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REVIEW

Advances in Antiviral Therapies Targeting Toll-like Receptors

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ABSTRACT

***Introduction*:** Organisms have evolved a rapid and non-specific way to defend themselves via Toll-like receptors (TLRs), which recognize specific signatures present on invading microbes and viruses. Once detected, these receptors flood the cell with cytokines and IFNs that not only help to eradicate the invading viruses but also activate the adaptive immune response. Owing to difficulties in viral detection, a whole class of TLRs is dedicated to sensing viral nucleic acids, while other TLRs detect viral coat proteins and aid in establishing antiviral immunity. To protect humans better, TLRs and their downstream mediators can be used as potential drug targets, which can be either activated or inhibited, to counter viral infections.

***Areas covered*:** The current review focuses on TLR-targeting investigational drugs developed to treat viral diseases and virus-induced complications.

***Expert opinion*:** TLRs are a good choice for eradicating viral infections because they can fine-tune the immune response. However, TLRs should be exploited carefully, as there have been instances where their activation has led to unwanted responses in terms of both immune and viral activation. Therefore, more focus should be placed on novel drugs that can induce significant and long-term immunity, while concomitantly alleviating side effects.

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1. Introduction

A defense system is vital for any organism to survive in harsh conditions where pathogens are always ready to sabotage the organism. To meet this requirement, evolution has equipped the organisms with sufficient resources and tactics to fend off invading pathogens.[1] Among these, pattern recognition receptors (PRRs) play a vital role in detecting the presence of a pathogen by interacting with their pathogen-associated molecular patterns (PAMPs) and initiating a coordinated network of signaling that initiates the secretion of cytokines and interferons (IFNs),[2] which eliminates the threat. The concept of PAMP-mediated signaling and protection of the host through PRRs was first proposed in 1989 and was later confirmed experimentally in *Drosophila*.[3,4] In humans, PRRs comprise many families, the most notable of which are the Toll-like receptors (TLRs), RIG-1-like receptors (RLR), and Nod-like receptors. TLRs are widely distributed among cell types, vary in their cellular location, and are involved in the detection of almost all PAMPs. TLRs are therefore widely studied in connection with pathogen detection, long-term immunity against pathogens, cancer biology, and other immune disorders.[5,6]

Viruses are the simplest form of organism that cannot replicate or survive without a host, and to fulfill this absolute requirement, they have evolved various strategies and mechanisms to evade and suppress an immune response and utilize the host's components for their replication.[7] However, most viruses can be detected at any stage of their life cycle by PRRs, such as TLRs, and because of this, TLRs can be effectively used to restrict viral infection.[8] In the current review, we will cover the TLRs that detect viral intrusion and mount an antiviral response, with a specific focus on the exploitation of these TLRs by ligands to eradicate viral infections efficiently. Moreover, drugs primarily targeting TLRs that are currently under investigation, or are in the testing phase, will also be discussed to highlight successes in the fight against viral diseases.

1.1. Structure and function of TLRs

TLRs are categorized as type 1 transmembrane proteins that are comprised of three distinct domains: the extracellular *N*-terminal domain, involved in the recognition of PAMPs; a transmembrane domain; and an intracellular Toll-interleukin (IL)-1 like (TIR) domain, which mediates signal-propagation by recruiting multiple other TIR domain-containing proteins.[9] Functional TLRs are variable in humans (10) and in mouse (12), as is their subcellular localization. In humans, TLR1, 2, 4–6, and 10 are functionally active and are present on the cell surface, whereas TLR3 and TLR7–9 are functionally active on endosomes, with the exception of TLR4 that can transmit a signal from both locations [10] (Figure 1).

The binding of a ligand to the ectodomain of a TLR initiates structural changes that allow the respective TLR to homo/heterodimerize. This dimerization conformationally rearranges the TIR-domain such that it becomes able to interact with

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Article highlights

- Viral invasion triggers a multitude of PRRs involving the TLR family to mount an effective, coordinated, and highly organized reaction, leading to a balanced response to counteract a viral challenge through innate and adaptive immunity.
- Besides mounting an effective antiviral response by activating PRRs, viruses adopt different strategies to attenuate their detection by masking themselves or enhancing the degradation of mediator molecules.
- A deeper understanding of the virus–TLR interaction has generated a wealth of information that has been effectively used to limit viral infections.
- TLRs induce an antiviral mechanism to restrict the viral disease, and the same effect can be achieved by employing a TLR agonist, for example, in cases where viruses have hampered the normal functioning of TLRs.
- In some cases, viral infection exploits TLR signaling for their own benefit. This necessitates the negative regulation of TLRs to counter viral pathogenesis.

Novel drugs that target TLRs are necessary to improve the management of viral diseases, since the clinical evaluation of many drugs has been terminated early owing to non-specific effects and safety concerns.

other TIR domain-containing adaptor proteins (TIRAPs) and initiate downstream signaling. The major signaling mediator proteins include myeloid differentiation protein (MyD) 88, TIRAP (also known as MyD88 adapter-like), TIR-domain-containing adapter-inducing INF-β (TRIF), and TRIF-related adaptor molecule. In general, TLRs can transduce a signal through two adaptor proteins, MyD88 and TRIF. TLR3 uses only the TRIF molecule as an adaptor, whereas all other TLRs signal though MyD88, with the exception of TLR4 that can signal using both adaptor proteins (Figure 1). TLR4 signals through MyD88 when present at the cell surface, but when it becomes internalized into endosomes, it is capable of signal induction using TRIF.[11]

Once the TIR-containing proteins have acquired the suitable conformation, they provide a docking site for other adaptor proteins, culminating in a protein–protein interaction network involving IL-1 receptor associated kinase, tumor necrosis factor receptor-associated factor-6 and transforming growth factor (TGF)-β-activated kinase (TAK)-1. This results in the phosphorylation of inhibitor of κB and unleashes nuclear factor (NF)-κB, which usually forms a dimer to induce the gene expression in the nucleus, resulting in inflammation mediated by cytokines such as ILs and TNF-α.[9] All TLRs activate Mitogen activated protein kinases (MAPKs) and NF-κB; however, IRF3 activation is achieved from endosomes by TLR3 and TLR4, while TLR7–9 are involved in IRF7 activation (Figure 1).[12] TLR signaling always culminates in the secretion of inflammatory molecules such as TNF-α, ILs (1, 6, 10, and 12), chemokines, and IFNs, and it modulates cellular physiology including proliferation, immune response, and apoptosis.

2. Viral infections and TLRs

The involvement of viruses in TLR signaling became apparent when it was observed that a respiratory syncytial virus (RSV) protein was able to induce IL-6 from mouse macrophages and for this, intact TLR4 signaling is indispensable.[13] To further reinforce this, it was observed that TLR signaling could be antagonized in cultured cells by proteins originating from the Vaccinia virus (VV).[14] Intracellular TLRs (3, 7–9) and RLRs are specialized for detection of viral nucleic acids,[15–46] whereas extracellular TLRs (1, 2, 4, and 6) detect viral proteins either on an intact viral particle or viral proteins that have been secreted into the extracellular matrix during viral replication [47–54] (Table 1). Unfortunately, it has also been shown that TLR activation can positively influence viral pathogenesis.[55,56]

Viral intrusion triggers a multitude of PRRs involving the TLR family in order to mount an effective antiviral immune response. This coordinated and highly organized reaction involving different receptors leads to a balanced response to counteract the viral challenge through innate and adaptive immunity.[58] Over time, viruses have evolved various ways to either avoid or exploit the human immune system for their own benefits.[59,60] For example, viruses can avoid the cellular response via direct inhibition of RLRs, by disguising the viral particles so as to prevent detection, by targeted inhibition of TIR-domain containing proteins, by hindering inhibitor of κB-kinase protein function, or by inducing a destructive mechanism that will degrade IRFs and prevent subsequent IFN production [61–94] (Figure 1, Table 2).

2.1. TLR2 (in combination with 1 and 6)

TLR2 is expressed by many different cell types, including dendritic cells (DCs), lymphocytes, and macrophages, and its agonists can manifest a variety of effects, including DC maturation, cytokine release from macrophages, and enhanced maturation and activation of B-cells and $CD8^+$ T-cells (CTL).[95,96] Because of these features, TLR2 ligands are particularly valuable for counteracting viruses, such as human cytomegalovirus, (HCMV) Epstein–Barr virus, herpes simplex virus (HSV), VV, RSV, Hepatitis C virus (HCV), and lymphocytic choriomeningitis virus (LCMV), and this antiviral response is generally cell-type dependent.[8] Activation of TLR2 can suppress viral infection; however, in some cases, its activation will actually support viral propagation.

2.2. TLR3

TLR3 is localized to the endosomes, where it senses single-/double-stranded (ss/ds) RNA and DNA viruses [55,56] and induces proinflammatory cytokines, chemokines, and Type 1 IFNs through NF-κB and IRF3, respectively. The dual role of TLR3 in viral infections has been demonstrated in numerous studies that necessitate the development of both activators and inhibitors of TLR3 in antiviral therapies in a context-dependent manner.[8] TLR3 signaling may not be beneficial in all types of viral infection. For example, a reduced viral infection was observed in $TLR3^{-/-}$ mice compared to wild-type mice when challenged with Influenza virus, Punta Toro virus (PTV), and VV.[20,23,24] However, conflicting reports have been published for West Nile virus (WNV), where TLR3 signaling may or may not be beneficial to the host.[18,31]

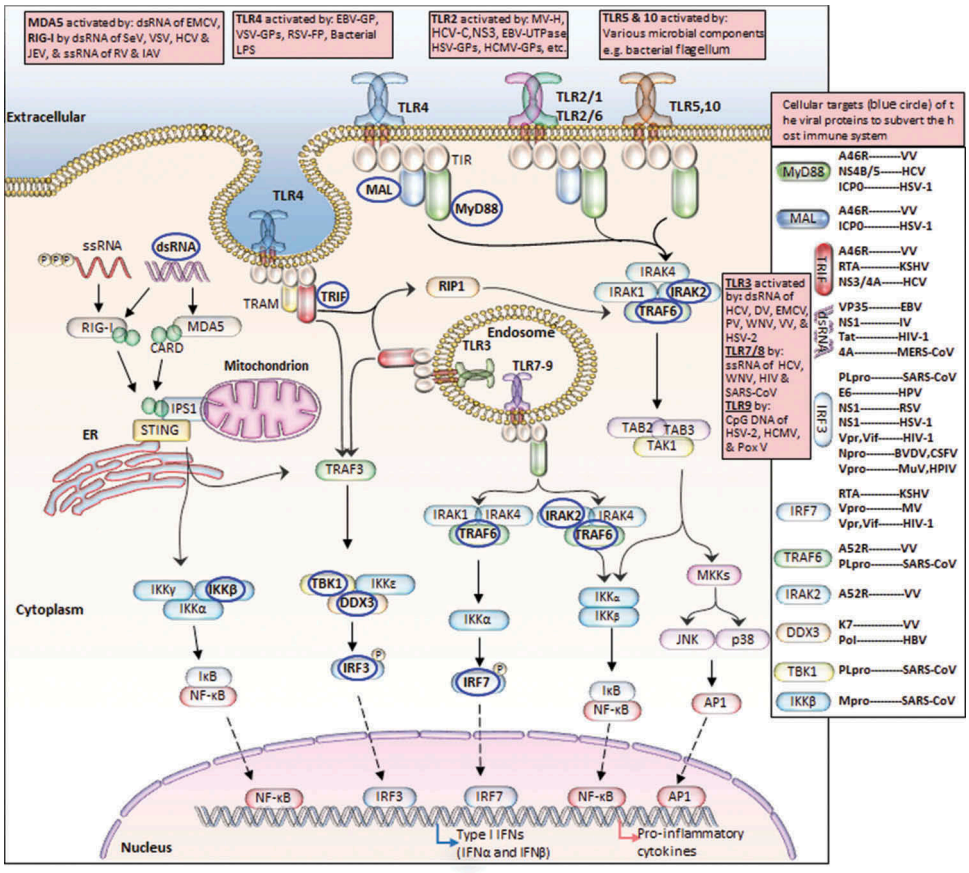

Figure 1. **Toll-like receptor signaling in viral infections.** Different Toll-like receptors (TLRs) are activated when a virus invades the host system. These TLRs sense different signatures, such as glycoproteins, fusion proteins, ss- and ds-RNAs on the viruses (orange boxes) and prepare the organism for fighting back. Primarily, an IFN response is generated by TLR3, 7-9 to attack the invading viruses and protect the uninfected cells. However, other TLRs, such as TLR4, 2/1, 2/6, 5 and 10 also participate in this process to enhance the antiviral response. All TLRs located on plasma membrane activate cytokines through a MyD88-dependent pathway except for TLR4. All endosomal TLRs and TLR4, after endocytosis, activate Type1 IFN induction through a TRIF-dependent pathway. Besides TLRs, RIG1-like receptors, including RIG-I and MDA5, also induce an antiviral response upon detection of viral nucleic acids. Viruses have also acquired specific defense measures to combat the immune response by targeting different cellular proteins, thereby enhancing their persistence and prolonging their pathogenicity. Some cellular proteins, which are targeted by viral-encoded proteins, are listed in the white box, and the mechanisms of their actions are briefly discussed in the text. AP1: Activated protein 1; BVDV; Bovine viral diarrhea virus; CARD: Caspase activation and recruitment domain; CSFV: Classical swine fever virus; DDX3: DEAD (Asp-Glu-Ala-Asp) box protein 3; EBV: Epstein-Barr virus; EMCV: Encephalomyocarditis virus; ER: Endoplasmic reticulum; GP: Glycoprotein; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HPIV: Human parainfluenza virus; HSV: Herpes simplex virus; IAV: Influenza A virus; IFN: Interferon; IKK: Inhibitor of κB-kinase; IPS1: IFN-β promoter stimulator 1; IRAK: Interleukin receptor associated kinase; IRF: interferon regulatory factor; JEV: Japanese encephalitis virus; JNK: c-Jun N-terminal Kinase; MAL: MyD88-adapter-like; MDA5: Melanoma differentiation-associated protein 5; MKK: MAP kinase kinase; MV: Measles virus; MyD88: Myeloid differentiation protein 88; NF-κB: Nuclear factor κ-light-chain-enhancer of activated B; NS: Non-structural; PLpro: Papain-like protease; Pol: Polymerase; RIG-I: Retinoic acid inducible gene-I; RIP1: Receptor-interacting protein 1; RSV: Respiratory syncytial virus; RTA: Replication and transcription activator; RV: Rabies virus; SeV: Sendai virus; STING: stimulator of IFN genes; TAB: TAK1-binding protein; TBK: TANK-binding kinase; TIR: Intracellular Toll-IL-1 like; TRAF: TNF-R-associated factor; TRAM: TRIF-related adaptor molecule; TRIF: TIR-domain-containing adapter-inducing interferon-β; Vpro: Viral protein; VSV: Vesicular stomatitis virus; VV: Vaccinia virus; WNV: West Nile virus.

2.3. TLR4

Among the TLRs, TLR4 has been widely studied since its discovery as a lipopolysaccharide (LPS) receptor, and it has also been shown to recognize viral PAMPs. The first report of TLR4-sensing viral-associated PAMPs was published by Kurt-Jones and colleagues, who discovered that cytokine production by the RSV fusion (F)-protein was TLR4-dependent.[13] It was later shown that, mice deficient in TLR4 showed defective natural killer (NK)-cell functions, IL-12 secretion, and viral clearance compared to wild-type mice.[22] Other viral proteins that initiate the TLR4 pathway include the glycoprotein of the Ebola virus, the mouse mammary tumor virus envelope protein, and the vesicular stomatitis virus glycoprotein G.[19,25,27]

2.4. TLR7/8 and TLR9

TLRs 7 and 8 that are located on the endosomes, are functionally similar, and detect viral origin guanosine-uridine (GU)- and adenosine-uridine (AU)-rich ssRNA sequences.[60] The expression pattern of these TLRs is different, in that TLR7 is expressed less commonly in plasmacytoid DCs (pDCs), macrophages, B-cells, and monocytes, whereas TLR8 is primarily expressed in macrophages, monocytes, and myeloid DCs (mDCs).

TLR9 is constitutively expressed in B-cells and pDCs, and its unique feature is that it recognizes the presence of unmethylated DNA in the microbial genome. When TLR9 comes in contact with unmethylated DNA, it induces the overexpression of Type 1 IFNs, which is a natural antiviral defense that halts viral replication and induces destruction of the infected cells, thus

Table 1. TLRs activation by different viruses and their reported outcomes.

NA	Virus	TLRs activating molecule	Receptor involved	PRR–virus interaction	Ref.
ss(–) RNA	RSV	Fusion protein	TLR4	Beneficial to host	[22]
	RSV	Unknown	TLR2/1,6	Beneficial to host	[52]
	Hanta virus	dsRNA	TLR3	Beneficial to host	[21]
	PTV	dsRNA	TLR3	Harmful to host	[20]
	Influenza	dsRNA	TLR3	Harmful to host	[24]
	Ebola virus	GP	TLR4	Harmful/Protective	[25]
	VSV	GP-G	TLR4	Beneficial to host	[19]
	SeV	dsRNA	RIG-I	Beneficial to host	[42]
	VSV	dsRNA	RIG-I	Beneficial to host	[42]
	RV	5′p genomic ssRNA	RIG-I	Beneficial to host	[40]
	IAV	5′p genomic ssRNA	RIG-I	Beneficial to host	[45]
	MV	Hemagglutinin	TLR2/1,6	Beneficial to host	[54]
ss(+) RNA	HCV	Core and NS3	TLR2/1,6	Harmful to host	[49]
	HCV	dsRNA	TLR3	Beneficial to host	[29]
	Dengue virus	dsRNA	TLR3	Beneficial to host	[30]
	EMCV	dsRNA	TLR3	Beneficial to host	[15]
	Coxsackievirus	dsRNA	TLR3	Beneficial to host	[28]
	Poliovirus	dsRNA	TLR3	Beneficial to host	[17]
	WNV	dsRNA	TLR3	Harmful/Protective	[18,31]
	HCV	ssRNA	TLR7/8	Beneficial to host	[32]
	WNV	ssRNA	TLR7/8	Protective/harmful	[37,57]
	HIV	ssRNA	TLR7/8	Favors viral growth	[35]
	HIV	CpG DNA	TLR9	Beneficial to host	[39]
	HIV-1	gp120	TLR9	Harmful to host	[43]
	SARS-CoV	ssRNA	TLR7/8	Beneficial to host	[44]
	HCV	dsRNA	RIG-I	Protective/Harmful	[42]
	JEV	dsRNA	RIG-I	Beneficial to host	[42]
	EMCV	dsRNA	MDA5	Beneficial to host	[38]
dsRNA	Rotavirus	nsp4	TLR2/1,6	Beneficial to host	[50]
	Rotavirus	dsRNA	TLR3	Beneficial to host	[26]
dsDNA	EBV	dsRNA	TLR3	Beneficial to host	[41]
	EBV	UTPase	TLR2/1,6	Beneficial to host	[47]
	HSV	GP gH/gL and gB	TLR2/1,6	Protective/Harmful	[51,53]
	HSV-2	dsRNA	TLR3	Beneficial to host	[16]
	HCMV	GP B/H	TLR2/1	Beneficial to host	[48]
	VV	dsRNA	TLR3	Harmful to host	[23]
	HSV-2	CpG DNA	TLR9	Beneficial to host	[34]
	HCMV	CpG DNA	TLR9	Beneficial to host	[33]
	Pox virus	CpG DNA	TLR9	Beneficial to host	[36]
	EBV	Small RNAs	RIG-I	Beneficial to host	[46]

EBV: Epstein–Barr virus; EMCV: Encephalomyocarditis virus; GP: Glycoprotein; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HSV: Herpes simplex virus; IAV: Influenza A virus; JEV: Japanese encephalitis virus; MV: Measles virus; NA: Nucleic acid; PRRs: Pathogen recognition receptors; PTV: Punta Toro virus; RSV: Respiratory syncytial virus; RV: Rabies virus; SeV: Sendai virus; VSV: Vesicular stomatitis virus; WNV: West Nile virus.

preventing the spread of virus. TLR9 also plays an important role in viral replication, primarily in HSV, adenovirus, Mouse cytomegalovirus (MCMV), and poxvirus infection.[97,98]

3. Antiviral therapies targeting TLRs

Emerging evidence regarding the functional importance of TLRs has led to the idea that these receptors could potentially be modified to treat inflammatory diseases and cancers; this perspective has been reinforced by informative studies into the positive and negative mediators of TLRs.[99] TLR agonists can mimic the natural ligands but are devoid of accessory molecules, thus generating low molecular weight molecules that can improve pharmacokinetics and pharmacodynamics compared to the parent molecules. These ligands are often used to improve vaccine efficacy and to treat type 1 allergy, cancer, and other infectious diseases (Figure 2A). In addition, TLRs antagonists are also useful in overcoming inflammatory and autoimmune diseases by suppressing excessive inflammation. Therefore, such ligands could potentially dictate a cell-mediated or antibody-based immune response that could alter the disease pathogenesis.

3.1. TLR activation for antiviral therapy

Since TLRs can induce a multitude of inflammatory cytokines and mediators, they play a major role in viral clearance. This has led to the discovery of TLR agonists that can be utilized to control viral infections. Here, we will present recent trends in TLR agonist research and their implications for viral diseases (Table 3).

3.1.1. TLR2 agonists

HSV-2 can predispose an individual to multiple sexually acquired infections in addition to the most prominent symptom of genital ulceration. Induction of the TLR2 pathway by the bacterial-derived ligand FSL-1 can create an antiherpetic environment that can effectively reduce the viral infection without any side effects, even when used at higher doses. [100] The protective effect of another TLR2 ligand, Pam2Cys, has also been evaluated, and it has been observed that intranasal application of this ligand can potentially reduce Influenza A virus (IAV) infection. When this ligand was administered, an immune stimulation was observed, resulting in the expression of IL-2, 6, 10, IFN-γ, monocyte chemo attractant

Table 2. Viruses and their proteins involved in immune evasion in viral diseases.

Virus	Viral protein	Target protein	Mechanism of immune suppression	Ref.
BVDV, CSFV	NPro	IRF3	Ubiquitin-mediated target degradation	[62,64]
Ebola virus	VP35	dsRNA	Impedes RLR signaling	[63]
EV-71	3C	IRF7, TRIF	Proteolytic cleavage of target protein	[67,68]
FMDV	3C	IKKγ	Proteolytic cleavage of IKKγ	[74]
HAV	3CD	TRIF	Cleavage of TRIF by 3CD	[71]
HBV	HBeAg	TIR	Abolishes TIR–TIR interaction	[66]
	Pol	DDX3	Hinders PRRs downstream signaling	[75]
HCV	NS3/4A	TRIF	Performs disintegration of TRIF	[69]
	NS4B/5	MyD88	Halts signaling propagation	[61]
HPV	E6	IRF3	Hinders activation	[72]
HSV-1	ICP0	MyD88, MAL	Disintegration of target proteins	[73]
	NS1	IRF3	Interacts with CBP	[70]
IAV	NS1	dsRNA	Impedes RLR signaling	[65]
KSHV	RTA	TRIF, IRF7	Disintegrates target proteins	[77]
	IRF Homologs	Inhibits IRFs	Suppress production of IFNs	[83]
HIV-1	Tat	dsRNA	Impedes RLR signaling	[93]
	Vpr, Vif	IRF3, IRF7	Attenuation of IFN production	[88]
MV	V Protein	Mimics IRF7	Pseudo-substrate of IKKα	[89]
MERS-CoV	NS4A	dsRNA	Impairs RLR signaling	[87]
	M, NS4A/4B/5	Multiple	Suppression of IFN production and ISRE promoter element signaling pathways	[94]
	PLpro	dsRNA	Enhance deubiquitination of target proteins	[86]
Mumps virus, HPIV-2/5	V Protein	Mimics IRF3	Pseudo-substrate for IKKε and TBK1 to prevent IFN production	[84]
RSV	NS1	IRF3	IRF3 mediated IFN-β production suppression	[90]
SARS-CoV	PLpro	IRF3	Hinders activation	[80]
	PLpro	TRAF6, TBK1	Removal of ubiquitin tag from target protein	[92]
	M Protein	IKKβ	Impairs NF-κB activation	[81]
VV	A46R	MyD88, TRIF, TRAM, MAL	Interrupt signaling mediators	[91]
	A52R	TRAF6, IRAK2	Hinders the functions of intermediate molecules	[82]
	A49	Mimics IκBα	Prevention of β-TrCP activity	[85]
	K7	DDX3	Inhibit the DDX3's activity	[79]
	E3L	Multiple	Modulation of immune related proteins	[78]

BVDV: Bovine viral diarrhea virus; CARD: Caspase activation and recruitment domain; CSFV: Classical swine fever virus; EBV: Epstein–Barr virus; EMCV: Encephalomyocarditis virus; EV: Enterovirus; FMDV: Foot-and-mouth disease virus; HAV: Hepatitis A virus; HBeAg: Hepatitis B virus e antigen; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HSV: Herpes simplex virus; IAV: Influenza A virus; IKK: Inhibitor of κB-kinase; IPS1: IFN-β promoter stimulator 1; IRAK: Interleukin receptor associated kinase; IRF: Interferon regulatory factor; JEV: Japanese encephalitis virus; MAL: MyD88-adapter-like; MKK: MAPK kinase; MV: Measles virus; MyD88: Myeloid differentiation protein 88; NF-κB: Nuclear factor κB; NPro: Nonstructural protein; NS: Non-structural; PLpro: Papain-like protease; Pol: Polymerase; RIP1: Receptor-interacting protein 1; RSV: Respiratory syncytial virus; RV: Rabies Virus; SeV: Sendai virus; STING: Stimulator of IFN genes; TAB: TAK1-binding protein; TBK: TANK-binding kinase; TIR: Intracellular Toll-IL-1 like; TRAF: TNF-R-associated factor; TRAM: TRIF-related adaptor molecule; TRIF: TIR-domain-containing adapter-inducing interferon-β; VV: Vaccinia virus; WNV: West Nile virus.

protein-1, and TNF-α, leading to an effective anti-influenza response.[101] Moreover, superinfection in IAV is a major cause of morbidity, in particular by *Pneumococcal pneumonia*, which can be effectively suppressed by macrophage-activating lipopeptide-2 (MALP-2). This ligand was partly derived from *Mycoplasma fermentans* and acts as an agonist of TLR2.[106] Intratracheal application of MALP-2 showed beneficial effects, with negligible side effects. Recently, one potent TLR1/2 agonist CU-T12-9 has been developed, which enhances TLR1 and TRL2 dimerization, activates the NF-κB pathway and upregulates proinflammatory cytokines *in vitro*.[102]

3.1.2. TLR2 adjuvants

A palmitic acid-conjugated Hepatitis B virus (HBV) vaccine (composed of HBV c-antigen + a palmitoylated helper T lymphocyte peptide) has been tested in chronic HBV-infected patients that showed good results.[107] Palmitic acid-conjugated HIV vaccine has also been evaluated in phase I and II clinical trials,[108] which unfortunately failed to boost HIV-specific CTL responses in HIV patients.[109,110] Multiple TLR2-targeting compounds conjugated with viral-peptide vaccines have been developed, which are still under clinical evaluation (Table 4).

3.1.3. TLR3 agonists

A synthetic mimic of dsRNA, polyriboinosinic:polyribocytidylic acid (poly[I:C]), is a TLR3-agonist [123] that has been widely used as a protective immune stimulant against HBV, IAV, coronaviruses, and some HIV strains.[124] It was also shown that when poly(I:C) was given to elderly mice through nasal route, it substantially counteracted a lethal dose of the Severe acquired respiratory syndrome (SARS) coronavirus and improved the animals' survival.[125] In addition to the induction of IFNs and proinflammatory cytokines, multiple pathways of adaptive immunity are initiated by poly(I:C), including virus-specific T-cell responses, NK cell cytotoxicity, and DC maturation, through TLR3, Melanoma differentiation-associated protein 5 (MDA5), and RIG1,[126–128] thus providing an attractive approach for DC-based vaccines to stimulate T-cell-mediated immune response. Poly(I:C12U) is a derivative of poly(I:C), in which uridylic acid is added (at a molar ratio of 12:1) during polycytidylic acid production. It has been shown to be safer than poly(I:C), and is also able to effectively induce an innate immune response; however, it could not protect $TLR3^{-/-}$ mice when they were challenged with PTV, nor did the mice produce IFN and IL-6, confirming its TLR3-dependent activity.[129] The efficacy of poly(I:C12U) is also currently being evaluated in HIV, HBV, influenza, and HCV infections. Another variant of poly(I:C), poly-ICLC, which consists of Poly(I:C), poly-L-lysine, and carboxymethyl cellulose, showed an improved safety profile and a broad spectrum of protection against avian-IAV, RSV, and SARS viruses.[130] Another study reported poly-ICLC-induced lung inflammation in a cotton rat model when an intranasal dose was administered.[131] In cases of sub-cutaneous administration in humans, poly-

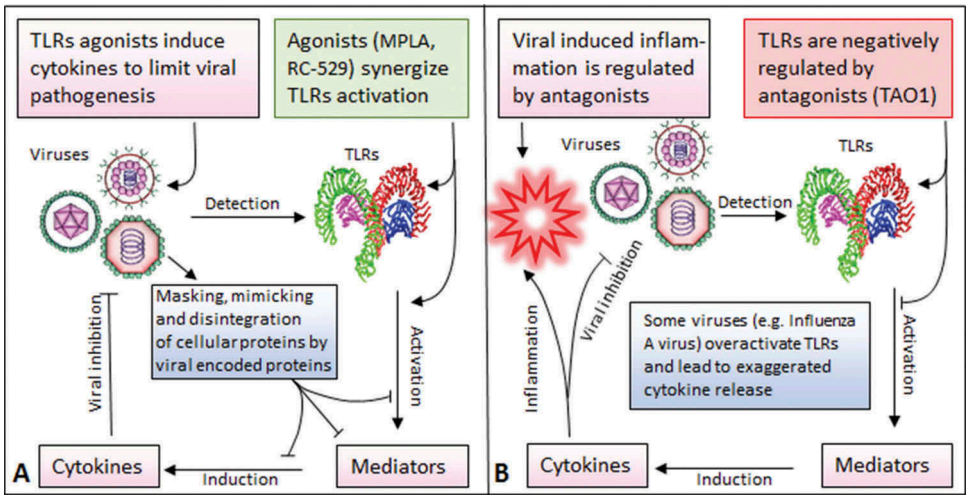

Figure 2. Mechanisms of TLRs targeting antiviral drugs and virus-mediated TLR activation and subversion. An antiviral innate immune response is initiated in cells when macromolecules (nucleic acids or proteins) of viruses are detected by TLRs. Cytokines, the ultimate result of TLR activation, help to restrict the viral infection as well as to activate adaptive immunity. Viruses also utilize their nonstructural proteins to enhance their pathogenesis after engaging a variety of cellular proteins in different ways, such as masking, disintegration, and mimicking. (**A**) TLR agonists reinforce the canonical cycle of viral-mediated cytokine induction to aid in clearing a viral infection. (**B**) TLRs have been negatively targeted to overcome viral-induced exaggerated inflammation to help prevent the development of inflammatory diseases.

Table 3. Promising compounds that activate TLRs to control viral infection.

TLR	Compound	Virus	Indication	Status	Ref; NCT
TLR2/ 1,6	FSL-1	HSV-2	Creates antiherpetic environment in mice vagina	Preclinical	[100]
	Pam2Cys	IAV	Activates TLR2 signaling	Preclinical	[101]
	CU-T12-9	–	Activates TLR2 signaling	Preclinical	[102]
TLR3	Poly(I:C12U)	HIV, IAV	Treatment of the listed viral infections (Ampligen)	Under CT	Hemispherx Biopharma
	Poly-ICLC or Hiltonol	HIV-1	To treat human HIV infection	Phase 1	Oncovir, Inc.; NCT02071095
	PIKA	IAV	Stimulation of innate immunity against influenza A virus	Preclinical	[103]
TLR4	MPL	HPV, HBV	Vaccine against HPV (Cervarix) and HBV (Fendrix)	Approved	GSK
	RC-529	HBV	Vaccination against HBV (Supervax)	Approved	Dynavax
	FimH	IAV	Stimulation of innate immunity against IAV	Preclinical	[76]
TLR7/8	Imiquimod	HPV	Topical treatment for HPV-induced warts	Approved	3M Pharma
	Resiquimod	HSV, HCV	Used topically for the treatment of genital HSV	Phase III*	3M Pharma
	CL097	HIV-1	Restore defective cytokine secretion in HIV infection	Preclinical	[104,105]
	PF-04878691	HCV, HPV	Developed for treatment against HCV and cervical cancer	Phase I (completed)	Pfizer; NCT00810758
	852A	HPV	Developed for treatment against cervical cancer	Phase II	NCT00319748
	RO6864018	HBV	To treat patients with chronic HBV infection	Phase II	NCT02391805
	ANA975	HCV	HCV treatment	Phase I*	Anadys
	ANA773	HCV	HCV treatment	Phase IIa	Anadys
	GS9620	HBV, HCV	For the treatment of HBV and HCV	Phase II	Gilead Sciences
TLR9	CPG10101	HCV	Treatment of chronic HCV infection	Phase II completed	Coley Pharma and Pfizer
	IMO-2125	HCV	Treatment of HCV infection	Phase I completed	Idera Pharma; NCT00728936
	MGN1706	HIV-1	TLR9 enhancement of antiviral immunity in chronic HIV-1 infection	Phase I/II	NCT02443935
	SD-101	HCV	Treatment of chronic HCV infection	Phase I completed	Dynavax; NCT00823862
	1018 ISS (HEPLISAV)	HBV	HBV	Phase III	Dynavax

*: Study suspended; EBV: Epstein–Barr virus; HAV: Hepatitis A virus; HBsAg: Hepatitis B virus surface antigen; CT: Clinical trial; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HSV: Herpes simplex virus; IAV: Influenza A virus; JEV: Japanese encephalitis virus; MPL: Monophosphoryl lipid A; MV: Measles virus; NCT: Clinical trial number; RSV: Respiratory syncytial virus; RV: Rabies virus; SeV: Sendai virus; VV: Vaccinia virus; WNV: West Nile virus.

ICLC produced immune responses of same level as that of a live viral vaccine.[132] It has also been shown to be therapeutically safe in a number of clinical trials, where it was either used alone or as an adjuvant. A derivative of poly(I:C), PIKA has found to reduce the viral loads of various influenza viruses in lungs.[103]

3.1.4. TLR3 adjuvants

Poly(I:C) has been used as an adjuvant in various experimental vaccine models. PIKA has been found to be an effective and potent adjuvant against HBV, where it assists in activating both lines of immunity.[133] Poly(I:C) inclusion in HIV vaccines has shown development of MHC class I-restricted $CD8^+$ cells *in vivo*,[112] and enhanced $CD4^+$ T-cell-mediated immunity.[113] Intranasal immunization with poly(I:C12U) as an adjuvant in a hemagglutinin (HA)-based H5N1 influenza vaccine induces higher levels of protective, specific mucosal IgA, and systemic IgG responses than the corresponding adjuvant-free vaccine.[111] A combined poly(I:C)-CAF01 (CAF01 itself is a liposome-based adjuvant)

Table 4. Promising clinical, preclinical TLR adjuvants used to treat viral diseases.

TLR	Adjuvants	Virus	Indication	Status	Ref; NCT
TLR2/ 1,6	Lipo-6+/− QS-21	HIV-1	HIV-specific B & T-cell responses in volunteers	Phase I/II	[108]
	Lipo-6T + IL-2/vCP1433	HIV-1	No impact on HIV-specific $CD4^+$ T-cells	Phase II	[109]
	Lipo-4, Lipo-6	HIV	72% (CD4) & 57% (CD8) response in HIV patients	Phase I & II	[110]
	Theradigm-HBV	HBV	HBV-specific CTL response	Phase I	[107]
TLR3	PIKA + HBsAg	HBV	Enhances B & T-cell responses in HBV infected mice	Clinical	[103]
	Poly(I:C) + Entecavir	HBV	Treatment of chronic hepatitis B	Phase IV	NCT02532413
	Poly(I:C12U) + H5N1	H5N1	Protected mice against H5N1 influenza virus	Phase III	[111]
	Poly(I:C) + HIV gp120	HIV-1	Development of MHC class I-restricted $CD8^+$ cells	Preclinical	[112,113]
	Poly(I:C) + CAF01	Multi	T- & B-cell responses in bacterial, viral & parasitic infections	Preclinical	[114,115]
TLR4	HBsAg + MPL	HBV	Enhances the efficacy of HBV vaccine	Approved	GSK
	HIV-1 vaccines + RC-529 (MPL)	HIV-1	Enhances immunogenicity of an HIV CTL multi-epitope peptide vaccine	Phase I	Pfizer, NCT00195234
	HPV-vaccines + Alum + RC-529	HPV	Prophylactic effect against hrHPV species, HPV16 and 18	Approved	GSK
	HBsAg + alum + RC-529	HBV	Seroprotection against HBV	Phase III	[116]
	AS02 (MPL + QS21) + HbsAg	HBV	Seroprotection against HBV	Phase I	[117]
	AS04 + gp350	EBV	Produce anti-gp350 antibodies	Phase II	[118]
TLR5	VAX125	IAV	Provide immunity against influenza viral strains	Phase II	VaxInnate NCT00966238
	VAX-102	IAV	Provide immunity against the IAV M2e antigen	Phase I	NCT00921206
	rGP5 + STF2 PRRSV		Capable of activating the innate immune response	Preclinical	[119]
TLR7/8	3M-012 + HIV-Gag	HIV	Enhanced the T helper 1 response	Preclinical	[120]
TLR9	HIV-1 + HYB2055	HIV-1	Therapeutic against HIV	Phase II	Idera/Immune Response Corp
	Pneumococcal vaccines + CpG 7909	HIV	Pneumococcal vaccination in HIV-infected adults	Phase II	NCT00562939
	HBsAg + CpG 1018	HBV	Enhance the efficacy of HBV vaccine	Phase III	Dynavax NCT00435812
	HBsAg + CpG 7909	HBV	Seroprotection achieved against HBV	Phase I/III	[121]
	Fluarix + CpG 7909	IAV	Immunity against influenza virus	Phase I	[122]

CT: Clinical trial; EBV: Epstein–Barr virus; HBsAg: Hepatitis B virus surface antigen; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HSV: Herpes simplex virus; IAV: Influenza A virus; JEV: Japanese encephalitis virus; MPL: Monophosphoryl lipid A; MV: Measles virus; NCT: Clinical trial number; PRRSV: Porcine reproductive and respiratory syndrome virus; RSV: Respiratory syncytial virus; RV: Rabies virus; SeV: Sendai virus; Theradigm-HBV; Palmitoylated CTL epitope (HBV core peptide) covalently linked to a HTL epitope (tetanus toxin peptide); VV: Vaccinia virus; WNV: West Nile virus.

adjuvant, called CAF05, favored T-cells and antibody responses in bacterial, viral, and parasitic infected animal models.[114,115]

3.1.5. TLR4 agonists

Monophosphoryl lipid A (MPL), is derived from *Salmonella minnesota* LPS, following a few chemical alterations that successfully complemented its adjuvant activity and showed negligible toxicity (~0.1%) compared to that of the parent LPS molecule. MPL can significantly mature human DCs, and upregulate leukocyte antigen-DR, CD40, CD80, CD86, and an activation marker, CD83.[134] Another derivative of LPS, RC529, has become a leading candidate for human vaccine adjuvant and shown great promise. RC529 activates TLR4 pathway in the same pattern as LPS and MPL, which results in the upregulation of chemokines, cytokines, and cell-surface co-stimulatory molecules. The upregulation of cytokine, IL-1, IL-6, IL-8, IL-10, and TNF-α were similar in peripheral blood mononuclear cells (PBMC) when treated with MPL and RC529.[135] Fimbriae H protein is a novel TLR4 ligand that can stimulate the innate immune system and elicit a protective response against influenza virus infections.[76]

3.1.6. TLR4 adjuvants

The primary focus of the worldwide public health field is to create safe and effective vaccines that can generate antibodies against multiple variants of viruses, and are capable of long-term immunity. This can be accomplished by supplementing vaccines with adjuvants. Aluminum hydroxide (alum) was the only choice for more than half a century because of its safety. However, the use of alum is not ideal due to its Th2-biased responses, instead of a Th1 response that primarily activates antibacterial and antiviral immunity.[136] Currently, new ways are being perused to design adjuvants that can provide effective, and durable humoral and T-cell mediated immunity with fewer side effects.

A combination of MPL plus alum has been used in HBV and Human papilloma virus (HPV) vaccines,[137] whereas combinations of MPL with liposomes, saponins, and emulsions have been evaluated clinically.[138] When used in vaccines, MPL has been shown to improve the duration and intensity of the adaptive immune response.[139] MPL has been combined with a variety of other adjuvants developed by GlaxoSmithKline, such as AS01 (liposomes) + QS21, AS02 (oil-in-water emulsion) + QS21, and AS04 (alum) [116–118] (Table 4). All these conjugates, AS01, AS02, and AS04 induce TLR4-dependent NF-κB activity and cytokine secretion, due to the activity of MPL.[140,141]

A previously rejected vaccine, FI-RSV, was tested on a cotton rat model that recapitulates the natural viral infection as seen in immunized children.[142,143] Upon RSV challenge, the cotton rats showed improved lung pathophysiology and a reduced viral load when immunized with FI-RSV plus MPL, compared to the experimental set of rats that were immunized with FI-RSV alone.[144,145] MPL also resulted in a Th1-skewed immune response when combined with nucleocapsid-depleted RSV membranes. Application of mucosal F1-RSV-MPL virosomes proved to be better at a live virus challenge, further

supporting the value of antiviral vaccines.[146] Finally, when F1-RSV protein was combined with MPL and administered initially intranasally and subsequently intradermally, it gave a protective effect with the fewest side effects.[147] However, when these clinical trials were extended to humans, a formalin-inactivated RSV vaccine actually worsened the disease condition when recipients acquired natural virus infection, leading to the demise of two recipients. Subunit and live-attenuated RSV vaccines were then considered as alternatives. However, subunit RSV vaccine generated type-2 symptoms and responses when used in a natural RSV infection treatment, whereas the attenuated viral vaccines displayed poor immunogenicity, residual virulence, and genetic instability [148,149]

3.1.7. TLR5 agonists and adjuvants

TLR5 is dedicated to detecting flagella during bacterial infection and helping to trigger immunity when provided with a good adjuvant target. Data regarding the involvement of TLR5 in viral detection and immune response are scarce. However, it can trigger an antiviral immune response and has been tested as adjuvant in IAV vaccine (Table 4). Genetically stable influenza A matrix2 protein (M2e) is a good candidate vaccine, but its immunogenicity is poor. When fused with the TLR5 agonist *Salmonella typhimurium fljB* flagellin (STF2), the fusion protein (M2e + STF2 = VAX-102) showed a potent antibody response. TLR5 agonists can also protect bone marrow transplant recipients from contracting cytomegalovirus (CMV) infection. VAX125, a recombinant fusion protein of STF2.HA1 Solomon Islands (SI) (which consists of STF2, fused at its C-terminus to HA antigen of influenza A HA1 SI) has been developed to enhance HA-antibodies in IAV patients.[150] Furthermore, by activating immunity through TLR5 using STF2 fused with Porcine reproductive and respiratory syndrome virus (PRRSV)-GP5 can protect pigs from respiratory syndrome, which may represent a novel adjuvant for human therapies.[119] Activation of TLR5 with CBLB502 (recombinant flagellin) can significantly protect mice from a subsequent MCMV challenge, as indicated by a reduced viral load and activation of cytotoxic NK cells.[151]

3.1.8. TLR7/8 agonists

TLR7 and TLR8 can be triggered by low molecular weight synthetic compounds imidazoquinolines and exhibit a strong antiviral activity.[152] Different family members differentially modulate TLR7 and TLR8 in different cell types, resulting in a variety of inflammatory profiles. TLR7 agonists, for example in human PBMCs, tend to induce Type 1 IFN and IFN-stimulated cytokines, whereas the agonists that specifically activate TLR8 generate inflammatory cytokines and chemokines that include IL-12 and TNF-α.[153]

Imiquimod, a member of the imidazoquinoline family, was developed to treat HPV-induced genital and perianal warts when applied topically. Its immunomodulatory potential was initially manifested by its interaction with TLR7 and the production of Type 1 IFN and other cytokines.[145] It was also approved for treating external genital warts, different types of skin malignancies, and it also exhibited variable efficiency in treating HSV.[154] Resiquimode, a TLR7/8 agonist, has been implicated in the oral treatment of HCV and in the topical treatment of HSV; however, the results were not convincing due to a loss of efficiency at low doses and profound unwanted side effects at higher doses.[154] In HIV infection, resiquimod was shown to limit HIV growth in cultured human monocytes.[155] CL097 is a TLR7/8 agonist that was shown to restore defective cytokine production from mDCs by triggering TLR7/8 from HIV-infected pregnant women.[104] PBMCs secreted a significant amount of granulocyte-colony stimulating factor after CL097 treatment, which may provide a new avenue for the treatment of neutropenia caused by IFN-α treatment of chronic HCV.[105] PF-04878691 has been developed as a potent TLR7 agonist, and its safety and tolerability were evaluated for HCV treatment in healthy volunteers. It was found that PF-04878691 induced immune biomarkers and a Type 1 IFN response in a dose-dependent manner. However, in a few volunteers, it caused hypotension, lymphopenia, and influenza-like symptoms, forcing investigators to terminate the study.[156] *In silico* techniques such as modeling and simulation were then conducted, and unfavorable results led to the recommendation that PF-04878691 be discontinued for HCV treatment.[157]

Isatoribine and its derivatives are guanosine analogs that also activate TLR7 and have been used for HCV treatment, where they were shown to significantly reduce the viral titer in the serum. Later, a prodrug, ANA795, that can be administered orally, was developed to overcome the gastrointestinal-based side effects of isatoribine. This prodrug showed better results, and it is efficiently absorbed into the blood stream, providing a better peak-plasma concentration than isatoribine and reducing the plasma RNA level of HCV.[158] However, it showed undesirable toxicity in animal studies that halted further testing. ANA773 is another prodrug of isatoribine that can be given orally and gave efficacious results in Type 1 IFN production with the fewest side effects and an appropriate tolerability in preclinical studies. Furthermore, in chronic HCV patients, ANA773 caused an increase in IFN production and a decrease in serum HCV RNA levels.[159] A transient drop in mDC and pDC levels was evident in HCV patients undergoing continuous treatment with ANA773, whereas a subset of patients who also responded to the drug showed reduced levels of HCV RNA and increased levels of IFN-α and IP-10.[160] 8-hydroxyadenine, which is structurally similar to isatoribine, and its derivatives were also investigated and higher IFN production was observed. In particular, oral administration of SM-276001 was successful at inducing IFN in monkeys and in mice, with superior efficiency to that of resiquimod.[161]

GS9620 is a specific TLR7 agonist that has been shown to be effective when administered orally. In preliminary studies, it caused a drastic reduction in HBV DNA and IFN-mediated innate immune activation in a chimpanzee model.[162] Furthermore, in preclinical trials that included double-blind studies and a placebo treatment, GS9620 was adequately absorbed, well-tolerated, and was very efficient at IFN and cytokine induction, all of which were achieved with doses well below the toxic concentration.[163] Due to these favorable results, GS962 has been the subject of further clinical trials for the treatment of HBV and HCV.

3.1.9. TLR7/8 adjuvants

The vaccine adjuvants of TLR7 and TLR8 agonists has not been developed yet; however, imiquimod and resiquimod have been extensively used in combination with topical cream used for HPV-induced warts, basal and squamous cell carcinoma, and actinic keratosis.[164] The TLR7/8 agonist, 3M-012, has been conjugated with HIV-Gag, this has dramatically enhanced the T helper 1 response [120] (Table 4).

3.1.10. TLR9 agonists

The specific molecules that initiate TLR9 signaling are DNA-based molecules including CPG10101, CPG7909, SD-101, and Immunomodulatory Oligonucleotide (IMO)-2125. DNA molecules with a canonical backbone are prone to degradation by cellular nucleases. However, this problem can be eliminated by replacing the phosphodiester bonds with phosphorothioate bonds. Pertinent to HIV, B-cells showed impaired TLR9 expression, leading to a defective immune response from B-cells, but treatment with CpG oligonucleotides (ODNs) restored and enhanced the B-cell immune response. [165] TLR9 agonist, CPG10101, showed encouraging effects in trials conducted on chronic HCV patients with tolerable side effects.[166] In a subsequent Phase Ib multicenter trial, CPG10101 activated the immune system in a dose-dependent manner and also reduced HCV RNA levels.[167]

Synthetic DNA analogs have been developed to meet the requirements of stability and broad-spectrum immune response. Such synthetic DNAs are known as IMOs that contain the dinucleotide immunostimulatory motifs CpR and/or RpG, where R is a synthetic analog of natural bases.[168,169] These IMOs have demonstrated superior metabolic stability, are safer to use, and can differentially induce cytokines and chemokines, as determined by the composition of the dinucleotide bases. One example is IMO-2125, which established an IFN response in animal models and hindered viral growth in clinical trials involving HCV patients. Further development lead to the synthesis of a next-generation TLR9 agonist, SD-101, which stimulated the secretion of IFN-α and IFN-λ up to 20-fold in human PBMCs. In a clinical trial involving HCV patients, it was shown that SD-101 was better-tolerated, produced greater effects, and reduced viral growth, all of which are essential for the treatment of viral diseases such as HCV.

3.1.11. TLR9 adjuvants

CpG ODN treatment triggers a Th1-type immune response through activation of TLR9. Therefore, CpG ODNs have been widely tested as an adjuvant for stimulating the immune system in various antiviral vaccines such as IAV, HIV, HBV, and in some cancers [121,122,164] (Table 4).

3.2. TLR inhibition for antiviral therapy

As it was discussed in the previous section, TLR activation is typically required to control viral infection; however, there are many conditions where TLRs activation actually worsen the disease pathology or make a patient prone to developing other serious conditions. Clearly, in these instances, it would be desirable to inhibit rather than induce the TLR signaling, and this can be achieved through the use of TLR antagonists (Figure 2B). In this section, we will cover TLR inhibitors and their implication in viral therapies [170–180] (Table 5).

3.2.1. TLR2 antagonists

The TLR2/1 heterodimer plays an important role in the regulation of innate immunity and provides an attractive target for the treatment of TLR2-related immune disorders. A novel compound, CU-CPT22, has been proven to specifically antagonize the effect of TLR2/1 and to repress TNF-α and IL-1β signaling in different immune disorders.[171] A high-throughput screening approach was employed to screen TLR2 antagonists, and compound E567 was found to efficiently inhibit the cytokine production induced by LCMV and HSV-1 in both human and mouse cell cultures.[181]

3.2.2. TLR3 antagonists

Negative regulation of TLR3 is desirable when its over-activation causes unwanted effects, as has been seen in numerous viral infections.[23,24] One promising method for blocking TLR3 activation is to hinder the interaction of poly(I:C) with

Table 5. Compounds that antagonize TLRs to control viral infection.

TLR	Compound	Virus	Indication	Status	Ref; NCT
TLR2/1	CU-CPT22	ND	TLR2/1 inhibitor	Preclinical	[171]
	E567	LCMV, HSV1	Inhibit LCMV, HSV1 infection	Preclinical	[181]
TLR3	TAO1	IAV	Treatment of viral upper respiratory tract infections	Phase II	NCT01651715
	CU-CPT4a	ND	Suppresses downstream signaling pathways of TLR3/dsRNA complex	Preclinical	[170]
TLR4	E5564	IAV	Controls inflammation associated with influenza	Preclinical	Eisai, Inc.
	TAK-242	ND	Septic shock	Phase III*	Akeda Pharma
	MUC1	ND	NSCLC	Phase III	Biomira/Merck
	OSL07	ND	Inhibition of TLR4 homodimerization	Preclinical	[175]
	SPA4	Lungs inflammation	SPA4 peptide inhibits LPS-induced lung inflammation	Preclinical	[176]
	STM28	TLR4 inhibitor	Inhibits LPS-induced activation of NF-κB and *in vivo* toxicity	Preclinical	[179]
TLR5	TH1020	ND	Antagonize TLR5 signaling	Preclinical	[180]
TLR7-9	2′-O-methyl-modified RNA	ND	Reduce IFN and cytokine production in TLR7 signaling	Preclinical	[177,178]
	ODNs antagonists	Inflammation	Inhibition of TLR7/8/9- pathways	Preclinical	[172]
	IRS-954 (DV-1079)	Autoimmune	Inhibition of TLR7 and TLR9	Preclinical	Dynavax/GSK
	CpG 52364	Autoimmune	TLR7 and 9 antagonist	Phase I	Coley Pharma
	E6446,AT791	Autoimmune	TLR7 and 9 antagonist	Preclinical	[173]
	CpG-ODN c41	Inflammation	Blocks TLR9 pathway	Preclinical	[174]

*:Study suspended; IAV: Influenza A virus; IFN: Interferon; LCMV: Lymphocytic choriomeningitis virus; LPS: Lipopolysaccharide; NCT: Clinical trial number; ND: Not define; NSCLC: Non-small cell lung cancer; NF-κB: Nuclear factor κB; ODNs: Oligonucleotide.

its receptor, and this has been successfully accomplished using ssDNA ODNs in PBMCs, epithelial cells, and DCs. [182,183] The ssDNA ODNs blocked poly(I:C)-induced cytokine expression when administered intranasally in *Macaca fascicularis*.[183] Recently, a study was carried out to identify small molecules that inhibit the binding of dsRNA to TLR3, one of these compounds, CU-CPT4a, showed a high binding affinity and specificity to TLR3.[170] TAO1, a medicinal product (containing antibodies (anti-TLR3) against a synthetic peptide selected from TLR3, is under clinical trials for treatment of viral upper respiratory tract infections such as common cold, influenza, or influenza-like illnesses (Table5). These studies have provided the basis for new therapeutic interventions for the treatment of aggravated inflammatory conditions involving TLR3.

3.2.3. TLR4 antagonists

TLR4 is a crucial component of innate immunity. It can signal through two pathways and therefore mounts a severe inflammatory response, when, for instance, LPS binds to TLR4.[175] The entire LPS structure is not required for toxicity, in fact the lipid A (LA) part is sufficient for signaling, making it an ideal target for drug development and therapy. Based on their structural similarity, LA antagonists have been derived from *Rhodobacter capsulatus* (E5531) and Eritoran tetrasodium (E5564) derived from *R. sphaeroides*. Both of these compounds showed protection against a gram-negative challenge in experimental models, and demonstrated an LPS-induced reduction in inflammation.[184] E5564 can occupy up to 90% of the hydrophobic pocket of MD2, as elucidated by a crystallography, blocking LPS from binding with MD2, and preventing LPS-induced septic shock.[25] However, in a phase III clinical trial, E5564 failed to protect against severe septic conditions.[185]

Antagonists are also necessary to block the harmful inflammation caused by certain viral pathogens. For example, the influenza virus can over-activate TLR3 and/or TLR4, generating an unnecessary response,[186] while $TLR4^{-/-}$ mice were protected against virus-induced lethality.[187] During microbial challenges, the host responds by generating a reactive oxygen species that can oxidize phospholipids, culminating in TLR4 activation that may damage the lungs.[186] These results highlight the crucial role of antagonists in managing the excessive inflammation induced by TLR4.

Recently, 18-amino-acid-long cyclic peptides, θ-defensins, have been implicated in TLR4 signaling inhibition in response to LPS or *Escherichia coli* challenge in mice.[188] They are naturally produced in non-humans primates, whereas in humans, their expression is hindered due to a stop codon. Therefore, synthetic θ-defensins, named retrocyclins, were generated by reverse-engineering a defensins pseudogene. [189–191] Both θ-defensins and retrocyclins exhibit strong antimicrobial and antitoxic characteristics and can control bacteria, fungi, and several viruses, including HSV and HIV. [189] Moreover, θ-defensins also suppress the inflammation caused by TLR4 signaling, and this can be exploited in inflammatory and autoimmune diseases. OSL07, a TLR4 antagonist, inhibits LPS-induced NF-κB and IRF3 activation by blocking TLR4 dimerization.[175] Small peptides are under investigation that can antagonize the over-activation of TLR4 in viral and microbial diseases, and have shown promising results. [176,179]

3.2.4. TLR7/8 and TLR9 antagonists

TLR7/8 occupies a vital position in recognizing viral RNAs and mounting an antiviral response in the form of IFNs and IFN-induced genes. However, continuous and prolonged activation of these TLRs may prone the system to developing detrimental disorders. In this respect, 2′-methyl-modified RNA significantly reduced IFN-α and IL-6 production in murine DCs, human PBMCs and in animal models that were pre-treated with a TLR7 agonist.[178] Other compounds have been derived from ODNs that have either a 7-deaza-dG or an anabino-G modification, and were also able to inhibit TLR7/8/9 in murine and human cell-based assays. These were also effective in animal models, providing a repertoire for treating autoimmune and inflammatory diseases.[172] Other compounds targeting TLR7 and TLR9 simultaneously or TLR9 alone, have been identified to treat autoimmune and inflammatory diseases,[173,174] these could also be helpful in controlling viral infection when inhibition of TLR7 and TLR9 is desired.

3.3. Safety of the TLR targeting drugs

In the history of drug development, safety and efficacy remained the first priority. Modulation of the drugs that target TLRs are necessary to improve the management of viral diseases, since the clinical evaluation of many drugs has been terminated earlier owing to nonspecific effects and safety concerns. From a safety perspective, clinical studies with polyI:C showed toxicity [192] and a recent trial by Anadys with its compound ANA975 (Isatoribine) for HCV has been put on hold, although no serious adverse events have been seen in its clinical trials to date. MPL was used as an adjuvant in RSV vaccines to evaluate its safety; however, when extended clinical trials to humans, a formalin-inactivated RSV worsened the disease condition, leading to the death of two recipients. PF-04878691 a potent TLR7 agonist, was evaluated for HCV treatment in healthy volunteers and showed Type 1 IFN response in a dose-dependent manner. However, in a few volunteers, it caused hypotension, lymphopenia, and influenza-like symptoms, forcing the investigators to terminate the study.[156] Resiquimode, a TLR7/8 agonist has been implicated in the oral treatment of HCV, and in the topical treatment of HSV; however, the results were not convincing due to the loss of efficiency at lower doses, and profound unwanted side effects at higher doses, which lead to the suspension of this study.[154] TAK-242 is a TLR4 inhibitor and its evaluation was stopped by Takeda pharma, because of its non-efficacious profile. However, this decision has not been influenced by any concerns over the safety of the compound. Topical administration of imiquimod (HPV treatment) in human vaccines trials was well tolerated with mild side effects; however, in phase I clinical trial of cancer patients, its oral administration was associated with sustained dose-related hematological toxicity. Thus formulating imiquimod for internal use or vaccine design

is likely to be problematic, nonetheless, its topical use is safe.[164]

4. Conclusion

TLRs are crucial to combating viral diseases; however, host–pathogen interactions, in the form of TLRs–virus interactions, are very complex and require further studies to delineate how the pathways interact. Most TLR signaling converges at NF-κB to execute the inflammation, and therefore, in principle, the TLR pathways are largely overlapping. For TLR3 (and partially TLR4), TRIF is the primary mediator that induces Type 1 IFN. However, there are still differences, owing to the differential expression of TLRs, activation of complementary pathways, participation of other receptors (MDA-5, RLRs), and the development of immune evasion strategies in viruses that altogether generate a unique pathophysiological response in different species and in different immune and nonimmune cells. Moreover, TLR4 can signal through MyD88 as well as TRIF, adding another layer of complexity to the already complex interactions. Therapeutics using ligands always result in some unwanted effects, and these side effects may become even more profound when the target signaling pathways are interlinked. Furthermore, the inherent negative regulation of TLRs may be disrupted, which would result in severe consequences.

In view of the growing number of viral infections, it is imperative to design novel ligands that can interfere with viral replication, viral spread, and can boost the immune system after it has been suppressed by viruses. Thus, the significance of antiviral ligands that can target TLRs is becoming increasingly recognized. Several drugs have been approved for therapy and as adjuvants, and many are currently in the evaluation process. With time, a better understanding of TLRs will lead to the development of new avenues that will confront viruses in an appropriate manner, without causing undesirable side effects.

5. Expert opinion

Activation and inhibition of TLRs are both vital to the effective treatment of viral infections. Therefore, both strategies must be considered to safely and efficiently eradicate the viruses. However, depending on the particular infection, virus type, infection status, and the TLR involved, the therapeutic strategy should be specifically designed to meet the necessary requirements. Since the over-activation or inhibition of TLRs can both predispose the patient to develop another disease condition, extreme caution is recommended when using either of these ligands for therapeutic purposes. As increasing amounts of data become available, our understanding of the TLR regulatory pathways is also improving, and opportunities are becoming available to exploit these pathways (Figure 2). TLRs not only induce inflammation but also induce the genes that regulate inflammation, and these pathways could be targeted for treating over-activation of immune system in viral infections.

Ligands that can disrupt TIR–TIR interactions would be an excellent choice for inhibiting multiple TLRs; however, they would have to cross the plasma membrane. This may disrupt the inflammatory balance in the body, but in certain cases where inflammation must be completely shut down, it would be an ideal target for an antiviral response. Furthermore, TLR activation not only produces inflammation, but also induces molecules that negatively regulate the inflammation [193] and if this balance is disrupted, it leads to either uncontrolled growth of the infecting organisms or to inflammatory disease. Recently, it has been reported that co-stimulation of TLR2, TLR4, and TLR7/8 induces inflammation, which severely hampered negative regulation.[194] The involvement of multiple TLRs in viral infection is common and poses many such challenges. To address this, using TLRs inhibitors together with redox modulators might be useful in treating such conditions. The identification of novel targets that are shared by multiple TLRs, and the ability to target them therapeutically, would mean that a single treatment could be used to treat multiple viral infections, particularly those that share common pathways. In addition to the induction of IFNs and proinflammatory cytokines, multiple pathways of adaptive immunity are initiated by poly(I:C), including virus-specific T-cell responses, NK cell cytotoxicity, and DC maturation, through TLR3, MDA5, and RIG1, thus providing an attractive approach for DC-based vaccines to stimulate the T-cell-mediated immune response. [126–128] It has also been reported that a combination of Pam2-ODN can activate both TLR2/6 and TLR9 pathway, protecting mice against influenza A and swine-origin H1N1 influenza viruses.[195]

NF-κB is a transcription factor that binds to DNA, leading to gene expression. This binding also requires the communication of many other proteins that form a stable complex on the promoter. For a given complex, a higher number of proteins offer a good target because disrupting any protein will destabilize the complex. Most of the focus of TLR research has been on ligands that can target the ectodomains of TLRs, offering an easier application and avoiding many complications. However, the NF-κB-DNA complex may also be a good choice, for example when competitively inhibiting TLRs is not a feasible option or when the reaction is slower, disrupting the NF-κB-DNA complex would be helpful. Moreover, NF-κB can potentially influence the expression of over 300 genes that include many types of molecules, ranging from cell surface receptors to transcription factors and from cytokines to intracellular mediators. Therefore, this should be considered when working with agonists that directly target TLRs to activate the downstream pathways. Most importantly, NF-κB can also trigger the expression of various viral proteins and may worsen the pathogenesis and aid in the viral spread.[196] Ligands that can modulate NF-κB activity, either by partially inhibiting it or sustaining its functions for longer, could also be targeted in virus-infected cells in order to more broadly manage viral infections.

Once injected, it is very difficult to control the interaction of a ligand with the cellular proteins. This usually results in minor to serious unwanted effects that severely hamper the potential application of most drugs. Since viruses are being detected by endosomal TLRs, the ligands should be enveloped in a way that would ensure direct delivery of the drugs into the endosomes of the target tissues. This would minimize unwanted

side effects of the drug and may enhance the effectiveness of treatment. Partial or weak TLR agonists can be used as vaccine adjuvants, where their use will not only reduce the number of vaccine doses, but will also improve its efficacy [164,197] (Table 4). However, for therapeutic purposes, a TLR ligand alone will not be effective and these ligands should instead be employed in combination with viral inhibitors to obtain their maximum therapeutic value.

An inflammatory response may vary under different physiological conditions, resulting in a number of different scenarios. The most prominent factors in this include obesity, preinfection, polymorphism or mutations, and the route of administration. These variations in inflammatory responses necessitate further studies into the development of novel ligands that can be fine-tuned during therapy. This is a difficult task, but by considering the route of administration, time and dosage of administration, drug formulations, and using temporary ligand-appendages that hydrolyze in a time-dependent manner, it may be possible to achieve and could be used successfully to treat a number of viral infections. A number of studies have been conducted, improving the efficacy of the TLR-targeting compounds by considering their route of administration and pharmacokinetics, to treat viral and non-viral TLR-related diseases.[198,199]

Any ligand that (in)activates TLR4 signaling can influence both cytokine and IFN-based responses. Novel ligands that target TLR4 should be of priority, and care should be sought in evaluating and employing such ligands. Moreover, because TLRs are heavily cross-linked, careful consideration of the combination of ligands that can modulate other receptors, resulting in improved effects and a beneficial outcome of the applied regime, would be highly valued. This would not only alleviate the problem of drug resistance, but would also improve the efficacy of the drugs.

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Articles of special interest are labeled as: • of interest; •• of special interest

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