of OC is typically immunosuppressive, efforts are ongoing to stimulate the immune population to effectively recognize and clear the tumor cells (12). In this regard, TLR activation in immune cells can favor the anti-tumor immune response, by increasing the capability of professional antigen-presenting cells (APCs) and facilitating the activation of anti-tumoral T cells (natural killer, NK cells; cytotoxic T lymphocytes, CTLs). In fact, the last decade of cancer immunology research has brought about several examples of the benefits of TLR activation in the immune cells surrounding the ovarian tumor milieu. Several clinical trials have been performed in an attempt to stimulate TLRs for OC therapy, including using TLR agonists in combination with other immunostimulating agents, such as DC vaccines (34, 35). Overall, these studies point to the potentially promising effects of TLR stimulation for OC patients with few efficacious treatment options available, especially if integrated with mainstream treatments or as adjuvants to other immunotherapies on a case-by-case basis.

In 2005, Adams et al. first described the rationale for TLR3 agonist therapy for advanced OC (35). In 2009, it was reported that TLR3 activation in DCs enhanced antigen processing and presentation by the APCs (24). Specifically, the authors described the inability of tumor-localized DCs to successfully activate anti-tumor immunity. Instead, they suppressed T cell function, although they were shown to be capable of processing tumor antigens. However, after stimulation with dsRNA (TLR3 ligand), with co-stimulation of CD40, DC function improved to trigger the desired tumor-eliminating inflammatory response. In these studies, this was indicated by the increase of interleukin 12 (IL-12) and type I IFN secretion by the DCs, as well as higher levels of co-stimulatory molecule expression and enhanced antigen-processing capability in both mouse and human OC samples. Furthermore, the treatment augmented the migratory capabilities of the DCs (to lymph nodes) and increased their antigen-presentation capability. These results point to the promising potential to re-structure the immunosuppressive OC environment to facilitate a robust anti-tumor response.

Earlier this year, Bellora and colleagues demonstrated that TLR activation in tumor-associated macrophages (TAMs) obtained from OC patients resulted in a shift from an M2 to an M1-polarization phenotype (36). This is significant, as M2-activated macrophages in the tumor environment are implicated in cancer growth, whereas M1-type (classically activated) macrophages are associated with better clinical outcome (37). M1-polarization is primarily immunostimulatory, characterized by the secretion of IL-12 and production of cytotoxic factors, such as nitric oxide (NO). M2-type or alternative macrophage activation largely results in immunosuppressive functions, and can be differentiated from M1-type activation by high levels of interleukin 10 (IL-10) secretion, as well as expression of specific markers, such as the mannose receptor (MR). In fact, the authors demonstrated that upon M1-polarization, the macrophages were able to induce cytolytic activity of NK cells (36). Thus, TLR activation

in TAMs may be of clinical benefit by shifting the M2-polarized, immunosuppressive macrophages to a more immunostimulatory, anti-tumor phenotype.

Recently, a TLR8-specific agonist, VTX-2337 (Venti-RX Pharmaceuticals), entered Phase II clinical trials for OC patients with chemotherapy-resistant and recurring disease (38). The Phase I clinical trial with this agent was conducted in 2011 and was shown to be well-tolerated while exhibiting a dose-dependent therapeutic activity (39). The rationale for the therapy is to activate TLR8 in immune cells, whereby its signaling has been shown to have a suppressive effect on Tregs (40). Although the mechanism for the TLR8-dependent inhibition of this immune cell population is unclear, it is known to occur independently of DCs (41). In addition, TLR8 signaling appears to affect the morphology of NK cells, increasing their IFN- γ secretion, thereby strengthening the innate immune response (42). Furthermore, there have been implications for the potential of TLR7 stimulation for OC treatment (41, 43). In 2010, Geller and colleagues were the first to administer a selective small-molecule TLR7 agonist, 852A, to a small group of breast, ovarian, and cervical cancer patients with recurrent disease (43). Although significant side effects were observed with ~30% of those enrolled in the study discontinuing the therapy prior to completion, the authors showed immune activation and stabilization of disease in 2 of the 15 patients.

TLR9 ligands have similarly received interest as potential treatments for OC, specifically in combination with other immunomodulatory agents (44). In 2009, it was reported that a combinational treatment of CpG oligodeoxynucleotides (CpGODN), TLR9 ligand, and LL-37 (cathelicidin peptide) resulted in a better therapeutic outcome in mice. The authors demonstrated that the dual treatment increased the uptake of the TLR9 agonist CpODN (as TLR9 is endosomal). It was shown that the treatment increased the expansion and activation of NK cells in the murine peritoneal space, indicating an activation of innate immunity. Furthermore, studies assessing the potential role of the NK cells in the tumor environment revealed that they were heavily implicated in the observed anti-cancer effects of the therapy.

TOLL-LIKE RECEPTOR SIGNALING IN OVARIAN CANCER CELLS

In 2009, Zhou and colleagues reported on the expression of TLRs in human ovarian tissue samples, including both normal and neoplastic (benign and malignant) tissue (26). It was concluded that TLR2, TLR3, TLR4, and TLR5 were found on the epithelium of healthy ovary tissue. Additionally, this subset of TLRs was also expressed in a variety of human epithelial tumors and in numerous OC cell lines. The authors also found differential expression of TLR6 and TLR8 on all the samples, as well as low levels of TLR1, TLR7, and TLR9. It was demonstrated that the TLRs expressed in the epithelial cells were functional and it was suggested that their activation may constitute a mechanism by which the cancerous epithelial cells can manipulate inflammatory pathways to encourage tumor growth. The last decade of research on TLRs in tumor cells indicates that TLR activation in cancer cells generally results in increased production of cell survival and angiogenic molecules, as well as up-regulation of T-cell-suppressive factors, facilitating immune evasion. TLR signaling in ovarian has been

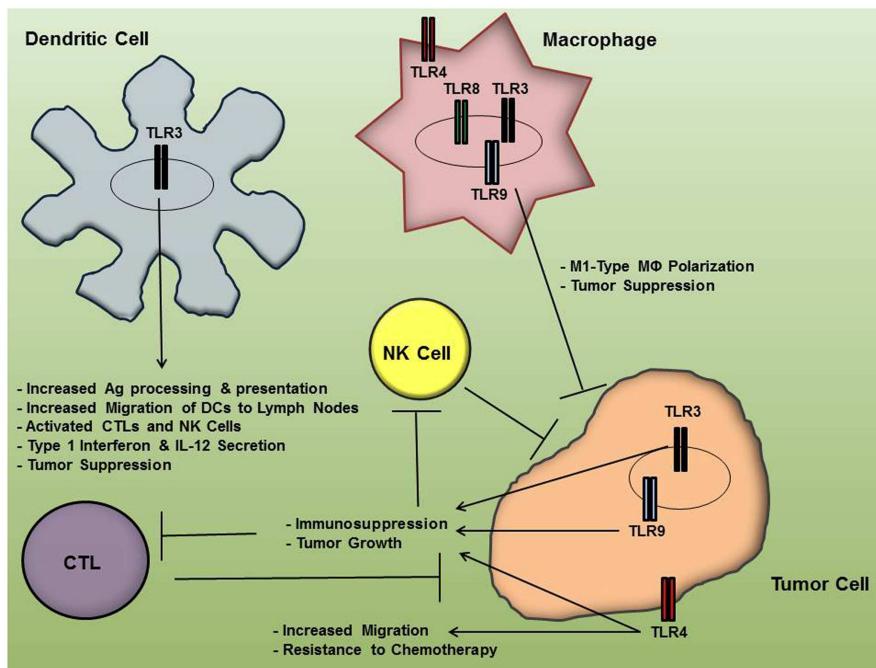


FIGURE 1 |Toll-like receptor (TLR) activation in ovarian cancer cells and immune cells results in differential effects on tumor progression. While TLR engagement in immune cells may facilitate an anti-tumor inflammatory

microenvironment, their signaling pathways in tumor cells may result in immunosuppression and resistance to chemotherapy, thereby furthering tumor growth.

attributed with more aggressive disease, potential for metastasis, and poorer end results in the clinic. Thus, specific inhibitors of TLRs (delivered to tumor cells) may be explored as potential therapeutic targets for some patients, especially in late-stage disease with fewer therapeutic options available (18). Recent research highlights the detrimental effects of TLR engagement in OC cells, indicating that inhibition of this receptor may be of benefit to the patient if targeted specifically in the cancer cells that overexpress the molecule.

The effects of TLR signaling in cancer cells have been extensively investigated for TLR4, perhaps the best-studied PRR. In 2005, Huang and colleagues reported on its expression and activation in numerous mouse cancer cell lines (45). They determined that TLR4 stimulation by LPS in tumor cells increased production of numerous soluble factors, such as IL-6, and ultimately inhibited the ability of CTLs to recognize and kill the cancer cells. It was also found that LPS treatment of the murine tumor cell supernatants impeded the proliferation of T cells and inhibited NK cell activity. Further, the authors demonstrated that inhibition of TLR4 signaling in tumor cells significantly increased survival in animal studies. The menacing effects of TLR4 activation specifically on human OC progression have also been reported (46). Kelly et al. demonstrated that TLR4 is upregulated in numerous ovarian epithelial tumors and that high expression correlates with increased tumor progression and likelihood of developing chemo-resistance to Paclitaxel. Additionally, TLR4 (and subsequent NF- κ B) activation has been demonstrated for human ovarian granulosa tumor cells (47). Thus, TLR4 inhibition in several types of OC cells may be therapeutically beneficial in conjunction

with standard chemotherapy in an effort to decrease the likelihood of drug resistance.

Similarly, TLR9 signaling by OC cells (as well as breast cancer cells) has been associated with disease aggressiveness and poor clinical outcome (48). Berger and colleagues determined that higher levels of TLR9 expression correlated with more severe tumor grade. Consistently, *in vitro* scratch assays revealed the increased migratory capabilities of tumor cells expressing higher TLR9 levels (in both ovarian and breast tumor cells). It was also reported that higher TLR9 expression was more common in poorly differentiated tumors (hormone-receptor-negative tumor cells were found to have more TLR9); thus, these tumors have fewer targeted therapeutic options. In addition, it was found that OC patients with metastatic disease had elevated levels of hypo-methylated DNA (TLR9 ligand) in their serum. Further, the authors offered even more evidence of the detrimental effects of TLR9 signaling in OC cells, showing the co-localization of TLR9 and its ligand, as well as NF- κ B activation, which was proportional to the levels of TLR9 expression. Significantly, NF- κ B appears to be constitutively activated in numerous cancer types, whereby it is associated with highly aggressive disease and poor disease outcome, highlighting the potential of TLR targeting to inhibit this important inflammatory switch in tumor cells (28, 49).

ENDOGENOUS TLR LIGANDS AND IMPLICATIONS FOR CANCER THERAPY

In addition to the PAMPs that can activate TLRs (e.g., LPS, viral RNA, etc.), endogenous ligands for these molecules have also been identified (50). For instance, TLR2 and TLR4 can be triggered by

biglycan and endoplasmin, while nucleic acid-sensing TLRs can bind to mRNA (TLR3), as well as siRNA (TLR7, TLR8). Additionally, damage-associated molecular patterns (DAMPs), molecules induced during cell stress or damage (e.g., HMGB1) can activate TLRs (51–53). As discussed, attempts to harness TLRs to promote cancer regression have been attempted in numerous trials, where the treatments are often used in combination with standard chemotherapy or radiation practices in an effort to maximize patient response. In fact, it appears likely that cell death (e.g., necrosis from standard therapy) can result in release of endogenous TLR ligands, which may activate nearby leukocytes, potentially improving the anti-tumor response (50). Continued characterization of ligands and determining downstream signaling will help elucidate the full function of TLRs in cancer progression and give more direction for novel therapeutic strategies for specific cancer types.

CONCLUDING REMARKS

The last decade of research on TLR activity and its implications in OC progression indicate that inhibition of certain TLRs in cancer cells and/or TLR stimulation in immune cells may be of therapeutic benefit in some patients. While immune activation by means of TLR stimulation can generate an anti-cancer effect, the cytokine profile following TLR activation in tumor cells typically favors an immunosuppression that can potentiate immune-tolerance and promote angiogenesis, furthering tumor growth. **Figure 1** summarizes the differential effects of TLR signaling by OC cells and immune cells. Undoubtedly, TLR targeting is a promising area of research for OC and other malignancies, although these pathways can produce such varying effects that exploitation of TLR pathways for cancer therapy has frequently been referred to as a “double-edged sword” (54, 55). Therefore, TLR targeting for OC therapy must be pursued with care and stimulating or inhibiting agents be delivered in a cell-specific manner. Given the complex nature of the effects of TLR activation in various cells, much remains to be investigated, including the multiple regulators of TLR expression and activation in the different cell types. For instance, miRNAs have recently been shown to be “fine-tuning” regulators of TLR signaling pathways; thus further research in this exciting area of study may yield even more targeting opportunities for TLR regulation that could be applied in cancer therapy (56, 57). Finally, future therapeutic strategies may be realized more effectively in conjunction with novel drug delivery mechanisms that allow for more cell-specific drug targeting.

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REFERENCES

- Cannistra SA. Cancer of the ovary. *N Engl J Med* (2004) **351**:2519–29. doi:10.1056/NEJMra041842
- Auersperg N, Wong A, Choi K, Kang S, Leung P. Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocr Rev* (2001) **22**:255–88. doi:10.1210/er.22.2.255
- Bast RCJ, Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer* (2009) **9**:415–28. doi:10.1038/nrc2644
- NCI. Seer Stat Fact Sheet: Ovary Cancer; 2003–2009; Surveillance Research Program, N. (ed.) (2013).
- Shah MA, Schwartz GK. Cell cycle-mediated drug resistance: an emerging concept in cancer therapy. *Clin Cancer Res* (2001) **7**:2168–81.
- de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. *Nat Rev* (2006) **6**:24–7. doi:10.1038/nrc1782
- Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nature* (2012) **12**:9. doi:10.1038/nrc3245
- Nelson D, Ganss R. Tumor growth or regression: powered by inflammation. *J Leukoc Biol* (2006) **80**:685–90. doi:10.1189/jlb.1105646
- Schreiber R, Old L, Smyth M. Cancer immunoediting: integrating immunity's roles in cancer suppression and progression. *Science* (2012) **331**:1565–70. doi:10.1126/science.1203486
- Benencia F, Muccioli M, Alnaeeli M. Perspectives on reprogramming cancer-associated dendritic cells for antitumor therapies. *Front Oncol* (2014) **4**:72. doi:10.3389/fonc.2014.00072
- Scarlett U, Cubillos-Ruiz JR, Nesbeth Y, Martinez D, Fields J, Gewitz A, et al. Immunosuppressive ovarian cancer-infiltrating dendritic cells can be transformed into immunostimulatory cells through *in situ* CD40 and toll-like receptor 3 stimulation. *Immunology* (2010) **184**:100–32.
- Lavoue V, Thedrez A, Leveque J, Foucher F, Hennet S, Jauffret V, et al. Immunity of human epithelial ovarian carcinoma: the paradigm of immune suppression in cancer. *J Transl Med* (2013) **11**:1–12. doi:10.1186/1479-5876-11-147
- Cubillos-Ruiz JR, Rutkowski M, Conejo-Garcia JR. Blocking ovarian cancer progression by targeting tumor microenvironmental leukocytes. *Cell Cycle* (2010) **9**:260–8. doi:10.4161/cc.9.2.10430
- Chen K, Huang J, Gong W, Iribarren P, Dunlop NM, Wang JM. Toll-like receptors in inflammation, infection, and cancer. *Int Immunopharmacol* (2007) **7**:13. doi:10.1016/j.intimp.2007.05.016
- Chen R, Alvero AB, Silasi DA, Steffensen KD, Mor G. Cancers take their toll – the function and regulation of toll-like receptors in cancer cells. *Oncology* (2008) **27**:225–33. doi:10.1038/sj.onc.1210907
- Chen R, Alvero AB, Silasi D-A, Mor G. Inflammation, cancer and chemoresistance: taking advantage of the toll-like receptor signaling pathway. *Am J Reprod Immunol* (2007) **57**:93–107. doi:10.1111/j.1600-0897.2006.00441.x
- Goutangy N, Estornes Y, Hasan U, Lebecque S, Caux C. Targeting pattern recognition receptors in cancer immunotherapy. *Target Oncol* (2012) **7**:29–54. doi:10.1007/s11523-012-0213-1
- Yu L, Chen S. Toll-like receptors expressed in tumor cells: targets for therapy. *Cancer Immunol Immunother* (2008) **57**:1271–8. doi:10.1007/s00262-008-0459-8
- Conforti R, Ma Y, Morel Y, Paturel C, Terme M, Viaud S, et al. Opposing effects of toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands. *Cancer Res* (2010) **70**:11. doi:10.1158/0008-5472.CAN-09-1890
- Underhill DM. Toll-like receptors: networking for success. *Eur J Immunol* (2003) **33**:9. doi:10.1002/eji.200324037
- Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* (2001) **8**:675–80. doi:10.1038/90609
- Takeda K, Akira S. TLR signaling pathways. *Semin Immunol* (2004) **16**:3–9. doi:10.1016/j.smim.2003.10.003
- Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* (2003) **21**:42. doi:10.1146/annurev.immunol.21.120601.141126
- Scarlett UK, Cubillos-Ruiz JR, Nesbeth Y, Martinez D, Engle X, Gewirtz AT, et al. In *situ* stimulation of CD40 and toll-like receptor 3 transforms ovarian cancer-infiltrating dendritic cells from immunosuppressive to immunostimulatory cells. *Cancer Res* (2009) **69**:7329–37. doi:10.1158/0008-5472.CAN-09-0835
- Scarlett UK, Rutkowski MR, Rauwerdink AM, Fields J, Escobar-Fadul X, Baird J, et al. Ovarian cancer progression is controlled by phenotypic changes in dendritic cells. *J Exp Med* (2012) **209**:495–506. doi:10.1084/jem.20111413

26. Zhou M, Macfarland-Mancini MM, Funk HM, Husseinzadeh N, Mounajed T, Drew AF. Toll-like receptor expression in normal ovary and ovarian tumors. *Cancer Immunol Immunother* (2009) **58**:1375–85. doi:10.1007/s00262-008-0650-y
27. Annunziata CM, Stavnes HT, Kleinberg L, Berner A, Hernandez LF, Birrer MJ, et al. Nuclear factor kappaB transcription factors are coexpressed and convey a poor outcome in ovarian cancer. *Cancer* (2010) **116**:9. doi:10.1002/cncr.25190
28. Hernandez L, Hsu SC, Davidson B, Birrer MJ, Kohn EC, Annunziata CM. Activation of NF-kappaB signaling by inhibitor of NF-kappaB kinase beta increases aggressiveness of ovarian cancer. *Cancer Res* (2010) **70**:10. doi:10.1158/0008-5472.CAN-09-3912
29. Sato Y, Goto Y, Narita N, Hoon DS. Cancer cells expressing toll-like receptors and the tumor microenvironment. *Cancer Microenviron* (2009) **2**:205–14. doi:10.1007/s12307-009-0022-y
30. Ruegg C. Leukocytes, inflammation, and angiogenesis in cancer: fatal attractions. *J Leukoc Biol* (2006) **80**:682–4. doi:10.1189/jlb.0606394
31. Conejo-Garcia JR, Benencia F, Courreges MC, Kang E, Mohamed-Hadley A, Bukanovich R, et al. Tumor-infiltrating dendritic cell precursors recruited by β -defensin contribute to vasculogenesis under the influence of Vgef-A. *Nat Med* (2004) **10**:950–8. doi:10.1038/nm1097
32. Conejo-Garcia JR, Buckanovich RJ, Benencia F, Courreges MC, Rubin SC, Carroll RG, et al. Vascular leukocytes contribute to tumor vascularization. *Blood* (2005) **105**:679–81. doi:10.1182/blood-2004-05-1906
33. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* (2010) **140**:883–99. doi:10.1016/j.cell.2010.01.025
34. Morse MA, Chapman R, Powderly J. Phase I study utilizing a novel antigen-presenting cell-targeted vaccine with toll-like receptor stimulation to induce immunity to self-antigens in cancer patients. *Clin Cancer Res* (2011) **17**:4844–53. doi:10.1158/1078-0432.CCR-11-0891
35. Adams M, Navabi H, Croston D, Coleman S, Tabi Z, Clayton A, et al. The rationale for combined chemo/immunotherapy using a toll-like receptor 3 (TLR3) agonist and tumour-derived exosomes in advanced ovarian cancer. *Vaccine* (2005) **23**:2374–8. doi:10.1016/j.vaccine.2005.01.014
36. Bellora F, Castriconi R, Dondero A, Pessino A, Nencioni A, Liggieri G, et al. TLR activation of tumor-associated macrophages from ovarian cancer patients triggers cytolytic activity of NK cells. *Eur J Immunol* (2014) **6**:1814–22. doi:10.1002/eji.201344130
37. Heusinkveld M, van der Burg S. Identification and manipulation of tumor associated macrophages in human cancers. *J Transl Med* (2011) **9**:1–13. doi:10.1186/1479-5876-9-216
38. Brueseke TJ, Tewari KS. Toll-like receptor 8: augmentation of innate immunity in platinum-resistant ovarian carcinoma. *Clin Pharmacol* (2013) **5**:13–9. doi:10.2147/CPAA.S40401
39. Cohen PA, Northfelt DW, Weiss GJ, Von Hoff DD, Manjarrez G, Dietsch G, et al. Phase I clinical trial of VTX-2337, a selective TLR8 agonist, in patients with advanced solid tumors. *J Clin Oncol* (2011) **29**:2557.
40. Peng G, Guo Z, Kiniwa Y, Voo KS, Peng W, Fu T, et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science* (2005) **309**:1380–4. doi:10.1126/science.1113401
41. Smits EL, Ponsaerts P, Berneman ZN, Van Tendeloo VF. The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. *Oncologist* (2008) **13**:859–75. doi:10.1634/theoncologist.2008-0097
42. Lu H, Dietsch GN, Matthews MH, Yang Y, Ghanekar S, Inokuma M, et al. VTX-2337 is a novel TLR8 agonist that activates NK cells and augments ADCC. *Clin Cancer Res* (2012) **18**:499–509. doi:10.1158/1078-0432.CCR-11-1625
43. Geller MA, Cooley S, Argenta PA, Downs L, Carson LF, Judson PL, et al. Toll-like receptor-7 agonist administered subcutaneously in a prolonged dosing schedule in heavily pretreated recurrent breast, ovarian, and cervix cancers. *Cancer Immunol Immunother* (2010) **59**:1877–84. doi:10.1007/s00262-010-0914-1
44. Chuang CM, Monie A, Wu A, Mao CP, Hung CF. Treatment with LL-37 peptide enhances antitumor effects induced by CpG oligodeoxynucleotides against ovarian cancer. *Hum Gene Ther* (2009) **20**:303–13. doi:10.1089/hum.2008.124
45. Huang B, Zhao J, Li H, He K, Chen Y, Mayer L, et al. Toll-like receptors on tumor cells facilitate evasion of immune surveillance. *Cancer Res* (2005) **65**:5009–14. doi:10.1158/0008-5472.CAN-05-0784
46. Kelly MG, Alvero AB, Chen R, Silasi D, Abrahams VM, Chan S, et al. TLR4 signaling promotes tumor growth and paclitaxel chemoresistance in ovarian cancer. *Cancer Res* (2006) **66**:10. doi:10.1158/0008-5472.CAN-05-3948
47. Woods DC, White YA, Dau C, Johnson AL. TLR4 activates NF-KB in human ovarian granulosa tumor cells. *Biochem Biophys Res Commun* (2011) **409**:675–80. doi:10.1016/j.bbrc.2011.05.063
48. Berger R, Fieg I, Goebel G, Obexer P, Ausserlechner M, Doppler W, et al. Toll-like receptor 9 expression in breast and ovarian cancer is associated with poorly differentiated tumors. *Cancer Sci* (2010) **101**:1059–66. doi:10.1111/j.1349-7006.2010.01491.x
49. Karin M, Cao Y, Greten FR, LI Z. NF- κ B in cancer: from innocent bystander to major culprit. *Nat Rev* (2002) **2**:301–10.
50. Yu L, Wang L, Chen S. Endogenous toll-like receptor ligands and their biological significance. *J Cell Mol Med* (2010) **14**:2592–603. doi:10.1111/j.1582-4934.2010.01127.x
51. Klune JR, Dhupar R, Cardinal J, Billiar TR, Tsung A. HMGB1: endogenous danger signaling. *Mol Med* (2008) **14**:476–84. doi:10.2119/2008-00034.Klune
52. Cheng N, He R, Tian J, Ye PP, Ye RD. Cutting edge: TLR2 is a functional receptor for acute-phase serum amyloid A. *J Immunol* (2008) **181**:22–6. doi:10.4049/jimmunol.181.1.22
53. Sandri S, Rodriguez D, Gomes E, Monteiro H, Russo M, Campa A. Is serum amyloid A and endogenous TLR4 agonist? *J Leukoc Biol* (2008) **88**:1174–80. doi:10.1189/jlb.0407203
54. Huang B, Zhao J, Unkeless JC, Feng ZH, Xiong H. TLR signaling by tumor and immune cells: a double-edged sword. *Oncogene* (2008) **27**:218–24. doi:10.1038/sj.onc.1210904
55. Killeen SD, Wang JH, Andrews EJ, Redmond HP. Exploitation of the toll-like receptor system in cancer: a doubled-edged sword? *Br J Cancer* (2006) **95**:247–52. doi:10.1038/sj.bjc.6603275
56. Chen R, Alvero AB, Silasi DA, Kelly MG, Fest S, Visintin I, et al. Regulation of IKKbeta by miR-199a adds NF-kappaB activity in ovarian cancer cells. *Oncogene* (2008) **27**:4712–23. doi:10.1038/onc.2008.112
57. O'Neill L, Sheedy FJ, McCoy CE. MicroRNAs: the fine-tuners of toll-like receptor signaling. *Nat Rev Immunol* (2011) **11**:163–75. doi:10.1038/nri2957

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Toll-like receptor 9 in breast cancer

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INTRODUCTION

Toll-like receptor 9 (TLR9) is a DNA receptor that recognizes microbial and vertebrate DNA (1–5). Initially, TLR9 was thought to recognize specifically the CpG sequence in DNA (1, 6). The sequence-requirement may, however, be relevant only for the synthetic, oligonucleotide TLR9-ligands in the phosphorothioate backbone, and also CpG sequence-independent TLR9 activation by DNA has been reported (6–8). Like the other TLRs that recognize nucleic acids (TLR3, TLRs 7–8, and TLR13), TLR9 is located at the endoplasmic reticulum in resting cells (9, 10). When DNA enters the cell, TLR9 translocates to the endosomal/lysosomal compartment where ligand recognition and binding takes place (9, 11). DNA recognition by TLR9 initiates a downstream signaling cascade, which includes the adaptor molecule MyD88 (12, 13). As an effector of the innate immune system, stimulation of TLR9 induces a NF-κB-mediated rapid inflammation, characterized by increased expression of various interleukins and cytokines. A common feature for the nucleotide-sensing TLRs is the induction of both antiviral and antitumoral type I interferons (IFNs) from plasmacytoid dendritic cells (pDCs) (14). Eventually, this inflammation also activates the adaptive immune system, which then results in the clearance of the invading pathogens and the infected cells (2, 15). A similar inflammatory response, mediated via TLRs, also takes place during sterile tissue damage (16–19). In addition to DNA, other biological molecules have also been suggested to induce TLR9-mediated responses. Such molecules include the malaria pigment hemozoin and histone proteins (17, 20–22). TLR9 was recently shown also to recognize RNA–DNA hybrids (23).

Toll-like receptor 9 (TLR9) is a cellular DNA receptor of the innate immune system. DNA recognition via TLR9 results in an inflammatory reaction, which eventually also activates a Th1-biased adaptive immune attack. In addition to cells of the immune system, TLR9 mRNA and protein are also widely expressed in breast cancer cell lines and in clinical breast cancer specimens. Although synthetic TLR9-ligands induce cancer cell invasion *in vitro*, the role of TLR9 in cancer pathophysiology has remained unclear. In the studies conducted so far, tumor TLR9 expression has been shown to have prognostic significance only in patients that have triple-negative breast cancer (TNBC). Specifically, high tumor TLR9 expression predicts good prognosis among TNBC patients. Pre-clinical studies suggest that TLR9 expression may affect tumor immunophenotype and contribute to the immunogenic benefit of chemotherapy. In this review, we discuss the possible contribution of tumor TLR9 to the pathogenesis and treatment responses in breast cancer.

Keywords: TLR9, breast cancer, invasion, inflammation, prognosis

TLR9 EXPRESSION IN BREAST CANCER

Toll-like receptor 9 expression has been detected in cells of breast milk (TLR9 mRNA) and also in normal epithelial cells of the mammary gland (TLR9 protein) (24, 25). TLR9 mRNA and protein are also widely expressed in various human cancer-cell lines as well as in clinical cancer specimens, including breast, prostate, brain, gastric, renal cell carcinoma, and esophageal tumors (24, 26–33). Specifically in breast cancer, TLR9 protein expression has been detected both in the epithelial cancer cells as well as in the fibroblast-like cells associated with the tumors (24, 26, 29, 34). Consistent with the endosomal/lysosomal localization of TLR9 at the subcellular level, in breast cancer cells *in vitro*, TLR9 appeared punctate in intracellular fluorescence staining, located especially in the perinuclear region, where these organelles are located (35). Of the five human TLR9 isoforms (A–E), mRNA expression of the TLR9 A and B isoforms has been studied and detected in breast cancer specimens (36, 37).

TLR9 AS A PROGNOSTIC FACTOR IN BREAST CANCER

The prognostic significance of TLR9 in cancers appears to be bimodal. In some cancers, such as glioma, prostate cancer, and esophageal adenocarcinoma, high tumor TLR9 expression has been associated with poor survival whereas in others, such as triple-negative breast cancer (TNBC) or renal cell carcinoma, low tumor TLR9 expression upon diagnosis predicts poor prognosis (27, 30, 32, 33, 38, 39). We demonstrated recently that although widely expressed in all clinical subtypes of breast cancer, TLR9 expression has significant, prognostic significance only in TNBC that lack the expression of estrogen (ER), progesterone (PR), and HER2 receptors. More specifically, low tumor TLR9 expression

upon diagnosis was associated with a significantly shortened disease-free-specific survival (29, 32). Furthermore, although we demonstrated that low-TLR9–TNBC cells become highly invasive in hypoxic conditions, it is currently unclear whether this mechanism contributes to the poor survival of the breast cancer patients that have hypoxic, low-TLR9–TNBC tumors. The mechanism for the increased invasion in hypoxia when TLR9 is absent is also not known (32). In addition to the actual tumor cells, the TLR9 expression status of tumor-associated fibroblast-like cells has also been shown to be of prognostic value in breast cancer. In this context, high TLR9 expression was associated with better prognosis (34). This study did not, however, assess triple-negative status of the cancers, and the exclusion of metastatic and neoadjuvant-treated patients probably counter selected against patients of the TNBC subtype.

EFFECTS OF TLR9 STIMULATION ON CELLULAR INVASION

Synthetic TLR9-ligands, the CpG sequence-containing oligonucleotides (CpG-ODNs, such as ODN M362) that mimic bacterial DNA, are strong inducers of inflammation in cells of the immune system (40, 41). These oligonucleotides mimic bacterial DNA based on their high CpG content and unmethylated cytosines. CpG-ODNs are taken up into cells via DEC-205, a multilectin cell surface receptor, which is expressed in various cell types (42). These same compounds induce cellular invasion in macrophages, mesenchymal stem cells, and in cancer cells of various origins *in vitro* (24, 28, 43, 44). In breast cancer cells, such synthetic TLR9 ligand-induced invasion has been detected both in ER-positive and ER-negative breast cancer cells (24, 28, 35). This invasive effect is mediated via TLR9, and it is blocked by chloroquine, an inhibitor of endosomal acidification and an inhibitor of TLR9 signaling. Downstream of TLR9, such invasion is mediated via TRAF6, but not MyD88 (24, 28, 35). At the proteolytic level, CpG-ODN-induced invasion is associated with down-regulation of tissue inhibitor of matrix metalloproteinases-3 (TIMP-3) and activation of matrix metalloproteinase-13 (MMP-13) (24, 28, 35, 44). Interestingly, although methylation of cytosines in CpGs has been shown to decrease their pro-inflammatory effects, the invasive effects of these molecules are independent of their methylation status (35, 40, 45). CpG-ODNs can form various secondary structures, including homopolymer duplexes and hairpins, containing stem loop structures. The stem loop secondary structure appears important for the invasive effects of the CpG-ODN (35). Furthermore, the invasive effects can also be seen with non-CpG sequence-containing ODNs that in inflammatory experiments act as TLR9 antagonists (24, 46). The synthetic, phosphorothioate-backbone-modified CpG-ODNs do not exist in nature. Thus, for this invasion to have physiological significance, it would have to be caused also by natural DNA in the phosphodiester backbone. In prostate cancer cell lines and in gastrointestinal cancer cell lines, bacterial DNA (purified from *Escherichia coli* or *Helicobacter pylori*, respectively) also has similar, stimulatory effects on invasion (28, 43). Whether microbe-derived DNA similarly induces invasion in breast cancer cells is not known. We, however, demonstrated recently that self-DNA, which is derived from chemotherapy-treated, dead cancer cells is rapidly taken up into surviving cancer cells, where it serves as an invasion-inducing

TLR9 ligand (47). This cellular uptake is possibly endocytosis or pinocytosis-mediated, since fluorescently labeled, dead cancer-cell-derived DNA, which was added to cell culture medium, was seen inside the recipient cells rapidly, within 15 min. However, similar to other reported TLR9-mediated effects of cell-derived self-DNA, complex formation of such cell-derived DNAs with the cationic antimicrobial peptide LL-37 enhanced DNA uptake into viable breast cancer cells, and was a requirement for the invasion-inducing effects (47, 48). This scenario may be physiologically relevant since LL-37 is expressed also in breast cancers (49, 50). Interestingly, the effects of cell-DNA on invasion are mediated via cathepsins and surprisingly, not via MMPs, which are the mediators for CpG-ODN-induced invasion (44, 47, 51, 52). DNA that was derived from intact, proliferating cancer cells did not induce invasion. This suggests that the invasive effect requires a certain DNA-structure, either alone or in complex with LL-37. Such DNA-structures could possibly be formed upon DNA degradation by nucleases. Whether self-DNA-induced and TLR9-mediated cancer cell invasion takes place *in vivo* in breast or any cancer is currently unknown. In principle, however, such DNA-induced and TLR9-mediated cancer cell invasion could represent a novel mechanism of treatment resistance. Since tumor growth is the sum of local proliferation and local invasion, such treatment resistance could theoretically manifest as no change or even increase in tumor size despite treatment. Finally, TLR9 appears to have also ligand-independent invasive activity. Down-regulation of TLR9 in MDA-MB-231 breast cancer cells through siRNA results in decreased *in vitro* invasion in the absence of exogenous DNA. The decreased invasion of the TLR9 siRNA cells was associated with decreased MMP activity and increased expression of TIMP-3 (32). Similar effects were also detected by TLR9 siRNA in brain cancer cells *in vitro* (53). These TLR9 expression-induced changes in the cellular invasive machinery suggest that TLR9, as a DNA-binding protein, might also have effects on gene transcription. TLR9 expression has indeed been detected in the nuclei of renal cell carcinoma tumor samples (30), but whether or not it can directly affect gene expression, requires further experimenting.

EFFECTS OF TLR9 STIMULATION ON INFLAMMATION

Toll-like receptor 9 agonists have various well documented pro-inflammatory effects in cells of the immune system (40, 41, 48, 54). Whether synthetic TLR9 agonists also induce the expression of inflammatory mediators in breast cancer cells, is not known. In cells of the immune system, a key characteristic of the TLR9-induced innate immune response is the promotion of a strong type I T helper cell (Th1) adaptive immune response. This includes both CD8⁺ T-cell responses and antigen-specific antibody responses (55). Since CD8⁺ T-cells are capable of immunologic tumor cell destruction, CpG-ODNs have been tested both as monotherapy and as an adjuvant for cancer vaccines, against various cancer types in pre-clinical cancer models, including breast cancer (55). In mouse models of breast cancer, CpG-ODN treatment resulted in the eradication of orthotopic tumors (56, 57). CpG-ODN treatment also induced an immunologic memory against tumor challenge, which was associated with an up-regulation of IFN- γ -positive CD4⁺ and CD8⁺ T-cells (56, 57). CpGs, when given as an adjuvant with a peptide vaccine,

also prevented the formation of spontaneous tumors in a mouse model of HER2-positive breast cancer (58). Although the direct growth inhibitory effects of CpG-ODNs on cancer cells are quite weak *in vitro*, certain modifications in the CpG structure have resulted in increased tumor growth inhibition, also in nude mouse models *in vivo*, suggesting direct tumor effects of these compounds (24, 59–61). Furthermore, when given in a combination, the immunomodulatory ODN was also shown to potentiate the efficacy of trastuzumab, an anti-HER2-antibody, in a mouse model of breast cancer (59). In conclusion, these pre-clinical experiments suggest that TLR9 ligands can directly inhibit the growth of breast cancer cells *in vitro* and *in vivo*, and they can enhance anti-tumor immunity, possibly via inducing a Th1 adaptive immune response. These studies have not, however, addressed the role of TLR9 expression in tumors vs. host in these responses. Despite the successful pre-clinical results, CpG treatment has demonstrated anti-tumor activity only in select patients in clinical trials. There are, however, no reports on their efficacy in breast cancer trials (55). Finally, the discrepancies between the *in vitro*-observed, unwanted tumor invasion-promoting effects and the favorable, most likely immune system-mediated anti-tumor effects of the synthetic TLR9-ligands are likely explained by the pharmacokinetics of these compounds. After s.c. and i.v. administration, highest concentrations of TLR9 ligands are detected in plasma, kidneys, and organs of the reticuloendothelial system, and much less so in tumor tissues (59).

Self-DNA has been shown to have TLR9-mediated inflammatory effects in other cell types, especially when complexed with LL-37, which is expressed in various tissues (16, 48, 52, 62). We demonstrated recently that self-DNA, which is derived from doxorubicin-killed breast cancer cells, induces mRNA expression of various inflammatory mediators in living, TLR9-expressing cells. Furthermore, while assessing treatment responses to doxorubicin in a mouse model of TNBC, we discovered that although the tumor response to treatment was similar in TLR9 siRNA and control siRNA TNBC groups, mice bearing TLR9 siRNA tumors lost significantly less weight than similarly treated mice with control siRNA tumors. Similar weights of the vehicle-treated mice suggested to us that TLR9 expression in the tumors may be an important determinant of chemotherapy-induced inflammation and activation of anti-tumor immunity (47). Inflammatory response to chemotherapy is gaining acceptance as an important mediator of treatment responses to standard cancer therapy (63). More specifically, we hypothesize that the tumor TLR9-dependent, post-treatment weight loss is actually a surrogate marker for self-DNA-induced and TLR9-mediated inflammation that takes place at the tumor site. Such tumor TLR9-mediated inflammation might then amplify the anti-tumor immune response, eradicate microscopic disease and through this mechanism, translate into cure (47). We predict that the lack of such immunogenic effect in tumors that have low-TLR9 expression indeed contributes to the described poor disease-specific survival in triple-negative disease (32). This hypothesis requires a detailed analysis of tumor TLR9-dependent immune response to chemotherapy in immune-competent pre-clinical cancer models. However, if true, it would mean that patients with low-TLR9–TNBC could especially benefit from adjuvant cancer immunotherapy. It is also

possible that TLR9 expression changes tumor immunophenotype independent of treatment and this aspect also requires further investigation.

TLR9 REGULATION IN BREAST CANCER

Several cancer-associated viruses have been shown to down-regulate TLR9 expression through their oncoproteins. For example, human papillomavirus (HPV), Epstein–Barr virus, and hepatitis B virus inhibit the expression and impair the function of TLR9 in infected target cells (64–66). Patients with chronic hepatitis B virus have decreased levels of TLR9 mRNA in peripheral blood mononuclear cells (67). The Merkel cell polyomavirus large T antigen down-regulates TLR9 expression in epithelial cells and in cells derived from Merkel cell carcinomas (68). For the HPV16, the mechanism behind TLR9 suppression was recently shown to involve the viral oncoprotein E7-induced formation of transcriptional inhibitory complex that includes NF- κ B p50–p65, ER α , and chromatin modifying enzymes. This complex induces epigenetic changes at the TLR9 promoter area (69). It is likely that these viral effects on TLR9 expression and function play an important role in viral persistence, through inhibition of host immune responses (64, 65, 67, 70, 71). Nevertheless, also opposite effects on microbial TLR9 regulation have been suggested (72, 73). Although breast cancer is not currently considered to have viral etiology, several viruses, including human papilloma viruses, have been detected in normal and cancerous human breast tissues (74–77). Whether or not these viral effects have a role in breast cancer development or pathophysiology is currently unknown.

Tumor microenvironment oxygen concentration is also an important regulator of TLRs. Similar with the effects of hypoxia on other TLRs in other cell types, hypoxia also up-regulates TLR9 expression in breast cancer cells *in vitro* and in orthotopic breast tumors *in vivo* (32, 51, 78). These hypoxia effects on TLR9 mRNA and protein expression were mediated via HIF-1 α in breast cancer cells *in vitro* (32). TNBCs are typically hypoxic (79). Therefore, understanding the mechanism on why tumor TLR9 expression levels remain low despite hypoxia in some TNBCs might open novel therapeutic possibilities that might also apply to renal cell carcinoma (30). It was also demonstrated recently that TLR9 expression is under the control of the circadian molecular clock (80). The significance of this finding for breast and other cancers is currently open.

Although TLR9 is expressed in all clinically relevant subtypes of breast cancer, we and others have discovered that there is an inverse correlation between tumor TLR9 and ER expression: ER-positive breast cancers have significantly lower levels of TLR9 expression, as compared with TNBCs (26, 29, 32, 36). The basal TLR9 expression is also significantly lower in human ER-positive breast cancer cells, as compared with human ER-negative breast cancer cell lines *in vitro*. Furthermore, transfection of ER α cDNA into TNBC cells suppresses TLR9 expression of the recipient cells (36). Both estradiol and testosterone induced TLR9 expression via their cognate receptors in breast cancer cells *in vitro*. Testosterone also augmented the pro-invasive effects of CpG-ODNs. Finally, bicalutamide, a commonly used hormonal treatment in prostate cancer, increased TLR9 expression in ER-positive breast cancer cells (36). This effect of bicalutamide on TLR9 expression might

be of therapeutic interest since a proportion of TNBC tumors express the androgen receptor that bicalutamide targets (81).

TLR9 POLYMORPHISM IN BREAST CANCER

The TLR9 gene is located on human chromosome 3 (82). Although TLR9 gene polymorphisms have been studied in other diseases, including infectious and autoimmune diseases and some cancers, very little is known about TLR9 gene polymorphism in breast cancer (83–86). A study conducted by Resler and coworkers using over 800 case and control samples, found that the single nucleotide polymorphism (SNP, rs352140) in *TLR9*, which does not alter protein amino acid sequence but might alter protein function or stability, was associated with breast cancer risk (OR 0.85, 95% CI 0.74–0.97) (87). The patients in this study were all post-menopausal (65–79 years) and 80% of the cases had hormone receptor-positive breast cancer. These results were in contrast to those of Etokebe et al., who found no association in the same TLR9 SNP with breast cancer risk in a small Croatian cohort, consisting of 130 breast cancer cases and 101 controls (88).

CONCLUSION

Although TLR9 is widely expressed in breast cancers, it appears that tumor TLR9 expression has prognostic significance only in TNBC. Especially, TNBC patients that have low tumor TLR9 expression upon diagnosis have a significantly shortened disease-specific survival, as compared with TNBC patients that have high tumor TLR9 expression. These findings, however, need to be repeated in larger and more diverse patient populations. TNBC tumors are typically hypoxic and low oxygen concentrations up-regulate TLR9 expression in TNBC cells in pre-clinical models. Understanding why TLR9 expression levels remain low in some TNBC tumors in the hypoxic tumor microenvironment might reveal novel therapeutic opportunities. It has been demonstrated recently that viral oncoproteins down-regulate TLR9 expression in various cancer tissues. Although breast cancer is not currently considered to have viral etiology, various viruses known to be capable of down-regulating TLR9 expression have also been detected in breast cancers. The contribution of these viral infections to low tumor TLR9 status in TNBC should therefore be addressed in future studies. Finally, the mechanisms how the lack of tumor TLR9 expression results in poor prognosis are unknown. Studies from pre-clinical TNBC models suggest that tumor TLR9 expression might affect tumor immunophenotype or be required for chemotherapy-induced anti-tumor immune response. If this is the case, then patients with low-TLR9–TNBC tumors might benefit from anti-cancer immune therapy. The specificity of the immune therapy requires, however, a clear understanding of how TLR9 expression affects tumor immunity. Synthetic TLR9 agonists, CpG-ODNs have demonstrated promising direct and immune system-mediated anti-cancer effects against breast cancer in pre-clinical models but they have not been studied in clinical breast cancer trials. It is clear that synthetic CpG-ODNs induce cancer-cell invasion *in vitro*. Whether this finding is relevant for the clinical situation, where such agonists are given in order to boost the anti-tumor immune response, remains to be resolved. Finally, aiming to increase tumor TLR9 expression prior to chemotherapy should

be considered a therapeutic opportunity in the TNBC patients that have low tumor TLR9.

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REFERENCES

1. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A toll-like receptor recognizes bacterial DNA. *Nature* (2000) **408**:740–5. doi:10.1038/35047123
2. Akira S, Hemmi H. Recognition of pathogen-associated molecular patterns by TLR family. *Immunol Lett* (2003) **85**:85–95. doi:10.1016/S0165-2478(02)00228-6
3. Bamboat ZM, Balachandran VP, Ocuin LM, Obaid H, Plitas G, DeMatteo RP. Toll-like receptor 9 inhibition confers protection from liver ischemia-reperfusion injury. *Hepatology* (2010) **51**:621–32. doi:10.1002/hep.23365
4. Rifkin IR, Leadbetter EA, Busconi L, Viglianti G, Marshak-Rothstein A. Toll-like receptors, endogenous ligands, and systemic autoimmune disease. *Immunol Rev* (2005) **204**:27–42. doi:10.1111/j.0105-2896.2005.00239.x
5. Barrat FJ, Meeker T, Gregorio J, Chan JH, Uematsu S, Akira S, et al. Nucleic acids of mammalian origin can act as endogenous ligands for toll-like receptors and may promote systemic lupus erythematosus. *J Exp Med* (2005) **202**:1131–9. doi:10.1084/jem.20050914
6. Bauer S, Kirschning CJ, Häcker H, Redecke V, Hausmann S, Akira S, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A* (2001) **98**:9237–42. doi:10.1073/pnas.161293498
7. Suwarti S, Yamazaki T, Svetlana C, Hanagata N. Recognition of CpG oligodeoxynucleotides by human toll-like receptor 9 and subsequent cytokine induction. *Biochem Biophys Res Commun* (2013) **430**:1234–9. doi:10.1016/j.bbrc.2012.12.068
8. Haas T, Schmitz F, Heit A, Wagner H. Sequence independent interferon-alpha induction by multimerized phosphodiester DNA depends on spatial regulation of toll-like receptor-9 activation in plasmacytoid dendritic cells. *Immunology* (2009) **126**:290–8. doi:10.1111/j.1365-2567.2008.02897.x
9. Leifer CA, Kennedy MN, Mazzoni A, Lee C, Krughak MJ, Segal DM. TLR9 is localized in the endoplasmic reticulum prior to stimulation. *J Immunol* (2004) **173**:1179–83. doi:10.4049/jimmunol.173.2.1179
10. Hidmark A, von Saint Paul A, Dalpke AH. Cutting edge: TLR13 is a receptor for bacterial RNA. *J Immunol* (2012) **189**(6):2717–21. doi:10.4049/jimmunol.1200898
11. Latz E, Schoenemeyer A, Visintin A, Fitzgerald KA, Monks BG, Knetter CF, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol* (2004) **5**:190–8. doi:10.1038/ni1028
12. Bauer S. Toll-like receptor 9 processing: the key event in toll-like receptor 9 activation? *Immunol Lett* (2013) **149**:85–7. doi:10.1016/j.imlet.2012.11.003
13. Blasius AL, Beutler B. Intracellular toll-like receptors. *Immunity* (2010) **32**:305–15. doi:10.1016/j.immuni.2010.03.012
14. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol* (2008) **8**:594–606. doi:10.1038/nri2358
15. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* (2001) **2**:675–80. doi:10.1038/90609
16. Hoque R, Malik AF, Gorelick F, Mehal WZ. Sterile inflammatory response in acute pancreatitis. *Pancreas* (2012) **41**:353–7. doi:10.1097/MPA.0b013e3182321500
17. Huang H, Evankovich J, Yan W, Nace G, Zhang L, Ross M, et al. Endogenous histones function as alarmins in sterile inflammatory liver injury through toll-like receptor 9 in mice. *Hepatology* (2011) **54**:999–1008. doi:10.1002/hep.24501
18. Imaeda AB, Watanabe A, Sohail MA, Mahmood S, Mohamadnejad M, Sutterwala FS, et al. Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. *J Clin Invest* (2009) **119**:305–14. doi:10.1172/JCI35958
19. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol* (2008) **8**:279–89. doi:10.1038/nri2215

20. Coban C, Ishii KJ, Kawai T, Hemmi H, Sato S, Uematsu S, et al. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J Exp Med* (2005) **201**:19–25. doi:10.1084/jem.20041836
21. Parroche P, Lauw FN, Goutagny N, Latz E, Monks BG, Visintin A, et al. Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to toll-like receptor 9. *Proc Natl Acad Sci U S A* (2007) **104**:1919–24. doi:10.1073/pnas.0608745104
22. Wagner H. Hemozoin: malaria's "built-in" adjuvant and TLR9 agonist. *Cell Host Microbe* (2010) **7**:5–6. doi:10.1016/j.chom.2010.01.002
23. Rigby RE, Webb LM, Mackenzie KJ, Li Y, Leitch A, Reijns MA, et al. RNA:DNA hybrids are a novel molecular pattern sensed by TLR9. *EMBO J* (2014) **33**:542–58. doi:10.1002/embj.201386117
24. Merrell MA, Ilvesaro JM, Lehtonen N, Sorsa T, Gehrs B, Rosenthal E, et al. Toll-like receptor 9 agonists promote cellular invasion by increasing matrix metalloproteinase activity. *Mol Cancer Res* (2006) **4**:437–47. doi:10.1158/1541-7786.MCR-06-0007
25. Armogida SA, Yannaras NM, Melton AL, Srivastava MD. Identification and quantification of innate immune system mediators in human breast milk. *Allergy Asthma Proc* (2004) **25**:297–304.
26. Berger R, Fiegl H, Goebel G, Obexer P, Ausserlechner M, Doppler W, et al. Toll-like receptor 9 expression in breast and ovarian cancer is associated with poorly differentiated tumors. *Cancer Sci* (2010) **101**:1059–66. doi:10.1111/j.1349-7006.2010.01491.x
27. Väistönen MR, Väistönen T, Jukkola-Vuorinen A, Vuopala KS, Desmond R, Selander KS, et al. Expression of toll-like receptor-9 is increased in poorly differentiated prostate tumors. *Prostate* (2010) **70**:817–24. doi:10.1002/pros.21115
28. Ilvesaro JM, Merrell MA, Swain TM, Davidson J, Zayzafoon M, Harris KW, et al. Toll like receptor-9 agonists stimulate prostate cancer invasion in vitro. *Prostate* (2007) **67**:774–81. doi:10.1002/pros.20562
29. Jukkola-Vuorinen A, Rahko E, Vuopala KS, Desmond R, Lehenkari PP, Harris KW, et al. Toll-like receptor-9 expression is inversely correlated with estrogen receptor status in breast cancer. *J Innate Immun* (2008) **1**:59–68. doi:10.1159/000151602
30. Ronkainen H, Hirvikoski P, Kauppila S, Vuopala KS, Paavonen TK, Selander KS, et al. Absent toll-like receptor-9 expression predicts poor prognosis in renal cell carcinoma. *J Exp Clin Cancer Res* (2011) **30**:84. doi:10.1186/1756-9966-30-84
31. Takala H, Kauppila JH, Soini Y, Selander KS, Vuopala KS, Lehenkari PP, et al. Toll-like receptor 9 is a novel biomarker for esophageal squamous cell dysplasia and squamous cell carcinoma progression. *J Innate Immun* (2011) **3**:631–8. doi:10.1159/000329115
32. Tuomela J, Sandholm J, Karihtala P, Ilvesaro J, Vuopala KS, Kauppila JH, et al. Low TLR9 expression defines an aggressive subtype of triple-negative breast cancer. *Breast Cancer Res Treat* (2012) **135**:481–93. doi:10.1007/s10549-012-2181-7
33. Väistönen MR, Jukkola-Vuorinen A, Vuopala KS, Selander KS, Vaarala MH. Expression of toll-like receptor-9 is associated with poor progression-free survival in prostate cancer. *Oncol Lett* (2013) **5**:1659–63.
34. González-Reyes S, Marín L, González L, González LO, del Casar JM, Lamelas ML, et al. Study of TLR3, TLR4 and TLR9 in breast carcinomas and their association with metastasis. *BMC Cancer* (2010) **10**:665. doi:10.1186/1471-2407-10-665
35. Ilvesaro JM, Merrell MA, Li L, Wakchoue S, Graves D, Brooks S, et al. Toll-like receptor 9 mediates CpG oligonucleotide-induced cellular invasion. *Mol Cancer Res* (2008) **6**:1534–43. doi:10.1158/1541-7786.MCR-07-2005
36. Sandholm J, Kauppila JH, Pressey C, Tuomela J, Jukkola-Vuorinen A, Vaarala M, et al. Estrogen receptor-alpha and sex steroid hormones regulate toll-like receptor-9 expression and invasive function in human breast cancer cells. *Breast Cancer Res Treat* (2012) **132**:411–9. doi:10.1007/s10549-011-1590-3
37. McKelvey KJ, Highton J, Hessian PA. Cell-specific expression of TLR9 isoforms in inflammation. *J Autoimmun* (2011) **36**(1):76–86. doi:10.1016/j.jaut.2010.11.001
38. Wang C, Cao S, Yan Y, Ying Q, Jiang T, Xu K, et al. TLR9 expression in glioma tissues correlated to glioma progression and the prognosis of GBM patients. *BMC Cancer* (2010) **10**:415. doi:10.1186/1471-2407-10-415
39. Kauppila JH, Takala H, Selander KS, Lehenkari PP, Saarnio J, Karttunen TJ. Increased toll-like receptor 9 expression indicates adverse prognosis in oesophageal adenocarcinoma. *Histopathology* (2011) **59**:643–9. doi:10.1111/j.1365-2559.2011.03991.x
40. Coch C, Busch N, Wimmenauer V, Hartmann E, Janke M, Abdel-Mottaleb MM, et al. Higher activation of TLR9 in plasmacytoid dendritic cells by microbial DNA compared with self-DNA based on CpG-specific recognition of phosphodiester DNA. *J Leukoc Biol* (2009) **86**:663–70. doi:10.1189/jlb.0509314
41. Qiao B, Li B, Yang X, Zhang H, Chu Y, Wang Y, et al. Specific siRNA downregulated TLR9 and altered cytokine expression pattern in macrophage after CpG DNA stimulation. *Cell Mol Immunol* (2005) **2**:130–5.
42. Lahoud MH, Ahmet F, Zhang JG, Meuter S, Policheni AN, Kitsoulis S, et al. DEC-205 is a cell surface receptor for CpG oligonucleotides. *Proc Natl Acad Sci U S A* (2012) **109**:16270–5. doi:10.1073/pnas.1208796109
43. Kauppila JH, Karttunen TJ, Saarnio J, Nyberg P, Salo T, Graves DE, et al. Short DNA sequences and bacterial DNA induce esophageal, gastric, and colorectal cancer cell invasion. *APMIS* (2013) **121**:511–22. doi:10.1111/apm.12016
44. Nurminniemi S, Kuvala P, Lehtonen S, Tiuraniemi S, Alahuhta I, Mattila RK, et al. Toll-like receptor 9 ligands enhance mesenchymal stem cell invasion and expression of matrix metalloprotease-13. *Exp Cell Res* (2010) **316**:2676–82. doi:10.1016/j.yexcr.2010.05.024
45. Jozsef L, Khreiss T, El Kebir D, Filep JG. Activation of TLR-9 induces IL-8 secretion through peroxynitrite signaling in human neutrophils. *J Immunol* (2006) **176**:1195–202. doi:10.4049/jimmunol.176.2.1195
46. Peter M, Bode K, Lipford GB, Eberle F, Heeg K, Dalpke AH. Characterization of suppressive oligodeoxynucleotides that inhibit toll-like receptor-9-mediated activation of innate immunity. *Immunology* (2008) **123**:118–28. doi:10.1111/j.1365-2567.2007.02718.x
47. Tuomela J, Sandholm J, Kaakinen M, Patel A, Kauppila JH, Ilvesaro J, et al. DNA from dead cancer cells induces TLR9-mediated invasion and inflammation in living cancer cells. *Breast Cancer Res Treat* (2013) **142**:477–87. doi:10.1007/s10549-013-2762-0
48. Lande R, Gregorio J, Facchinetto V, Chatterjee B, Wang YH, Homey B, et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* (2007) **449**:564–9. doi:10.1038/nature06116
49. Weber G, Chamorro CI, Granath F, Liljegegn A, Zreika S, Saidak Z, et al. Human antimicrobial protein hCAP18/LL-37 promotes a metastatic phenotype in breast cancer. *Breast Cancer Res* (2009) **11**:R6. doi:10.1186/bcr2221
50. Heilborn JD, Nilsson MF, Jimenez CI, Sandstedt B, Borregaard N, Tham E, et al. Antimicrobial protein hCAP18/LL-37 is highly expressed in breast cancer and is a putative growth factor for epithelial cells. *Int J Cancer* (2005) **114**:713–9. doi:10.1002/ijc.20795
51. Kuhlicke J, Frick JS, Morote-Garcia JC, Rosenberger P, Eltzschig HK. Hypoxia inducible factor (HIF)-1 coordinates induction of toll-like receptors TLR2 and TLR6 during hypoxia. *PLoS One* (2007) **2**:e1364. doi:10.1371/journal.pone.0001364
52. Oka T, Hikoso S, Yamaguchi O, Taneike M, Takeda T, Tamai T, et al. Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. *Nature* (2012) **485**:251–5. doi:10.1038/nature10992
53. Sandholm J, Tuomela J, Kauppila JH, Harris KW, Graves D, Selander KS. Hypoxia regulates toll-like receptor-9 expression and invasive function in human brain cancer cells in vitro. *Oncol Lett* (2014) **8**(1):266–74.
54. De Nardo D, De Nardo CM, Nguyen T, Hamilton JA, Scholz GM. Signaling crosstalk during sequential TLR4 and TLR9 activation amplifies the inflammatory response of mouse macrophages. *J Immunol* (2009) **183**:8110–8. doi:10.4049/jimmunol.0901031
55. Krieg AM. CpG still rocks! Update on an accidental drug. *Nucleic Acid Ther* (2012) **22**:77–89. doi:10.1089/nat.2012.0340
56. Xiong Z, Gharagozlu S, Vengco I, Chen W, Ohlfest JR. Effective CpG immunotherapy of breast carcinoma prevents but fails to eradicate established brain metastasis. *Clin Cancer Res* (2008) **14**:5484–93. doi:10.1158/1078-0432.CCR-07-4139
57. Cai Q, Kublo L, Cumberland R, Gooding W, Baar J. Optimized systemic dosing with CpG DNA enhances dendritic cell-mediated rejection of a poorly immunogenic mammary tumor in BALB/c mice. *Clin Transl Sci* (2009) **2**:62–6. doi:10.1111/j.1752-8062.2008.00073.x
58. Nava-Parada P, Forni G, Knutson KL, Pease LR, Celis E. Peptide vaccine given with a toll-like receptor agonist is effective for the treatment and prevention of spontaneous breast tumors. *Cancer Res* (2007) **67**:1326–34. doi:10.1158/0008-5472.CAN-06-3290

59. Wang H, Rayburn ER, Wang W, Kandimalla ER, Agrawal S, Zhang R. Immunomodulatory oligonucleotides as novel therapy for breast cancer: pharmacokinetics, in vitro and in vivo anticancer activity, and potentiation of antibody therapy. *Mol Cancer Ther* (2006) **5**:2106–14. doi:10.1158/1535-7163.MCT-06-0158
60. Yang L, Wu X, Wan M, Yu Y, Yu Y, Wang L. CpG oligodeoxynucleotides with double stem-loops show strong immunostimulatory activity. *Int Immunopharmacol* (2013) **15**:89–96. doi:10.1016/j.intimp.2012.10.020
61. Yu D, Zhu FG, Bhagat L, Wang H, Kandimalla ER, Zhang R, et al. Potent CpG oligonucleotides containing phosphodiester linkages: in vitro and in vivo immunostimulatory properties. *Biochem Biophys Res Commun* (2002) **297**:83–90. doi:10.1016/S0006-291X(02)02127-7
62. Hurtado P, Peh CA. LL-37 promotes rapid sensing of CpG oligodeoxynucleotides by B lymphocytes and plasmacytoid dendritic cells. *J Immunol* (2010) **184**:1425–35. doi:10.4049/jimmunol.0902305
63. de la Cruz-Merino L, Barco-Sánchez A, Henao Carrasco F, Nogales Fernández E, Vallejo Benítez A, Brugal Molina J, et al. New insights into the role of the immune microenvironment in breast carcinoma. *Clin Dev Immunol* (2013) **2013**:785317. doi:10.1155/2013/785317
64. Hasan UA, Bates E, Takeshita F, Biliato A, Accardi R, Bouvard V, et al. TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. *J Immunol* (2007) **178**:3186–97. doi:10.4049/jimmunol.178.5.3186
65. Fathallah I, Parroche P, Gruffat H, Zannetti C, Johansson H, Yue J, et al. EBV latent membrane protein 1 is a negative regulator of TLR9. *J Immunol* (2010) **185**:6439–47. doi:10.4049/jimmunol.0903459
66. Vincent IE, Zannetti C, Lucifora J, Norder H, Protzer U, Hainaut P, et al. Hepatitis B virus impairs TLR9 expression and function in plasmacytoid dendritic cells. *PLoS One* (2011) **6**:e26315. doi:10.1371/journal.pone.0026315
67. Sajadi SM, Mirzaei V, Hassanshahi G, Khorramdelazad H, Daredor HY, Hosseini SM, et al. Decreased expressions of toll-like receptor 9 and its signaling molecules in chronic hepatitis B virus-infected patients. *Arch Pathol Lab Med* (2013) **137**:1674–9. doi:10.5858/arpa.2012-0415-OA
68. Shahzad N, Shuda M, Gheit T, Kwon HJ, Cornet I, Saidi D, et al. The T antigen locus of Merkel cell polyomavirus downregulates human toll-like receptor 9 expression. *J Virol* (2013) **87**:13009–19. doi:10.1128/JVI.01786-13
69. Hasan UA, Zannetti C, Parroche P, Goutagny N, Malfroy M, Roblot G, et al. The human papillomavirus type 16 E7 oncoprotein induces a transcriptional repressor complex on the toll-like receptor 9 promoter. *J Exp Med* (2013) **210**:1369–87. doi:10.1084/jem.20122394
70. van Gent M, Griffin BD, Berkhoff EG, van Leeuwen D, Boer IG, Buisson M, et al. EBV lytic-phase protein BGLF5 contributes to TLR9 downregulation during productive infection. *J Immunol* (2011) **186**:1694–702. doi:10.4049/jimmunol.0903120
71. Xu N, Yao HP, Lv GC, Chen Z. Downregulation of TLR7/9 leads to deficient production of IFN-alpha from plasmacytoid dendritic cells in chronic hepatitis B. *Inflamm Res* (2012) **61**:997–1004. doi:10.1007/s00011-012-0493-z
72. Hasim A, Ge L, Li QZ, Zhang RP, Guo X. Expressions of toll-like receptors 3, 4, 7, and 9 in cervical lesions and their correlation with HPV16 infection in Uighur women. *Chin J Cancer* (2011) **30**:344–50. doi:10.5732/cjc.010.10456
73. Lagunes-Servin H, Torres J, Maldonado-Bernal C, Pérez-Rodríguez M, Huerta-Yépez S, Madrazo de la Garza A, et al. Toll-like receptors and cytokines are upregulated during Helicobacter pylori infection in children. *Helicobacter* (2013) **18**:423–32. doi:10.1111/hel.12067
74. Buehring GC, Shen HM, Jensen HM, Choi KY, Sun D, Nuovo G. Bovine leukemia virus DNA in human breast tissue. *Emerg Infect Dis* (2014) **20**:772–82. doi:10.3201/eid2005.131298
75. Lv YR, Wang JL, Zhang K, Gao HD, Sun JZ, Gong YY, et al. Human papilloma viruses (HPVs) no co-existence in breast cancer and cervical cells in the same patient. *Chin J Physiol* (2014) **57**(2):105–6. doi:10.4077/CJP.2014.BAC207
76. Manzouri L, Salehi R, Shariatipanahi S, Rezaie P. Prevalence of human papilloma virus among women with breast cancer since 2005–2009 in Isfahan. *Adv Biomed Res* (2014) **3**:75. doi:10.4103/2277-9175.125873
77. Alibek K, Kakpenova A, Mussabekova A, Sypabekova M, Karataeva N. Role of viruses in the development of breast cancer. *Infect Agent Cancer* (2013) **8**:32. doi:10.1186/1750-9378-8-32
78. Kim SY, Choi YJ, Joung SM, Lee BH, Jung YS, Lee JY. Hypoxic stress up-regulates the expression of toll-like receptor 4 in macrophages via hypoxia-inducible factor. *Immunology* (2010) **129**:516–24. doi:10.1111/j.1365-2567.2009.03203.x
79. Piccolo S, Enzo E, Montagner M. p63, Sharp1, and HIFs: master regulators of metastasis in triple-negative breast cancer. *Cancer Res* (2013) **73**:4978–81. doi:10.1158/0008-5472.CAN-13-0962
80. Silver AC, Arjona A, Walker WE, Fikrig E. The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity. *Immunity* (2012) **36**:251–61. doi:10.1016/j.jimmuni.2011.12.017
81. Fioretti FM, Sita-Lumsden A, Bevan CL, Brooke G. Revising the role of the androgen receptor in breast cancer. *J Mol Endocrinol* (2014) **52**(3):R257–65. doi:10.1530/JME-14-0030
82. Du X, Poltorak A, Wei Y, Beutler B. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. *Eur Cytokine Netw* (2000) **11**:362–71.
83. Torres-García D, Cruz-Lagunas A, García-Sancho Figueroa MC, Fernández-Plata R, Baez-Saldaña R, Mendoza-Milla C, et al. Variants in toll-like receptor 9 gene influence susceptibility to tuberculosis in a Mexican population. *J Transl Med* (2013) **11**:220. doi:10.1186/1479-5876-11-220
84. Zhang J, Zhu Q, Meng F, Lei H, Zhao Y. Association study of TLR-9 polymorphisms and systemic lupus erythematosus in northern Chinese Han population. *Gene* (2014) **533**:385–8. doi:10.1016/j.gene.2013.08.051
85. Laska MJ, Troldborg A, Hansen B, Stengaard-Pedersen K, Junker P, Nexø BA, et al. Polymorphisms within toll-like receptors are associated with systemic lupus erythematosus in a cohort of Danish females. *Rheumatology (Oxford)* (2014) **53**:48–55. doi:10.1093/rheumatology/ket316
86. Wang X, Xue L, Yang Y, Xu L, Zhang G. TLR9 promoter polymorphism is associated with both an increased susceptibility to gastric carcinoma and poor prognosis. *PLoS One* (2013) **8**:e65731. doi:10.1371/journal.pone.0065731
87. Resler AJ, Malone KE, Johnson LG, Malkki M, Petersdorf EW, McKnight B, et al. Genetic variation in TLR or NFκB pathways and the risk of breast cancer: a case-control study. *BMC Cancer* (2013) **13**:219. doi:10.1186/1471-2407-13-219
88. Etokebe GE, Knezevic J, Petricevic B, Pavelic J, Vrbanec D, Dembic Z. Single-nucleotide polymorphisms in genes encoding toll-like receptor -2, -3, -4, and -9 in case-control study with breast cancer. *Genet Test Mol Biomarkers* (2009) **13**:729–34. doi:10.1089/gtmb.2009.0045

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The role of pattern-recognition receptors in graft-versus-host disease and graft-versus-leukemia after allogeneic stem cell transplantation

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Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only treatment with curative potential for certain aggressive hematopoietic malignancies. Its success is limited by acute graft-versus-host disease (GVHD), a life-threatening complication that occurs when allo-reactive donor T cells attack recipient organs. There is growing evidence that microbes and innate pattern-recognition receptors (PRRs) such as toll-like receptors (TLR) and nod-like receptors (NLR) are critically involved in the pathogenesis of acute GVHD. Currently, a widely accepted model postulates that intensive chemotherapy and/or total-body irradiation during pre-transplant conditioning results in tissue damage and a loss of epithelial barrier function. Subsequent translocation of bacterial components as well as release of endogenous danger molecules stimulate PRRs of host antigen-presenting cells to trigger the production of pro-inflammatory cytokines (cytokine storm) that modulate T cell allo-reactivity against host tissues, but eventually also the beneficial graft-versus-leukemia (GVL) effect. Given the limitations of existing immunosuppressive therapies, a better understanding of the molecular mechanisms that govern GVHD versus GVL is urgently needed. This may ultimately allow to design modulators, which protect from GvHD but preserve donor T-cell attack on hematologic malignancies. Here, we will briefly summarize current knowledge about the role of innate immunity in the pathogenesis of GVHD and GVL following allo-HSCT.

Keywords: graft-versus-host disease, allogenic hematopoietic stem cell transplantation, pattern-recognition receptors, inflammsome, microbiota, danger molecules

INTRODUCTION

Allo-HSCT is an established treatment modality for aggressive hematological malignancies and is performed in more than 30,000 patients annually worldwide (1). Donor-derived T cells in the graft can maintain remission after induction therapy by attacking residual tumor cells in a process known as graft-versus-leukemia (GVL). Unfortunately, beneficial GVL effects are tightly associated with the pathogenesis of acute graft-versus-host disease (GVHD). Allogeneic donor T cells recognize mismatches in major or minor histocompatibility antigens present in non-malignant host tissues and subsequently induce immune-mediated damage to target organs such as the gastrointestinal tract, skin, liver, and lungs (2). Acute GVHD occurs in 40–50% of all allo-HSCT patients and accounts for considerable morbidity and mortality (3). Depletion of T cells from the allograft can decrease the incidence of acute GVHD, but comes at the cost of greater risk of graft failure, reduced GVL activity, and increased incidence of leukemic relapse (4). As a current standard of care for GVHD, glucocorticoids and other immunosuppressive drugs are used to inhibit T-cell activation and proliferation, which similarly affects GVL activity. A better understanding of the underlying molecular mechanisms may help to design measures to prevent GVHD but preserve donor T-cell responses and GVL activity, thus allowing for a broader

application of allo-HSCT in the future. Here, we discuss how the innate immune system and its environmental triggers shape the clinical course and outcome of allo-HSCT in patients and corresponding animal models.

The biology and function of pattern-recognition receptors (PRRs) is reviewed in detail within this research topic issue (5, 6). In brief, PRRs are germ line-encoded receptors that detect conserved molecular structures that are specific to invading microbes but are absent on host cells under homeostatic conditions. Ligation of such pathogen-associated molecular patterns (PAMPs) leads to activation and maturation of antigen-presenting cells (APCs), release of pro-inflammatory cytokines and, eventually, the initiation of an adaptive immune response. PRRs are expressed on different cell types of the innate and adaptive immune systems as well as non-hematopoietic cells such as endo- and epithelial cells.

IMPORTANCE OF HOST MICROBIOTA AND THE EMERGING ROLE OF INNATE IMMUNITY IN GVHD

Primary target organs of acute GVHD such as the gastrointestinal tract, skin, liver, and lungs all form epithelial linings that constantly interact with commensal and pathogenic bacteria, either through the epidermis, intestinal, or airway mucosa or the portal circulation. Consistently, there is growing evidence that bacteria

and innate PRRs are critically involved in the pathogenesis of acute GVHD. Landmark studies by van Bekkum and colleagues in mice demonstrated that bacterial decontamination or utilization of germ-free mice lead to less severe intestinal GVHD (7, 8). Reduction of intestinal microbiota by antibiotic treatment not only mitigated intestinal but also skin GVHD, suggesting a systemic effect of gut decontamination (9). Similarly, antibiotic decontamination in patients undergoing allo-HSCT seemed to confer robust protection from acute GVHD (10, 11). Lipopolysaccharide (LPS) derived from Gram-negative bacteria was identified as a driver of GVHD pathogenesis. In experimental models, allo-HSCT recipients that were treated either with anti-endotoxin neutralizing antibodies (12, 13) or an oral LPS inhibitor (14) showed reduced GVHD severity associated with preserved GVL effects and improved overall survival. These findings launched widespread use of prophylactic antibiotic treatment to reduce the bacterial burden prior to allo-HSCT, now routinely performed in many transplantation centers worldwide (15). Interestingly, modification of the intestinal microbiota using the probiotic microorganism *Lactobacillus rhamnosus* resulted in reduced translocation of enteric bacteria to the mesenteric lymph nodes, associated with improved survival and reduced acute GVHD in mice (16). Furthermore, intestinal inflammation during GVHD in mice and humans is associated with major shifts in the composition of the intestinal microbiota. In one report, GVHD-associated loss of paneth cells resulted in reduced production of antimicrobial peptides and a loss of microbial diversity with outgrowth of *Escherichia coli*. Antibiotic treatment prevented outgrowth of *E. coli* and ameliorated the course of GVHD (17). Another study showed a marked expansion of *Lactobacillales* in murine GVHD. Elimination of this species from the flora of mice before allo-HSCT aggravated GVHD, whereas its reintroduction mediated significant protection, indicating that the microbiota can modulate the severity of intestinal inflammation (18). A recent study suggested that not only bacteria but also host fungal communities (mycobiome) can critically shape acute GVHD (19). Patients colonized with candida species suffered from more severe GVHD and showed more frequent intestinal involvement (33 versus 19%). Interestingly, candida colonization was more frequent in patients bearing a loss-of-function single nucleotide polymorphism (SNP) that is associated with impaired function of the innate PRR Dectin-1, a member of the C-type lectin family of receptors that detect carbohydrates constituent of fungal cell walls, thus playing an important role in the initiation of antifungal immunity (20).

With increasing knowledge on how PRRs detect conserved microbial and danger-associated molecular patterns (DAMPs) and initiate adaptive immune responses, their role in the pathogenesis of acute GVHD has become a focus of intense research. A widely accepted model (depicted in **Figure 1**) postulates that intensive chemotherapy and/or total-body irradiation (TBI) during pre-transplant conditioning results in tissue damage and loss of epithelial barrier function. Bacterial components translocated across the barrier as well as endogenous danger molecules released from damaged cells are sensed by PRRs on host and/or donor APCs such as dendritic cells (DCs), which produce pro-inflammatory cytokines and prime allo-reactive donor-derived T cells (21). This model is supported by mouse studies, which demonstrate that

intensified TBI increases epithelial damage and is associated with more severe GVHD (14, 22). Intriguingly, innate lymphoid cell-derived IL-22 protects both the intestinal stem cell compartment and the mature intestinal epithelium from inflammatory tissue damage (23) in line with the general concept that IL-22 can maintain epithelial integrity under inflammatory conditions (24). The enhanced intestinal barrier function thus may limit LPS translocation and subsequent PRR activation. Consistently, genetic deficiency for IL-22 results in impaired gut epithelial integrity and increased tissue damage and mortality from acute GVHD (23). Along these lines, prophylactic treatment with recombinant keratinocyte growth factor protected mice from the development of lethal acute GVHD, presumably via reduction of intestinal epithelial apoptosis and diminished LPS-mediated pro-inflammatory cytokine release (25). However, administration of the recombinant human keratinocyte growth factor palifermin before and after allo-HSCT in a phase I/II placebo-controlled clinical trial had no significant effect on the incidence and severity of acute GVHD and short-term survival (26), presumably due to pleiotropic effects of palifermin.

TOLL-LIKE RECEPTORS IN GVHD PATHOGENESIS

Toll-like receptors (TLRs) constitute a family of transmembrane PRRs that are broadly expressed in hematopoietic and non-hematopoietic cells (27). TLR ligation by a variety of microbial components leads to activation of APCs, production of pro-inflammatory cytokines, and release of chemokines. One of the best-studied TLRs in the context of GVHD is TLR4, which detects LPS in the cell wall of Gram-negative bacteria. The importance of LPS translocation and subsequent release of pro-inflammatory cytokines such as TNF- α for the pathogenesis of acute GVHD have been clearly documented (14). Moreover, genetic deficiency for TLR4 in either donor or recipient cells resulted in reduced DC activation, dampened allogenic T-cell proliferation, and less severe acute GVHD (28). However, signaling through TLR4 seems not to be absolutely required for the development of GVHD in all cases. Accordingly, in another study TLR4-deficient recipient mice showed GVHD severity comparable to wild-type mice (29), suggesting that alternative pathways in the absence of TLR4 signaling can lead to the activation of host APCs and subsequent donor T-cell stimulation. Genetic association studies in patients undergoing allo-HSCT have shown inconsistent results concerning the role of TLR4 in the pathogenesis of GVHD. Patients showed reduced frequency of severe GVHD when they or their sibling donors carried at least one of two SNPs that are associated with reduced TLR4 responsiveness to LPS (odds ratio of 0.63 and 0.88, respectively) (30). A second study showed that if both patient and donor carry the SNP Thr399Ile, the incidence of severe acute GVHD was significantly increased but overall survival was not influenced (31). These contrasting results may be attributable to differences in patient cohorts, conditioning regimens and antimicrobial treatment routines.

Other members of the TLR family have been associated with immunomodulatory capacities and suppression of GVHD. Pre-treatment of mice with the TLR5 ligand flagellin resulted in reduced GVHD and improved overall survival (32). Interestingly,

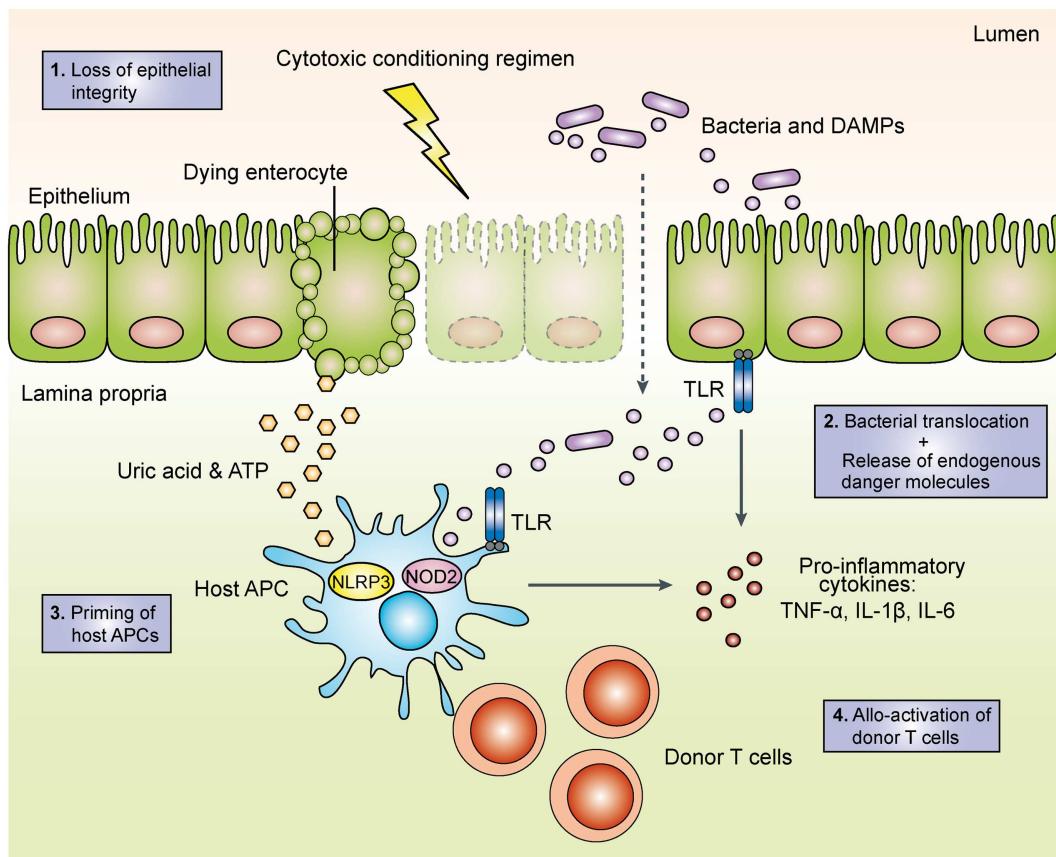


FIGURE 1 | Schematic overview of the initiation phase of acute graft-versus-host disease. During the toxic conditioning regimen with total-body irradiation and/or chemotherapy, the destruction of intestinal epithelial cells leads to the loss of the epithelial barrier function. The subsequent translocation of luminal bacteria as well as the release of endogenous danger molecules such as adenosine triphosphate (ATP) and uric acid result in the

production of pro-inflammatory cytokines. Activated host and/or donor antigen-presenting cells then prime allo-reactive donor T cells, which perpetuate acute GVHD. TLR, toll-like receptor; APC, antigen-presenting cell; DAMP, danger-associated molecular pattern; TNF, tumor necrosis factor; IL, interleukin; NOD2, nucleotide-binding oligomerization domain; NLRP3, NACHT, LRR, and PYD domains-containing protein 3.

in a clinical study of adoptively transferred immunosuppressive regulatory T cells to allo-HSCT recipients, patients who developed GVHD showed significantly increased TLR5 mRNA expression in peripheral blood mononuclear cells (33), whereas patients that did not show GVHD had reduced TLR5 mRNA expression. These results in the human system are difficult to interpret but may indirectly suggest a pro-inflammatory role of TLR5 in allo-HSCT recipients, contrary to the mouse study cited above.

Furthermore, it was shown that tissue inflammation induced by TLR ligation can modulate the development of GVHD at a local level (34). In this regard, the authors created mixed chimeras by transplanting B6 bone marrow cells into lethally irradiated BALB/c mice. After establishment of the B6 allograft, they transferred additional B6 donor T cells, which mimic the clinical use of donor lymphocyte infusions. Transplantation of donor T cells into established mixed chimeras did not induce GVHD, as donor T cells did not enter target tissues despite undergoing allo-activation, expansion, and up-regulation of homing molecules. Strikingly, topical application of R-848, a synthetic TLR7 agonist, unleashed massive

skin infiltration of donor T cells, and development of localized GVHD. Using a different TLR7 ligand (3M-011), another group demonstrated that the timing of TLR activation has important consequences for the pathogenesis of GVHD. While repetitive applications of 3M-011 after allo-HSCT aggravated GVHD severity (35), a single treatment timed between TBI and allo-HSCT induced expression of the immunoinhibitory enzyme indoleamine 2,3-dioxygenase (IDO) in host APCs, which resulted in reduced lethal intestinal GVHD (36).

In addition, signaling via TLR9 that detects microbial CpG-DNA motifs has been implicated in the pathogenesis of acute GVHD. Studies in TLR9 deficient mice showed reduced GVHD and improved survival (29, 37). Repetitive application of CpG-DNA following allo-HSCT results in increased GVHD mortality (35). This effect was dependent on TLR9 signaling and subsequent IFN- γ release in host hematopoietic cells. Less consistent results come from human studies: Transplant patients who carry gene variants associated with reduced TLR9 expression showed GVDH occurrence similar to control patients (38). A recent report analyzed two alternative SNPs that have been described to interfere

with the TLR signaling pathway (39). While patients receiving stem cells from an unrelated donor with the A1174G variant experienced severe acute GVHD more frequently (49.5 vs. 20.7%), the T1635C variant in donor cells was associated with protective effect against severe acute GVHD (16.7 vs. 49.1%).

Taken together, TLR signaling can both aggravate and attenuate the development of local and systemic GVHD; critical factors seem to be the cell type primarily affected (e.g., hematopoietic versus non-hematopoietic) and the time point of TLR ligation. Thus, the role of TLRs in the pathophysiology of GVHD remains controversial. Recipient mice that are genetically deficient for either the TLR signaling adaptor molecules MyD88 or TRIF were found to show less severe intestinal GVHD (37). In a contrasting report, bone marrow chimeric recipient mice deficient for MyD88 and/or TRIF only in hematopoietic cells developed GVHD comparable to wild-type controls (40). Other than by differences in the experimental setting between institutions (e.g., microbiota and conditioning regime), these differences might be explained by alternative (non-TLR) pathways in APCs or epithelial cells, leading to allo-activation and proliferation of donor T cells in the absence of TLR signaling.

NOD-LIKE RECEPTORS IN GVHD

Another family of PRRs with relevance to GVHD is the cytoplasmic NOD-like receptors (NLRs). NOD1 and NOD2 detect peptidoglycans as components of the bacterial cell wall (6). Both receptors have been extensively studied in the context of Crohn's disease, a chronic inflammatory bowel disease that shares several immunopathogenic features with intestinal GVHD. Reduced NOD2 activity was found to be associated with impaired epithelial barrier function and aggravated intestinal inflammation (41). Similarly, following allo-HSCT, NOD2-deficient mice showed signs of exacerbated GVHD (42). Another study with bone marrow chimeric mice that lacked NOD2 activity only in hematopoietic cells showed that NOD2 negatively regulates the development of GVHD through its inhibitory effect on host APCs. The presence of different SNPs in the *NOD2* coding region resulting in impaired downstream signaling via the pro-inflammatory transcription factor NF- κ B in either the patient, donor or both was associated with more severe GVHD (43). Two follow-up reports confirmed *NOD2* mutations as independent risk factor for transplant-related mortality (44, 45). However, several studies proposed contrasting data as they could not find an impact of *NOD2* polymorphisms on GVHD severity and outcome after allo-HSCT (46–48).

Several members of the NLR family not only detect microbial invaders but also survey cellular homeostasis and sense endogenous danger signals (6). Examples of such DAMPs are adenosine triphosphate (ATP), uric acid crystals, and double-stranded DNA released from dying cells. Activation of specific members of the NLR family by DAMPs results in the formation of cytosolic multi-protein complexes called inflammasomes, whose exact composition depends on the activator initiating their assembly (49). Inflammasome activation leads to the cleavage of pro-caspase-1 and the subsequent processing of the bioactive form of IL-1 β and IL-18. These downstream effector molecules have been shown to modulate GVHD as antibody-mediated neutralization of IL-1 β resulted in less severe acute GVHD in mice (50, 51). In a phase I/II clinical trial, blockade of IL-1 signaling attenuated GVHD in 8 out

of 14 patients with glucocorticoid-refractory disease (52). In contrast, a larger randomized study showed no effect of a recombinant IL-1 receptor antagonist on GVHD severity and overall survival (53). However, timing and way of administration of IL-1 receptor blockade may be critical. Novel IL-1 β specific antibodies await clinical testing in the setting of allo-HSCT.

The NLRP3-inflammasome is an essential platform for caspase-1 activation in response to multiple distinct exogenous and endogenous danger signals (6) and its function can be regarded as a guardian of intracellular homeostasis. NLRP3 utilizes the adapter protein ASC for activation of caspase-1 and subsequent cleavage of the precursor protein pro-IL-1 β into its active form. Binding of the endogenous danger molecule ATP to the purinergic receptor P2X₇ leads to potassium efflux and subsequent activation of the NLRP3-inflammasome. In mice and humans undergoing allo-HSCT, increased extracellular levels of ATP were found after TBI and during the development of GVHD (54). ATP released from damaged or dying cells induces activation of host APCs and priming of allo-reactive donor T cells. Pharmacological metabolism of ATP using apyrase resulted in less severe GVHD (54). Chimeric mice that were genetically deficient for the purinoceptor P2X₇ in hematopoietic cells were partially protected from GVHD. Reconstitution with wild-type DCs resulted in restored GVHD development, demonstrating a critical role for host DCs in sensing ATP and the subsequent induction of GVHD. However, significantly reduced overall survival but no alterations in GVHD severity were found in patients or corresponding donors with a loss-of-function SNP in the *P2X₇* receptor gene (55). After conditioning therapy in mice, intestinal commensal bacteria and uric acid contribute to NLRP3-inflammasome-mediated IL-1 β processing, and gastrointestinal decontamination or enzymatic uric acid depletion led to reduced GVHD severity (51). NLRP3 and the adapter protein ASC, which are both required for pro-IL-1 β cleavage, were critical for the full manifestation of GVHD. In transplanted mice, IL-1 β exerted its effects on both DCs and T cells, which preferably differentiated into IL-17A-producing Th17 cells (51), a CD4 $^+$ T-cell subpopulation that has been causally linked to instances of aggravated GVHD after allo-HSCT (56). Donors carrying one of two genetic alterations in the non-coding regions of the *NLRP3* gene are associated with increased disease relapse and reduced overall survival but no alterations in GVHD severity in allo-HSCT patients (57). Thus, directed therapies targeting the NLRP3-inflammasome or depletion of specific DAMPs remain promising therapeutic options to reduce the level of systemic inflammation in the setting of allo-HSCT, but data reported so far are somewhat controversial and await further clarification.

In summary, NOD2 signaling in hematopoietic cells appears to protect from acute GVHD. Conflicting data from genetic association studies in humans are most likely attributable to differences in frequency of *NOD2* SNPs between patient cohorts, and differences with conditioning, immune suppression, and antibiotic protocols (44). We refer to Ref. (58) for a more detailed discussion of NOD2 in GVHD. Data on inflammasomes in allo-HSCT are not yet abundant, but NLRP3 and possibly other inflammasomes that sense endogenous danger signals such as ATP and uric acid and induce IL-1 β release seems to have a role in the pathogenesis of acute GVHD.

INNATE PATTERN-RECOGNITION RECEPTORS AND THE GRAFT-VERSUS-LEUKEMIA EFFECT

Many studies have highlighted the fact that innate PRRs contribute to the inflammatory processes that lead to activation of allo-reactive T cells and the pathogenesis of GVHD. In contrast, the molecular details that shape the beneficial GVL effect remain poorly understood. Yet, only a detailed molecular understanding of the GVL effect will allow for the discrimination between GVL-pathways and allo-immune reactions that drive clinical GVHD, a prerequisite for broader application of allo-HSCT in the future. Unspecific depletion or proliferative inhibition of donor T cells is believed to come at the cost of increased relapse of the underlying malignant disease (59). However, recent data challenge that view, since T-cell depletion via selection of CD34⁺ cells in the allograft was found to be associated with markedly reduced GVHD but no differences in the rate of leukemic relapse (60, 61). Yet, data on the role of PRRs in GVL remain scarce. Studies that showed an association between loss-of-function SNPs in the *NOD2* gene and the severity of GVHD found no impact on the relapse rate by these same mutations (43, 45). Thus, *NOD2* would seem to be an attractive pharmacological target to attenuate GVHD without interfering with the GVL effect. However, other studies that investigated the same *NOD2* SNPs in transplant patients and corresponding donors could not confirm their effect on GVHD pathogenesis (52), or showed an increased risk of relapse and death if recipients and/or donors were carrying such an alteration in the *NOD2* gene (62, 63). These contrasting results emphasize that data on differential regulation of GVHD versus GVL by PRRs on a systemic level are still premature and do not yet allow for systemic modulation of PRRs as a general treatment approach. In contrast, as PRRs can control the development of GVHD at a local level (34), their pharmacological manipulation in specific immune compartments seems to be a more promising approach. Interfering with PRR signaling in GVHD target tissues, such as intestine and skin, but sparing lymphoid organs and bone marrow, where residual hematologic malignancies reside, may allow to efficiently target GVHD but leaving GVL intact.

CONCLUSION AND FUTURE DIRECTIONS

Toll-like receptors and NLRs respond to a variety of microbial and endogenous danger signals and there is increasing evidence that they influence the development of acute GVHD. Yet, the role of TLRs in the pathophysiology of GVHD remains controversial, as studies with TLR4- and MyD88-deficient mice demonstrated that TLR signaling may not be absolutely required for the development of GVHD. Loss-of-function mutations in the *NOD2* gene, on the other hand, correlated in some studies with adverse allo-HSCT outcome in humans, suggesting a protective role of *NOD2*. Furthermore, activation of the NLRP3-inflammasome during early conditioning in mice contributes to the development of acute GVHD. Other receptors involved in the local control of microbiota will be the focus of future studies. Type I interferon has been shown to play an important role in defining the balance between GVHD and GVL responses (64). Thus, PRRs that detect cytosolic nucleic acids and lead to the production of large amounts of type I interferon such as the family of RIG-I-like helicases (5) or the recently discovered cytosolic DNA receptor cyclic GAMP synthase (cGAS)

and its adapter STING (65) are of particular interest. Unraveling their role in acute GVHD will not only boost our understanding of this major complication after allo-HSCT, but may allow for novel therapeutic approaches to GVHD and related disorders like inflammatory bowel disease.

In light of the contradicting data regarding the role of some PRRs in acute GVHD, we would like to point out some of the major obstacles in the field of allo-HSCT research. Mouse models of GVHD are heterogeneous, with different subsets of immune cells being the main drivers of respective GVHD pathologies. In addition, innate and adaptive immunity are influenced by intestinal microbiota, which can vary critically between different breeding facilities. The effect of a given genetic alteration or therapeutic intervention may therefore differ between models and breeding facilities, and interpretation of such data must be undertaken with caution. Parts of the existing data may have to be revised in light of these new perceptions. Awareness of these difficulties together with increasing knowledge of graft and host immune and microbial physiology will, however, make this task easier in the future.

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REFERENCES

1. Gratwohl A, Baldomero H, Aljurf M, Pasquini MC, Bouzas LF, Yoshimi A, et al. Hematopoietic stem cell transplantation: a global perspective. *JAMA* (2010) **303**(16):1617–24. doi:10.1001/jama.2010.491
2. Shlomchik WD. Graft-versus-host disease. *Nat Rev Immunol* (2007) **7**(5):340–52. doi:10.1038/nri2000
3. Jacobsohn DA, Vogelsang GB. Acute graft versus host disease. *Orphanet J Rare Dis* (2007) **2**:35. doi:10.1186/1750-1172-2-35
4. Horowitz MM, Gale RP, Sondel PM, Goldman JM, Kersey J, Kolb HJ, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood* (1990) **75**(3):555–62.
5. Reikine S, Nguyen JB, Modis Y. Pattern recognition and signaling mechanisms of RIG-I and MDA5. *Front Immunol* (2014) **5**:342. doi:10.3389/fimmu.2014.00342
6. Saxena M, Yeretssian G. NOD-like receptors: master regulators of inflammation and cancer. *Front Immunol* (2014) **5**:327. doi:10.3389/fimmu.2014.00327
7. van Bekkum DW, Knaan S. Role of bacterial microflora in development of intestinal lesions from graft-versus-host reaction. *J Natl Cancer Inst* (1977) **58**(3):787–90.
8. van Bekkum DW, Roodenburg J, Heidt PJ, van der Waaij D. Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. *J Natl Cancer Inst* (1974) **52**(2):401–4.
9. Lampert IA, Moore RH, Huby R, Cohen J. Observations on the role of endotoxin in graft-versus-host disease. *Prog Clin Biol Res* (1988) **272**:351–9.
10. Storb R, Prentice RL, Buckner CD, Clift RA, Appelbaum F, Deeg J, et al. Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Beneficial effect of a protective environment. *N Engl J Med* (1983) **308**(6):302–7.
11. Beelen D, Haralambie E, Brandt H, Linzenmeier G, Muller K, Quabeck K, et al. Evidence that sustained growth suppression of intestinal anaerobic bacteria reduces the risk of acute graft-versus-host disease after sibling marrow transplantation. *Blood* (1992) **80**(10):2668–76.
12. Bayston K, Baumgartner JD, Clark P, Cohen J. Anti-endotoxin antibody for prevention of acute GVHD. *Bone Marrow Transplant* (1991) **8**(5):426–7.
13. Cohen J, Moore RH, Al Hashimi S, Jones L, Apperley JF, Aber VR. Antibody titres to a rough-mutant strain of *Escherichia coli* in patients undergoing allogeneic bone-marrow transplantation. Evidence of a protective effect against graft-versus-host disease. *Lancet* (1987) **1**(8523):8–11.

14. Cooke KR, Gerbitz A, Crawford JM, Teshima T, Hill GR, Tesolin A, et al. LPS antagonism reduces graft-versus-host disease and preserves graft-versus-leukemia activity after experimental bone marrow transplantation. *J Clin Invest* (2001) **107**(12):1581–9. doi:10.1172/JCI12156
15. Krüger WH, Bohlius J, Cornely OA, Einsele H, Hebart H, Massenkeil G, et al. Antimicrobial prophylaxis in allogeneic bone marrow transplantation. Guidelines of the Infectious Diseases Working Party (AGIHO) of the German Society of Haematology and Oncology. *Ann Oncol* (2005) **16**(8):1381–90. doi:10.1093/annonc/mdi238
16. Gerbitz A, Schultz M, Wilke A, Linde HJ, Scholmerich J, Andreesen R, et al. Probiotic effects on experimental graft-versus-host disease: let them eat yogurt. *Blood* (2004) **103**(11):4365–7. doi:10.1182/blood-2003-11-3769
17. Eriguchi Y, Takashima S, Oka H, Shimoji S, Nakamura K, Uryu H, et al. Graft-versus-host disease disrupts intestinal microbial ecology by inhibiting Paneth cell production of alpha-defensins. *Blood* (2012) **120**(1):223–31. doi:10.1182/blood-2011-12-401166
18. Jenq RR, Ubeda C, Taur Y, Menezes CC, Khanin R, Dudakov JA, et al. Regulation of intestinal inflammation by microbiota following allogeneic bone marrow transplantation. *J Exp Med* (2012) **209**(5):903–11. doi:10.1084/jem.20112408
19. van der Velden WJ, Netea MG, de Haan AF, Huls GA, Donnelly JP, Blijlevens NM. Role of the mycobiome in human acute graft-versus-host disease. *Biol Blood Marrow Transplant* (2013) **19**(2):329–32. doi:10.1016/j.bbmt.2012.11.008
20. Drummond RA, Brown GD. Signalling C-type lectins in antimicrobial immunity. *PLoS Pathog* (2013) **9**(7):e1003417. doi:10.1371/journal.ppat.1003417
21. Blazar BR, Murphy WI, Abedi M. Advances in graft-versus-host disease biology and therapy. *Nat Rev Immunol* (2012) **12**(6):443–58. doi:10.1038/nri3212
22. Hill GR, Ferrara JLM. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: Rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood* (2000) **95**(9):2754–9.
23. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. *Immunity* (2012) **37**(2):339–50. doi:10.1016/j.immuni.2012.05.028
24. Rutz S, Eidenschenk C, Ouyang W. IL-22, not simply a Th17 cytokine. *Immunol Rev* (2013) **252**(1):116–32. doi:10.1111/imr.12027
25. Ellison CA, Natuik SA, Fischer JM, McIntosh AR, Scully SA, Bow EJ, et al. Effect of recombinant human keratinocyte growth factor (rHuKGF) on the immunopathogenesis of intestinal graft-vs.-host disease induced without a preconditioning regimen. *J Clin Immunol* (2004) **24**(2):197–211. doi:10.1023/B:JOCI.0000019785.35850.a5
26. Blazar BR, Weisdorf DJ, Defor T, Goldman A, Braun T, Silver S, et al. Phase 1/2 randomized, placebo-control trial of palifermin to prevent graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HSCT). *Blood* (2006) **108**(9):3216–22. doi:10.1182/blood-2006-04-017780
27. O'Neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors – redefining innate immunity. *Nat Rev Immunol* (2013) **13**(6):453–60. doi:10.1038/nri3446
28. Zhao Y, Liu Q, Yang L, He D, Wang L, Tian J, et al. TLR4 inactivation protects from graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. *Cell Mol Immunol* (2013) **10**(2):165–75. doi:10.1038/cmi.2012.58
29. Calcaterra C, Sfondrini L, Rossini A, Sommariva M, Rumio C, Menard S, et al. Critical role of TLR9 in acute graft-versus-host disease. *J Immunol* (2008) **181**(9):6132–9. doi:10.4049/jimmunol.181.9.6132
30. Lorenz E, Schwartz DA, Martin PJ, Gooley T, Lin MT, Chien JW, et al. Association of TLR4 mutations and the risk for acute GVHD after HLA-matched-sibling hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* (2001) **7**(7):384–7. doi:10.1053/bbmt.2001.v7.pm11529488
31. Elmaagaci AH, Koldehoff M, Hindahl H, Steckel NK, Trenschel R, Peceny R, et al. Mutations in innate immune system NOD2/CARD 15 and TLR-4 (Thr399Ile) genes influence the risk for severe acute graft-versus-host disease in patients who underwent an allogeneic transplantation. *Transplantation* (2006) **81**(2):247–54. doi:10.1097/01.tp.0000188671.94646.16
32. Hossain MS, Jaye DL, Pollack BP, Farris AB, Tselenyane ML, David E, et al. Flagellin, a TLR5 agonist, reduces graft-versus-host disease in allogeneic hematopoietic stem cell transplantation recipients while enhancing antiviral immunity. *J Immunol* (2011) **187**(10):5130–40. doi:10.4049/jimmunol.1101334
33. Sawitzki B, Brunstein C, Meisel C, Schumann J, Vogt K, Appelt C, et al. Prevention of graft-versus-host disease by adoptive T regulatory therapy is associated with active repression of peripheral blood toll-like receptor 5 mRNA expression. *Biol Blood Marrow Transplant* (2014) **20**(2):173–82. doi:10.1016/j.bbmt.2013.10.022
34. Chakraverty R, Cote D, Buchli J, Cotter P, Hsu R, Zhao G, et al. An inflammatory checkpoint regulates recruitment of graft-versus-host reactive T cells to peripheral tissues. *J Exp Med* (2006) **203**(8):2021–31. doi:10.1084/jem.20060376
35. Taylor PA, Ehrhardt MJ, Lees CJ, Panoskaltsis-Mortari A, Krieg AM, Sharpe AH, et al. TLR agonists regulate alloresponses and uncover a critical role for donor APCs in allogeneic bone marrow rejection. *Blood* (2008) **112**(8):3508–16. doi:10.1182/blood-2007-09-113670
36. Jasperson LK, Bucher C, Panoskaltsis-Mortari A, Mellor AL, Munn DH, Blazar BR. Inducing the tryptophan catabolic pathway, indoleamine 2,3-dioxygenase (IDO), for suppression of graft-versus-host disease (GVHD) lethality. *Blood* (2009) **114**(24):5062–70. doi:10.1182/blood-2009-06-227587
37. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Lodenkemper C, et al. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. *Gut* (2010) **59**(8):1079–87. doi:10.1136/gut.2009.197434
38. Elmaagaci AH, Koldehoff M, Beelen DW. Improved outcome of hematopoietic SCT in patients with homozygous gene variant of toll-like receptor 9. *Bone Marrow Transplant* (2009) **44**(5):295–302. doi:10.1038/bmt.2009.32
39. Xiao HW, Luo Y, Lai XY, Shi JM, Tan YM, He JS, et al. Donor TLR9 gene tagSNPs influence susceptibility to aGVHD and CMV reactivation in the allo-HSCT setting without polymorphisms in the TLR4 and NOD2 genes. *Bone Marrow Transplant* (2014) **49**(2):241–7. doi:10.1038/bmt.2013.160
40. Li H, Matte-Martone C, Tan HS, Venkatesan S, McNiff J, Demetris AJ, et al. Graft-versus-host disease is independent of innate signaling pathways triggered by pathogens in host hematopoietic cells. *J Immunol* (2011) **186**(1):230–41. doi:10.4049/jimmunol.1002965
41. de Zoete MR, Flavell RA. Interactions between nod-like receptors and intestinal bacteria. *Front Immunol* (2013) **4**:462. doi:10.3389/fimmu.2013.00462
42. Penack O, Smith OM, Cunningham-Bussel A, Liu X, Rao U, Yim N, et al. NOD2 regulates hematopoietic cell function during graft-versus-host disease. *J Exp Med* (2009) **206**(10):2101–10. doi:10.1084/jem.20090623
43. Holler E, Rogler G, Herfarth H, Brenmoehl J, Wild PJ, Hahn J, et al. Both donor and recipient NOD2/CARD15 mutations associate with transplant-related mortality and GvHD following allogeneic stem cell transplantation. *Blood* (2004) **104**(3):889–94. doi:10.1182/blood-2003-10-3543
44. Holler E, Rogler G, Brenmoehl J, Hahn J, Herfarth H, Greinix H, et al. Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination. *Blood* (2006) **107**(10):4189–93. doi:10.1182/blood-2005-09-3741
45. van der Velden WJ, Blijlevens NM, Maas FM, Schaap NP, Jansen JH, van der Reijden BA, et al. NOD2 polymorphisms predict severe acute graft-versus-host and treatment-related mortality in T-cell-depleted haematopoietic stem cell transplantation. *Bone Marrow Transplant* (2009) **44**(4):243–8. doi:10.1038/bmt.2009.21
46. Nguyen Y, Al-Lehibi A, Gorbe E, Li E, Haagenson M, Wang T, et al. Insufficient evidence for association of NOD2/CARD15 or other inflammatory bowel disease-associated markers on GVHD incidence or other adverse outcomes in T-replete, unrelated donor transplantation. *Blood* (2010) **115**(17):3625–31. doi:10.1182/blood-2009-09-243840
47. Sairafi D, Uzunel M, Remberger M, Ringden O, Mattsson J. No impact of NOD2/CARD15 on outcome after SCT. *Bone Marrow Transplant* (2008) **41**(11):961–4. doi:10.1038/bmt.2008.9
48. van der Straaten HM, Paquay MM, Tilanus MG, van Geloven N, Verdonck LF, Huisman C. NOD2/CARD15 variants are not a risk factor for clinical outcome after nonmyeloablative allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* (2011) **17**(8):1231–6. doi:10.1016/j.bbmt.2010.12.709
49. Franchi L, Munoz-Planillo R, Nunez G. Sensing and reacting to microbes through the inflammasomes. *Nat Immunol* (2012) **13**(4):325–32. doi:10.1038/ni.2231
50. Hill GR, Teshima T, Gerbitz A, Pan L, Cooke KR, Brinson YS, et al. Differential roles of IL-1 and TNF-alpha on graft-versus-host disease and graft versus leukemia. *J Clin Invest* (1999) **104**(4):459–67. doi:10.1172/JCI6896
51. Jankovic D, Ganesan J, Bscheider M, Stickel N, Weber FC, Guarda G, et al. The Nlrp3 inflammasome regulates acute graft-versus-host disease. *J Exp Med* (2013) **210**(10):1899–910. doi:10.1084/jem.20130084

52. McCarthy PL Jr, Williams L, Harris-Bacile M, Yen J, Przepiorka D, Ippoliti C, et al. A clinical phase I/II study of recombinant human interleukin-1 receptor in glucocorticoid-resistant graft-versus-host disease. *Transplantation* (1996) **62**(5):626–31. doi:10.1097/00007890-199609150-00015
53. Antin JH, Weisdorf D, Neuberg D, Nicklow R, Clouthier S, Lee SJ, et al. Interleukin-1 blockade does not prevent acute graft-versus-host disease: results of a randomized, double-blind, placebo-controlled trial of interleukin-1 receptor antagonist in allogeneic bone marrow transplantation. *Blood* (2002) **100**(10):3479–82. doi:10.1182/blood-2002-03-0985
54. Wilhelm K, Ganeshan J, Muller T, Durr C, Grimm M, Beilhack A, et al. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. *Nat Med* (2010) **16**(12):1434–8. doi:10.1038/nm.2242
55. Lee KH, Park SS, Kim I, Kim JH, Ra EK, Yoon SS, et al. P2X7 receptor polymorphism and clinical outcomes in HLA-matched sibling allogeneic hematopoietic stem cell transplantation. *Haematologica* (2007) **92**(5):651–7. doi:10.3324/haematol.10810
56. Fulton LM, Carlson MJ, Coghill JM, Ott LE, West ML, Panoskalsis-Mortari A, et al. Attenuation of Acute Graft-versus-Host Disease in the Absence of the Transcription Factor ROR γ t. *J Immunol* (2012) **189**(4):1765–72. doi:10.4049/jimmunol.1200858
57. Granell M, Urbano-Ispizua A, Pons A, Arostegui JI, Gel B, Navarro A, et al. Common variants in NLRP2 and NLRP3 genes are strong prognostic factors for the outcome of HLA-identical sibling allogeneic stem cell transplantation. *Blood* (2008) **112**(10):4337–42. doi:10.1182/blood-2007-12-129247
58. Holler E, Landfried K, Meier J, Hausmann M, Rogler G. The role of bacteria and pattern recognition receptors in GVHD. *Int J Inflam* (2010) **2010**:814326. doi:10.4061/2010/814326
59. Warren EH, Deeg HJ. Dissecting graft-versus-leukemia from graft-versus-host-disease using novel strategies. *Tissue Antigens* (2013) **81**(4):183–93. doi:10.1111/tan.12090
60. Bayraktar UD, de Lima M, Saliba RM, Maloy M, Castro-Malaspina HR, Chen J, et al. Ex vivo T cell-depleted versus unmodified allografts in patients with acute myeloid leukemia in first complete remission. *Biol Blood Marrow Transplant* (2013) **19**(6):898–903. doi:10.1016/j.bbmt.2013.02.018
61. Pasquini MC, Devine S, Mendizabal A, Baden LR, Wingard JR, Lazarus HM, et al. Comparative outcomes of donor graft CD34+ selection and immune suppressive therapy as graft-versus-host disease prophylaxis for patients with acute myeloid leukemia in complete remission undergoing HLA-matched sibling allogeneic hematopoietic cell transplantation. *J Clin Oncol* (2012) **30**(26):3194–201. doi:10.1200/JCO.2012.41.7071
62. Granell M, Urbano-Ispizua A, Arostegui JI, Fernandez-Aviles F, Martinez C, Rovira M, et al. Effect of NOD2/CARD15 variants in T-cell depleted allogeneic stem cell transplantation. *Haematologica* (2006) **91**(10):1372–6.
63. Mayor NP, Shaw BE, Hughes DA, Maldonado-Torres H, Madrigal JA, Keshav S, et al. Single nucleotide polymorphisms in the NOD2/CARD15 gene are associated with an increased risk of relapse and death for patients with acute leukemia after hematopoietic stem-cell transplantation with unrelated donors. *J Clin Oncol* (2007) **25**(27):4262–9. doi:10.1200/JCO.2007.12.1897
64. Robb RJ, Kreijveld E, Kuns RD, Wilson YA, Oliver SD, Don AL, et al. Type I-IFNs control GVHD and GVL responses after transplantation. *Blood* (2011) **118**(12):3399–409. doi:10.1182/blood-2010-12-325746
65. Bhat N, Fitzgerald KA. Recognition of cytosolic DNA by cGAS and other STING-dependent sensors. *Eur J Immunol* (2014) **44**(3):634–40. doi:10.1002/eji.201344127

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Toll-like receptor-4 modulation for cancer immunotherapy

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INTRODUCTION

Toll-like receptors (TLRs) are evolutionarily conserved pattern recognition molecules. Since the discovery of the Toll pathway cascade (1, 2), our knowledge about the structure, function, and mechanics of TLRs in infectious and inflammatory conditions has increased remarkably. The role of TLR4 as a pathogen-pattern recognition receptor has been studied extensively. We now know that TLR4 recognizes pathogen-associated molecular patterns (PAMPs), such as Gram-negative bacterial lipopolysaccharide (LPS) and endogenous damage-associated molecular patterns (DAMPs) like fibronectin and hyaluronan, which are released during infectious and non-infectious inflammatory conditions. Some chronic infections and inflammatory conditions are known to promote carcinogenesis. For example, *Helicobacter pylori* (3) and viral hepatitis (4) infections lead to gastric and liver cancers, respectively. Also, in inflammatory bowel disease, non-infectious inflammation promotes the development of colorectal cancer (5). Evidence from recent reports suggests that increased expression and activity of TLR4 in chronic infectious and inflammatory conditions is associated with cancer progression (6–8). At the same time, additional studies suggest the protective role of TLR4 in cancer (9–14). The role of TLR4 in cancer has only recently been studied. This review article provides a brief summary of the current understanding of TLR4-signaling, its pro- and anti-cancer effects, and the therapeutic potential of TLR4 immunomodulation in the prevention and treatment of cancer.

LIGAND RECOGNITION AND ACTIVATION OF TLR4

Activation of TLR4 and downstream intracellular signaling involves interaction with

TLR4 ligands, dimerization, and assembly of the TLR4-complex with its adaptor and co-receptor molecules. Our understanding of TLR4-signaling is based on the results of the studies focused on the interaction of TLR4 with *Escherichia coli*-derived LPS. It has been demonstrated that the LPS binding protein (LBP) transfers the LPS to CD14 that is present in soluble form or linked to the cell surface by a glycosylphosphatidylinositol anchor. LPS is then transferred from CD14 to the myeloid differentiation (MD2) protein (6). CD14 and MD2 do not have cytoplasmic tails and are unable to transduce signals on their own. The fatty acyl chains of lipid A of LPS are integrated into the hydrophobic pocket of MD2, and negatively charged phosphate groups on the diglucosamine backbone of lipid A interact with the charged residues at the opening of the binding pocket of MD2. The LPS-MD2 interaction with TLR4 then causes dimerization of the TLR4-MD2 in 1:1 ratio and assembly of TLR4-MD2-LPS complex. The crystal structure of human TLR4-MD2-LPS complex shows the formation of an “m”-shaped oligomer made up of two molecules of TLR4 and two molecules of MD2 (15). **Figure 1A** provides a pictorial representation of the steps involved in the formation of the TLR4-MD2-LPS complex.

TLR4-SIGNALING AND HOST DEFENSE MECHANISMS

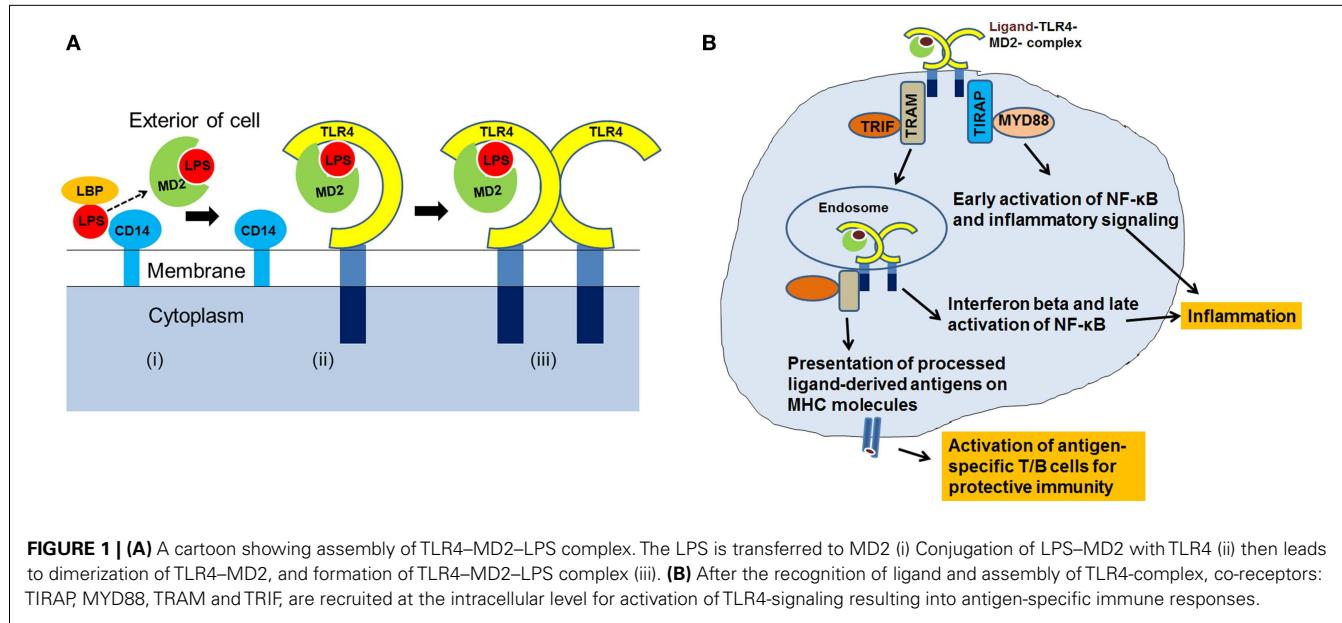
After the TLR4-MD2-LPS complex formation, TLR4 signals through myeloid differentiation primary response protein (MYD88)-dependent and Toll/IL-1R domain-containing adaptor inducing interferon-beta (TRIF)-dependent pathways. Dimerization of TLR4-MD2 recruits TIRAP (Toll/IL-1R domain-containing adaptor protein) and MYD88, leading to intracellular signaling, activation of

transcription factors, and production of pro-inflammatory cytokines. TLR4 also recruits additional proteins, TRIF-related adaptor molecule (TRAM) and TRIF, and induces production of type I interferons. Type I interferons are associated with immune responses elicited by T and NK cells, important effector cells of adaptive immunity (16). During the late phase of signaling, activation of TRIF can also induce NF- κ B transcription factor.

Immune response to any pathogenic stimuli includes activation of innate immunity, inflammation, and adaptive immunity. TLR4-signaling can eventually lead to a multitude of cellular effects (17). It is well-established that during the innate phase of immune response, TLR4 recognizes its ligands (pathogens, PAMPs, or DAMPs), and facilitates their uptake, intracellular processing, and the inflammatory response (18–21). After the TLR4 ligands are internalized and processed, the antigens are loaded onto the major histocompatibility complex (MHC) molecules for presentation to naïve lymphocytes. Published reports support the role of TLR4 in antigen-presentation and activation of cellular and humoral immune responses (22–25). **Figure 1B** summarizes the recognition of ligands by TLR4, TLR4-signaling through MYD88 and TRIF, and its role in inflammation and antigen-presentation. Thus, it is apparent that TLR4 is involved directly or indirectly with different arms of the host defense system (21, 26).

TLR4 AND CANCER

TLR4 is associated with cancer in several ways. Diverse cell lines and tissue samples derived from patients with head and neck, esophageal, gastric, colorectal, liver, pancreatic, skin, breast, ovarian, cervical, and breast cancer have been shown to express increased amounts of TLR4 (27).



Constitutive expression of some TLR4 genetic variants has also been linked to cancer (28–32). These characteristics are therefore being considered for their prognostic value in cancer treatment (32–34). In these scenarios of established cancer, TLR4 facilitates an environment that is suitable for continued cancer cell proliferation. Pro-cancer mechanisms could include the evasion of cancer cells from immune surveillance (35–38).

Persistent activation of TLR4-induced inflammatory signaling in chronic inflammatory conditions can also contribute to carcinogenesis (39). Experimental evidence suggests that cancer cell migration and invasion are induced by triggering of TLR4–NF- κ B under inflammatory conditions (40–42). LPS-induced TLR4-signaling also promotes cancer cell survival and proliferation in hepatocellular carcinoma (43, 44). Moreover, the blockade of TLR4 by siRNA and NF- κ B inhibitors decreases the invasive ability of cancer cells. Correspondingly, TLR4 silencing has been shown to decrease tumor burden in a murine model of colorectal metastasis and hepatic steatosis (45).

At the same time, published data suggest that TLR4 is required for protective immune response and killing of cancer cells. For example, TLR4-deficient mice developed more tumors after oral gavage with polyaromatic hydrocarbon 7,12-dimethylbenz(a)anthracene than did

wild-type mice (46). Similarly, silencing of TLR4 increased breast cancer metastasis (47). Although mechanism is not fully understood, TLR4 can induce an efficient cancer antigen-specific cytotoxic T cell immune response (48). The cytotoxic T cells will eventually kill the cancer cells. The dynamics of the TLR4-induced immune parameters in the tumor microenvironment could be complex, and is not well studied. It is possible that TLR4 exerts pro- or anti-cancer effects, depending on the prevailing conditions in the tissue microenvironment during different phases of cancer development or metastasis.

CURRENTLY AVAILABLE TLR4 IMMUNOMODULATORY AGENTS

A number of immunomodulators, which target TLR4 have been developed. These modulators (antagonists or agonists) have been grouped based on their binding and sequestration of LPS, antagonizing LBP and CD14/LPS interactions, and targeting of MD2, TLR4–MD2, or TLR4.

Monophosphoryl lipid A (MPLA), a chemically modified derivative of LPS, is less toxic, and retains most of the immunostimulatory activity of LPS. MPLA serves as a TLR4 agonist. It has been approved in Europe as a vaccine adjuvant, and is a component of Hepatitis B and Human Papillomavirus Virus vaccines (49). Another lipid-based agonist, E6020 (Eisai/Sanofi Pasteur), has

also been developed as a vaccine adjuvant (50, 51). Other lipid molecules are being investigated for their potential to target the CD14–LPS interaction and antagonistic activity (52). Eritoran (E5564), developed by Eisai (Tokyo, Japan), directly binds to the hydrophobic pocket of MD2, competitively inhibits LPS from binding to MD2, and prevents the dimerization of TLR4, as well as TLR4-signaling (53). TAK-242, a cyclohexene derivative, was later developed by Takeda Pharmaceuticals (Tokyo, Japan) to target the TLR4 on the cellular membrane. Both TAK-242 and Eritoran (E5564) have been investigated in clinical trials as possible treatments for sepsis (54). Ibudilast (AV4II), a TLR4 antagonist, has been shown to suppress pro-inflammatory cytokines, such as TNF- α and IL-6, in neuroinflammation (55). Antibodies that target TLR4, NI-0101, and IA6 (NovImmune, Geneva, Switzerland), are being investigated for the treatment of acute and chronic inflammation. Glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE; Immune design, Seattle, WA, USA), is also being studied (<http://www.clinicaltrials.gov>). Although Eritoran and TAK-242 did not show efficacy for treatment of sepsis, a complicated clinical problem, studies with these modulators have clearly improved our understanding of the structural aspects of TLR4-complex formation and signaling.

POTENTIAL OF TLR4 IMMUNOMODULATION FOR THE PREVENTION OR TREATMENT OF CANCER

Agents with TLR4-antagonistic activity have been shown to reduce inflammation-induced carcinogenesis by suppressing the TLR4-induced NF- κ B signaling. Curcumin, the main constituent of the spice turmeric, has been found to most likely bind to MD2, thus competing with LPS (56). A number of synthetic curcuminoids, such as EF24, have also been found to have anti-inflammatory activity (57–60). Our lab recently developed TLR4-interacting surfactant protein-A (SP-A) peptide, called SPA4, which binds to TLR4 protein in complex with MD2, and is effective therapeutically in cell culture systems and in a mouse model (61, 62). In the initial studies, our results showed that the TLR4-interacting SPA4 peptide suppresses LPS-TLR4-induced migration and invasion of colon cancer cells (63). More studies are warranted to understand the mechanism of SPA4 peptide activity. Other agents, including resveratrol (64), NI-0101 antibody (65), and paeoniflorin (66), have also shown suppression of inflammation-induced carcinogenesis.

While TLR4 antagonists could help reduce progression of inflammation-induced carcinogenesis or metastasis, TLR4 agonists have been shown to induce anti-tumor immunity in patients and models of established cancer. Lipid A-based TLR4 agonists, known as OM-174 and AS15, exhibit anti-cancer effects (67–70). Incorporation of the LPS and E6020 to Paclitaxel, whole cell tumor cell vector, and Trastuzumab improved the anti-tumor immunity in mouse models (71–73). Picibanil (OK-432) targets both TLR2 and TLR4 and suppresses cancer (74). Vacchelli et al. recently published a detailed review of the ongoing clinical trials on TLR modulators, including TLR4 agonists. While the results from the ongoing clinical trials are pending, there is currently a significant emphasis on the design and development of novel TLR4 immunomodulators.

Although the potential of TLR4 immunomodulation for cancer immunotherapy has not been explored extensively, initial results from pre-clinical and clinical studies look promising. It is reasonable

to imagine a TLR4 immunomodulatory agent that reduces inflammatory response, but promotes anti-tumor immunity. This could be beneficial in controlling multiple stages of cancer. Comprehensive studies are therefore needed to understand the mechanism of action of TLR4 immunomodulators in appropriate *in vitro* and *in vivo* models of cancer.

INFORMATION ABOUT PATENT APPLICATIONS PERTAINING TO TLR4 IMMUNOMODULATION BY SURFACTANT PROTEIN-A (SP-A) DERIVED PEPTIDES

Patent applications have been filed on the concept of TLR4-interacting SP-A peptides for immunomodulation with United States Patent and Trademark Office (USPTO), World Intellectual Property Organization, European, Canadian, and Australian Patent agencies. A patent was recently issued by the USPTO (US 8,623,832; Inventor: Shajana Awasthi; Assigned to the Board of Regents of the University of Oklahoma, Norman, Oklahoma).

REFERENCES

- Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to *Drosophila* Toll. *Proc Natl Acad Sci U S A* (1998) **95**(2):588–93. doi:10.1073/pnas.95.2.588
- Poltorak A, He X, Smirnova I, Liu MY, Van Hufel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in TLR4 gene. *Science* (1998) **282**(5396):2085–8. doi:10.1126/science.282.5396.2085
- Wang F, Meng W, Wang B, Qiao L. *Helicobacter pylori*-induced gastric inflammation and gastric cancer. *Cancer Lett* (2014) **345**(2):196–202. doi:10.1016/j.canlet.2013.08.016
- Xu JH, Fu JJ, Wang XL, Zhu JY, Ye XH, Chen SD. Hepatitis B or C viral infection and risk of pancreatic cancer: a meta-analysis of observational studies. *World J Gastroenterol* (2013) **19**(26):4234–41. doi:10.3748/wjg.v19.i26.4234
- Rogler G. Chronic ulcerative colitis and colorectal cancer. *Cancer Lett* (2014) **345**(2):235–41. doi:10.1016/j.canlet.2013.07.032
- Oblak A, Jerala R. Toll-like receptor 4 activation in cancer progression and therapy. *Clin Dev Immunol* (2011) **2011**:609579. doi:10.1155/2011/609579
- Pradere JP, Dapito DH, Schwabe RF. The Yin and Yang of Toll-like receptors in cancer. *Oncogene* (2013) **33**(27):3485–95. doi:10.1038/onc.2013.302
- Wolska A, Lech-Maranda E, Robak T. Toll-like receptors and their role in carcinogenesis and anti-tumor treatment. *Cell Mol Biol Lett* (2009) **14**(2):248–72. doi:10.2478/s11658-008-0048-z
- Higgins SC, Jarnicki AG, Lavelle EC, Mills KH. TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. *J Immunol* (2006) **177**(11):7980–9. doi:10.4049/jimmunol.177.11.7980
- Kerepesi LA, Hess JA, Leon O, Nolan TJ, Schad GA, Abraham D. Toll-like receptor 4 (TLR4) is required for protective immunity to larval *Strongyloides stercoralis* in mice. *Microbes Infect* (2007) **9**(1):28–34. doi:10.1016/j.micinf.2006.10.003
- Imado T, Iwasaki T, Kitano S, Satake A, Kuroiwa T, Tsunemi S, et al. The protective role of host Toll-like receptor-4 in acute graft-versus-host disease. *Transplantation* (2010) **90**(10):1063–70. doi:10.1097/TP.0b013e3181f86947
- Jordan JM, Woods ME, Olano J, Walker DH. The absence of Toll-like receptor 4 signaling in C3H/HeJ mice predisposes them to overwhelming rickettsial infection and decreased protective Th1 responses. *Infect Immun* (2008) **76**(8):3717–24. doi:10.1128/IAI.00311-08
- Supajatura V, Ushio H, Nakao A, Okumura K, Ra C, Ogawa H. Protective roles of mast cells against enterobacterial infection are mediated by Toll-like receptor 4. *J Immunol* (2001) **167**(4):2250–6. doi:10.4049/jimmunol.167.4.2250
- Nunez NG, Andreani V, Crespo MI, Nocera DA, Breser ML, Moron G, et al. IFN β produced by TLR4-activated tumor cells is involved in improving the antitumoral immune response. *Cancer Res* (2012) **72**(3):592–603. doi:10.1158/0008-5472.CAN-11-0534
- Park BS, Lee JO. Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med* (2013) **45**:e66. doi:10.1038/emm.2013.97
- Le Bon A, Tough DF. Links between innate and adaptive immunity via type I interferon. *Curr Opin Immunol* (2002) **14**(4):432–6. doi:10.1016/S0952-7915(02)00354-0
- Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. *Cytokine* (2008) **42**(2):145–51. doi:10.1016/j.cyto.2008.01.006
- Chen YJ, Hsieh MY, Chang MY, Chen HC, Jan MS, Maa MC, et al. Eps8 protein facilitates phagocytosis by increasing TLR4-MyD88 protein interaction in lipopolysaccharide-stimulated macrophages. *J Biol Chem* (2012) **287**(22):18806–19. doi:10.1074/jbc.M112.340935
- Jain V, Halle A, Halmen KA, Lien E, Charrel-Dennis M, Ram S, et al. Phagocytosis and intracellular killing of MD-2 opsonized gram-negative bacteria depend on TLR4 signaling. *Blood* (2008) **111**(9):4637–45. doi:10.1182/blood-2007-11-126862
- Neal MD, Leaphart C, Levy R, Prince J, Biliar TR, Watkins S, et al. Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. *J Immunol* (2006) **176**(5):3070–9. doi:10.4049/jimmunol.176.5.3070
- Underhill DM, Goodridge HS. Information processing during phagocytosis. *Nat Rev Immunol* (2012) **12**(7):492–502. doi:10.1038/nri3244
- Ni Gabhann J, Spence S, Wynne C, Smith S, Byrne JC, Coffey B, et al. Defects in acute responses to TLR4 in Btk-deficient mice result in impaired dendritic cell-induced IFN-gamma production by natural killer cells. *Clin Immunol* (2012) **142**(3):373–82. doi:10.1016/j.clim.2011.12.009
- Wagner CS, Cresswell P. TLR and nucleotide-binding oligomerization domain-like receptor signals differentially regulate exogenous antigen

- presentation. *J Immunol* (2012) **188**(2):686–93. doi:10.4049/jimmunol.1102214
24. Pufnock JS, Cigal M, Rolczynski LS, Andersen-Nissen E, Wolff M, McElrath MJ, et al. Priming CD8+ T cells with dendritic cells matured using TLR4 and TLR7/8 ligands together enhances generation of CD8+ T cells retaining CD28. *Blood* (2011) **117**(24):6542–51. doi:10.1182/blood-2010-11-317966
25. Park HJ, Qin H, Cha SC, Sharma R, Chung Y, Schluns KS, et al. Induction of TLR4-dependent CD8+ T cell immunity by murine beta-defensin 2 fusion protein vaccines. *Vaccine* (2011) **29**(18):3476–82. doi:10.1016/j.vaccine.2011.02.061
26. Siegmund S, Sauer K. Balancing pro- and anti-inflammatory TLR4 signaling. *Nat Immunol* (2012) **13**(11):1031–3. doi:10.1038/ni.2452
27. Mai CW, Kang YB, Pichika MR. Should a Toll-like receptor4 (TLR-4) agonist or antagonist be designed to treat cancer? TLR-4: its expression and effects in the ten most common cancers. *Oncotargets Ther* (2013) **6**:1573–87. doi:10.2147/OTT.S50838
28. Stevens VL, Hsing AW, Talbot JT, Zheng SL, Sun J, Chen J, et al. Genetic variation in the toll-like receptor gene cluster (TLR10-TLR1-TLR6) and prostate cancer risk. *Int J Cancer* (2008) **123**(11):2644–50. doi:10.1002/ijc.23826
29. Zhang K, Zhou B, Wang Y, Rao L, Zhang L. The TLR4 gene polymorphisms and susceptibility to cancer: a systematic review and meta-analysis. *Eur J Cancer* (2013) **49**(4):946–54. doi:10.1016/j.ejca.2012.09.022
30. Zou TH, Wang ZH, Fang JY. Positive association between Toll-like receptor 4 gene +896A/G polymorphism and susceptibility to gastric carcinogenesis: a meta-analysis. *Tumour Biol* (2013) **34**(4):2441–50. doi:10.1007/s13277-013-0795-y
31. Huang L, Yuan K, Liu J, Ren X, Dong X, Tian W, et al. Polymorphisms of the TLR4 gene and risk of gastric cancer. *Gene* (2014) **537**(1):46–50. doi:10.1016/j.gene.2013.12.030
32. Yang CX, Li CY, Feng W. Toll-like receptor 4 genetic variants and prognosis of breast cancer. *Tissue Antigens* (2013) **81**(4):221–6. doi:10.1111/tan.12096
33. Slattery ML, Herrick JS, Bondurant KL, Wolff RK. Toll-like receptor genes and their association with colon and rectal cancer development and prognosis. *Int J Cancer* (2012) **130**(12):2974–80. doi:10.1002/ijc.26314
34. Ehsan N, Murad S, Ashiq T, Mansoor MU, Gul S, Khalid S, et al. Significant correlation of TLR4 expression with the clinicopathological features of invasive ductal carcinoma of the breast. *Tumour Biol* (2013) **34**(2):1053–9. doi:10.1007/s13277-013-0645-y
35. Wang L, Zhao Y, Qian J, Sun L, Lu Y, Li H, et al. Toll-like receptor-4 signaling in mantle cell lymphoma: effects on tumor growth and immune evasion. *Cancer* (2013) **119**(4):782–91. doi:10.1002/cncr.27792
36. Huang B, Zhao J, Li H, He KL, Chen Y, Chen SH, et al. Toll-like receptors on tumor cells facilitate evasion of immune surveillance. *Cancer Res* (2005) **65**(12):5009–14. doi:10.1158/0008-5472.CAN-05-0784
37. Fu HY, Li C, Yang W, Gai XD, Jia T, Lei YM, et al. FOXP3 and TLR4 protein expression are correlated in non-small cell lung cancer: implications for tumor progression and escape. *Acta Histochem* (2013) **115**(2):151–7. doi:10.1016/j.acthis.2012.06.002
38. Tang X, Zhu Y. TLR4 signaling promotes immune escape of human colon cancer cells by inducing immunosuppressive cytokines and apoptosis resistance. *Oncol Res* (2012) **20**(1):15–24. doi:10.3727/096504012X13425470196092
39. Fukata M, Shang L, Santaolalla R, Sotolongo J, Pastorini C, Espana C, et al. Constitutive activation of epithelial TLR4 augments inflammatory responses to mucosal injury and drives colitis-associated tumorigenesis. *Inflamm Bowel Dis* (2011) **17**(7):1464–73. doi:10.1002/ibd.21527
40. Ikebe M, Kitaura Y, Nakamura M, Tanaka H, Yamasaki A, Nagai S, et al. Lipopolysaccharide (LPS) increases the invasive ability of pancreatic cancer cells through the TLR4/MyD88 signaling pathway. *J Surg Oncol* (2009) **100**(8):725–31. doi:10.1002/jso.21392
41. Liao SJ, Zhou YH, Yuan Y, Li D, Wu FH, Wang Q, et al. Triggering of Toll-like receptor 4 on metastatic breast cancer cells promotes alphavbeta3-mediated adhesion and invasive migration. *Breast Cancer Res Treat* (2012) **133**(3):853–63. doi:10.1007/s10549-011-1844-0
42. Kelsh RM, McKeown-Longo PJ. Topographical changes in extracellular matrix: activation of TLR4 signaling and solid tumor progression. *Trends Cancer Res* (2013) **9**:1–13.
43. Wang L, Zhu R, Huang Z, Li H, Zhu H. Lipopolysaccharide-induced toll-like receptor 4 signaling in cancer cells promotes cell survival and proliferation in hepatocellular carcinoma. *Dig Dis Sci* (2013) **58**(8):2223–36. doi:10.1007/s10620-013-2745-3
44. Yuan X, Zhou Y, Wang W, Li J, Xie G, Zhao Y, et al. Activation of TLR4 signaling promotes gastric cancer progression by inducing mitochondrial ROS production. *Cell Death Dis* (2013) **4**:e794. doi:10.1038/cddis.2013.334
45. Earl TM, Nicoud IB, Pierce JM, Wright JP, Majoras NE, Rubin JE, et al. Silencing of TLR4 decreases liver tumor burden in a murine model of colorectal metastasis and hepatic steatosis. *Ann Surg Oncol* (2009) **16**(4):1043–50. doi:10.1245/s10434-009-0325-8
46. Naseemuddin M, Iqbal A, Nasti TH, Ghandhi JL, Kapadia AD, Yusuf N. Cell mediated immune responses through TLR4 prevents DMBA-induced mammary carcinogenesis in mice. *Int J Cancer* (2012) **130**(4):765–74. doi:10.1002/ijc.26100
47. Ahmed A, Wang JH, Redmond HP. Silencing of TLR4 increases tumor progression and lung metastasis in a murine model of breast cancer. *Ann Surg Oncol* (2013) **20**(Suppl 3):S389–96. doi:10.1245/s10434-012-2595-9
48. Fang H, Ang B, Xu X, Huang X, Wu Y, Sun Y, et al. TLR4 is essential for dendritic cell activation and anti-tumor T-cell response enhancement by DAMPs released from chemically stressed cancer cells. *Cell Mol Immunol* (2014) **11**(2):150–9. doi:10.1038/cmi.2013.59
49. Krieg AM. Toll-free vaccines? *Nat Biotechnol* (2007) **25**(3):303–5. doi:10.1038/nbt0307-303
50. Ishizaka ST, Hawkins LD. E6020: a synthetic Toll-like receptor 4 agonist as a vaccine adjuvant. *Expert Rev Vaccines* (2007) **6**(5):773–84. doi:10.1586/14760584.6.5.773
51. Dumonteil E, Bottazzi ME, Zhan B, Heffernan MJ, Jones K, Valenzuela JG, et al. Accelerating the development of a therapeutic vaccine for human Chagas disease: rationale and prospects. *Expert Rev Vaccines* (2012) **11**(9):1043–55. doi:10.1586/erv.12.85
52. Piazza M, Rossini C, Della Fiorentina S, Pozzi C, Comelli F, Betttoni I, et al. Glycolipids and benzylammonium lipids as novel antisepsis agents: synthesis and biological characterization. *J Med Chem* (2009) **52**(4):1209–13. doi:10.1021/jm801333m
53. Hawkins LD, Ishizaka ST, McGuinness P, Zhang H, Gavin W, DeCosta B, et al. A novel class of endotoxin receptor agonists with simplified structure, toll-like receptor 4-dependent immunostimulatory action, and adjuvant activity. *J Pharmacol Exp Ther* (2002) **300**(2):655–61. doi:10.1124/jpet.300.2.655
54. Savva A, Roger T. Targeting toll-like receptors: promising therapeutic strategies for the management of sepsis-associated pathology and infectious diseases. *Front Immunol* (2013) **4**:387. doi:10.3389/fimmu.2013.00387
55. Leedeboer A, Mahoney JH, Milligan ED, Martin D, Maier SF, Watkins LR. Spinal cord glia and interleukin-1 do not appear to mediate persistent allodynia induced by intramuscular acidic saline in rats. *J Pain* (2006) **7**(10):757–67. doi:10.1016/j.jpain.2006.04.001
56. Gradiash H, Keber MM, Pristovsek P, Jerala R. MD-2 as the target of curcumin in the inhibition of response to LPS. *J Leukoc Biol* (2007) **82**(4):968–74. doi:10.1189/jlb.1206727
57. Hossain DM, Bhattacharyya S, Das T, Sa G. Curcumin: the multi-targeted therapy for cancer regression. *Front Biosci (Schol Ed)* (2012) **4**:335–55. doi:10.2741/S272
58. Anand P, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB. Curcumin and cancer: an “old-age” disease with an “age-old” solution. *Cancer Lett* (2008) **267**(1):133–64. doi:10.1016/j.canlet.2008.03.025
59. Zhu HT, Bian C, Yuan JC, Chu WH, Xiang X, Chen F, et al. Curcumin attenuates acute inflammatory injury by inhibiting the TLR4/MyD88/NF-kappaB signaling pathway in experimental traumatic brain injury. *J Neuroinflammation* (2014) **11**:59. doi:10.1186/1742-2094-11-59
60. Vilekar P, Awasthi S, Natarajan A, Anant S, Awasthi V. EF24 suppresses maturation and inflammatory response in dendritic cells. *Int Immunopharmacol* (2012) **24**(7):455–64. doi:10.1093/intimm/dxr121
61. Ramani V, Madhusoodhanan R, Kosanke S, Awasthi S. A TLR4-interacting SPA4 peptide inhibits LPS-induced lung inflammation. *Innate Immun* (2013) **19**(6):596–610. doi:10.1177/1753425912474851
62. Awasthi S, Brown K, King C, Awasthi V, Bondugula R. A toll-like receptor-4-interacting surfactant protein-A-derived peptide suppresses tumor necrosis factor-alpha release from mouse JAWS II dendritic cells. *J Pharmacol Exp Ther* (2011) **336**(3):672–81. doi:10.1124/jpet.110.173765

63. Madhusoodhanan R, Moriasi C, Ramani V, Anant S, Awasthi S. A TLR4-interacting peptide inhibits lipopolysaccharide-stimulated inflammatory responses, migration and invasion of colon cancer SW480 cells. *Oncoimmunology* (2012) **1**(9):1495–506. doi:10.4161/onci.22089
64. Panaro MA, Carofiglio V, Acquafredda A, Cavallo P, Cianciulli A. Anti-inflammatory effects of resveratrol occur via inhibition of lipopolysaccharide-induced NF-kappaB activation in Caco-2 and SW480 human colon cancer cells. *Br J Nutr* (2012) **108**(9):1623–32. doi:10.1017/S0007114511007227
65. Hodgkinson L. Digestive disease week 2010. Turning science into medicine – part 2. *IDrugs* (2010) **13**(7):424–6.
66. Zhang J, Dou W, Zhang E, Sun A, Ding L, Wei X, et al. Paeoniflorin abrogates DSS-induced colitis via a TLR4-dependent pathway. *Am J Physiol Gastrointest Liver Physiol* (2014) **306**(1):G27–36. doi:10.1152/ajpgi.00465.2012
67. Onier N, Hilpert S, Arnould L, Saint-Giorgio V, Davies JG, Jeannin JF, et al. Cure of colon cancer metastasis in rats with the new lipid A OM 174. Apoptosis of tumor cells and immunization of rats. *Clin Exp Metastasis* (1999) **17**(4):299–306. doi:10.1023/A:1006663017149
68. Garay RP, Viens P, Bauer J, Normier G, Bardou M, Jeannin JF, et al. Cancer relapse under chemotherapy: why TLR2/4 receptor agonists can help. *Eur J Pharmacol* (2007) **563**(1–3):1–17. doi:10.1016/j.ejphar.2007.02.018
69. Cluff CW. Monophosphoryl lipid A (MPL) as an adjuvant for anti-cancer vaccines: clinical results. *Adv Exp Med Biol* (2010) **667**:111–23. doi:10.1007/978-1-4419-1603-7_10
70. Gerard C, Baudson N, Ory T, Louahed J. Tumor mouse model confirms MAGE-A3 cancer immunotherapeutic as an efficient inducer of long-lasting anti-tumoral responses. *PLoS One* (2014) **9**(5):e94883. doi:10.1371/journal.pone.0094883
71. Wang S, Astsaturov IA, Bingham CA, McCarthy KM, von Mehren M, Xu W, et al. Effective antibody therapy induces host-protective antitumor immunity that is augmented by TLR4 agonist treatment. *Cancer Immunol Immunother* (2012) **61**(1):49–61. doi:10.1007/s00262-011-1090-7
72. Davis MB, Vasquez-Dunddel D, Fu J, Albesiano E, Pardoll D, Kim YJ. Intratumoral administration of TLR4 agonist absorbed into a cellular vector improves antitumor responses. *Clin Cancer Res* (2011) **17**(12):3984–92. doi:10.1158/1078-0432.CCR-10-3262
73. Roy A, Singh MS, Upadhyay P, Bhaskar S. Nanoparticle mediated co-delivery of paclitaxel and a TLR-4 agonist results in tumor regression and enhanced immune response in the tumor microenvironment of a mouse model. *Int J Pharm* (2013) **445**(1–2):171–80. doi:10.1016/j.ijpharm.2013.01.045
74. Akeda T, Yamanaka K, Kitagawa H, Kawabata E, Tsuda K, Kakeda M, et al. Intratumoral injection of OK-432 suppresses metastatic squamous cell carcinoma lesion inducing interferon-gamma and tumour necrosis factor-alpha. *Clin Exp Dermatol* (2012) **37**(2):193–4. doi:10.1111/j.1365-2230.2011.04151.x

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