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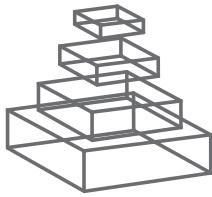
PROCEEDINGS OF ICI MILAN 2013

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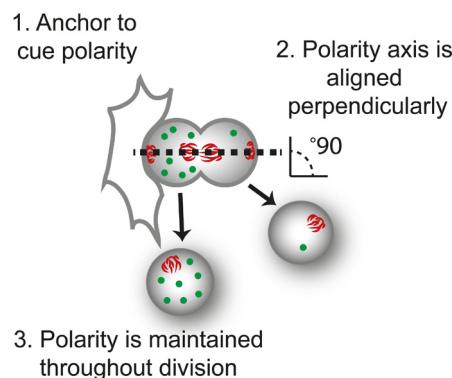
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Asymmetric cell division in hematopoietic cells. It is now clear that asymmetric cell division (ACD) occurs during development and function of immune cells, but the functional role of ACD has not been resolved. In other well studied models of ACD, control of proliferation, death and differentiation of the progeny involves 3 conditions: (1) an anchor dictates the axis of polarity; (2) the dividing cell is aligned along the axis of polarity (usually perpendicular to the anchor, shown by the alignment of the mitotic spindle, red); and (3) asymmetry of cell fate determinants is maintained thought division such that the fate determinants are differentially inherited by the daughter cells. To understand how ACD dictates fate in hematopoietic cells, we need to understand the mechanisms by which fate determinants are inherited differently by the two daughter cells of a dividing cell, and how this impacts upon the fate of each daughter cell and its progeny
(Copyright: Kim Pham, Faruk Sacirbegovic and Sarah M. Russell).

This Research Topic covers all of the major lectures and symposia addresses delivered by invited speakers at the 2013 International Congress in Immunology (ICI) at Milan, Italy, August 22-27, 2013.

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Articles from the 15th ICI meeting in Milan, Italy

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The First International Congress in Immunology was held in 1971 in Washington, DC, USA. Since that initial gathering of worldwide immunologists, we have evolved considerably as the articles selected from presentations at the 15th ICI and compiled here attest. By 1971, the two main systems involved in adaptive immunity had been described and the two major types of lymphocytes, B cells and T cells, recognized by their surface expression of immunoglobulin molecules and theta antigen had just been described. Years later, I was riding in a bus at an immunology meeting that was headed to the conference banquet. My companion in the seat next to me happened to mention that he was present at the first ICI, and I replied that I was also there. My seatmate then went on to say that everyone that he spoke to at that inaugural meeting felt that the major mysteries in immunology had been uncovered and now we just needed to dot the I's and cross the T's. Needless to say that we have all come a long way in the past 42 years, and great strides have been

made in delineating the characteristics of the immune system, as exemplified here in this compilation of contributions stemming from the 15th ICI.

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Skin-resident antigen-presenting cells: instruction manual for vaccine development

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The induction of antigen-specific effector T cells is driven by proper antigen presentation and co-stimulation by dendritic cells (DCs). For this reason strategies have been developed to instruct DCs for the induction of CD4⁺ and CD8⁺ T cell responses. Since DCs are localized, amongst other locations, in peripheral tissues such as the skin, new vaccines are aiming at targeting antigens to DCs *in situ*. Optimal skin-DC targeting in combination with adequate adjuvant delivery facilitates DC maturation and migration to draining lymph nodes and enhances antigen cross-presentation and T cell priming. In this review we describe what DC subsets populate the human skin, as well as current vaccination strategies based on targeting strategies and alternative administration for the induction of robust long-lived anti-cancer effector T cells.

Keywords: skin, antigen-presenting cells, vaccination, microneedles, C-type lectin receptors, glycans

INTRODUCTION

Immature dendritic cells (DCs) patrol the tissues to sense for pathogens. Recognition of pathogens through specific innate receptors allows antigen uptake and processing for presentation on MHC class I and II molecules, while DCs migrate to secondary lymphoid organs. Upon entry in the lymph nodes, DCs display a mature phenotype, characterized by high expression levels of co-stimulatory molecules and the production of pro-inflammatory cytokines. The presentation of antigenic peptides on MHC class I or II molecules combined with the signals derived from mature DCs allows the initiation of antigen-specific humoral and cellular immune responses (Ueno et al., 2007). Besides triggering activating immune responses aiming at the clearance of pathogens, DCs are also capable to down-modulate unwanted auto-immune reactions by maintaining immune tolerance via the induction of regulatory T cells (Maldonado and von Andrian, 2010). Consequently, as DCs are able to induce both activating as well as suppressive immune responses, this makes them ideal targets for vaccination strategies against cancer or autoimmune disorders.

During the past years, extensive research has focused on the development of DC-targeting vaccination strategies against cancer. First attempts focused on pulsing DCs *ex vivo* with tumor antigens and maturation agents (Tacken et al., 2007). This strategy involved the generation of DCs from monocytes or CD34⁺ precursors and the subsequent re-injection into the patient to generate effective T and B cell responses against the tumor. Although this system is well tolerated by the patients and have shown modest clinical responses (Galluzzi et al., 2012), there are limitations to *ex vivo* culture and antigen-loading of DCs. Firstly, it has been shown that *ex vivo* cultured DCs exhibited reduced migratory potential and secondly, the development of personalized vaccines is costly and difficult to standardize. By comparison, higher objective clinical response rates have been obtained by immunotherapies

based on adoptive transfer of tumor-specific T cells [either *ex vivo* expanded tumor infiltrating lymphocytes (TILs) or T cells transduced with high affinity TAA-specific TCR or chimeric antigen receptors (CARs)] (Gattinoni et al., 2012; Restifo et al., 2012; Turtle et al., 2012). The success of this type of immunotherapy is shown to depend on the number and differentiation status of adoptively transferred T cells (Gattinoni et al., 2005; Klebanoff et al., 2011). Additionally, several studies point to improved anti-tumor efficacy when the T cells are activated immediately prior to or directly after adoptive transfer. The latter could be accomplished by co-administration of a tumor-antigen vaccine (Overwijk et al., 2003; de Witte et al., 2008a,b; Klebanoff et al., 2009). However, also this therapy is very costly and since effective TILs seem to be restricted to melanoma and genetically engineered T cells only possess monoclonal specificity, targeting DCs directly *in vivo* therefore provides an attractive alternative.

The goal of *in vivo* DC-targeting vaccines is twofold, accumulating antigens to DCs in a cell-specific manner while promoting antigen uptake, cross-presentation, and DC maturation. In order to achieve this, knowledge on the selective expression of antigen-uptake receptors on various DC subsets is required, as well as which DC subsets has superior cross-presentation capacity. DCs express a multitude of pattern recognition receptors, such as Toll-like receptors (TLRs) and C-type lectins (CLRs). While TLRs play a crucial role in pathogen recognition, the induction of DC maturation, and the production of inflammatory cytokines, CLRs have been shown to have a subset-specific expression pattern and are able to mediate antigen uptake and cross-presentation. Already more than 10 years ago, work from the group of Steinman showed that the CLR DEC-205 mediated the uptake of ovalbumin coupled to a DEC-205 antibody, resulting in increased CD4⁺ and CD8⁺ T cell activation (Bonifaz et al., 2002, 2004). Since then, the targeting of several CLRs with antigens conjugated to monoclonal antibodies (mAbs),

including mAbs against mannose receptor (MR), CLEC9A, DC-SIGN, and Langerin, has been explored (Caminschi and Shortman, 2012). While some of these CLRs are not considered DC specific, such as DEC-205 and MR, others such as DC-SIGN, CLEC9A, and Langerin are expressed on specific DC subsets [myeloid DCs (mDC), plasmacytoid DCs (pDCs), and Langerhans' cells, respectively]. Although several studies in search of the most efficient cross-presenting DC subset have demonstrated the CLEC9A⁺ pDC as the most potent one in cross-presenting soluble antigen compared to LC and mDC, it may still be that for certain glycosylated antigens that target Langerin⁺ LC and DC-SIGN⁺ mDC, these subsets might have similar potential as the CLEC9A⁺ pDC to cross-present (Tel et al., 2012, 2013; Unger et al., 2012). In general, it has become clear that only the simultaneous delivery of CLR-targeting antigens together with a potent adjuvant will lead to the generation of efficient CD4⁺ and CD8⁺ T cell responses, especially in the context of anti-tumor immune therapies.

In addition, DC subsets present in the various tissues and lymphoid organs do not all express the same level and variety of CLRs and not all DC subsets are equally potent in activating CD4⁺ and CD8⁺ T cells. By deliberate selection of the right DC subset and/or through targeting of CLRs specifically expressed by DC subsets, an optimal induction of cellular immune responses (either immunity or tolerance) can be achieved. Although a lot of data has been accumulated on DC targeting strategies in *in vivo* systems in mice, still little is known on the efficacy of DC-targeting vaccines in human skin. Here, we review recent knowledge on DC subsets residing in the human skin, including their CLR and TLR expression, capacity to cross-present antigens, and to respond to adjuvants for migration to draining lymph nodes. Finally, new developments on the strategies used to selectively deliver vaccines to specific layers within the skin, as well as how to overcome potential side effects of the immune suppressive skin micromilieu will be discussed.

DC SUBSETS: DIFFERENCES IN FUNCTION AND CLR AND TLR EXPRESSION

The human skin has been classically divided in two main compartments: the epidermis and the dermis. The epidermis is the outer layer that provides the barrier function to the skin. Within the epidermis frequently dividing keratinocytes are located, melanocytes, which produce the pigment melanin, and LCs, which are the main epidermis-resident antigen-presenting cell (APC) and are characterized by the expression of the CLR Langerin (**Figure 1**). In addition, T cells, mainly CD8⁺ T cells, can be found in the epidermis. Whereas the epidermis has a relatively simple histology, the underlying dermis is anatomically more complex and accumulates greater cell diversity. The dermis is rich in many specialized immune cells, including dermal DCs, CD4⁺ T helper (Th) cells, $\gamma\delta$ T cells, and natural killer T (NKT) cells. Moreover, macrophages, mast cells, fibroblasts, and nerve-related cell types are also present. The dermis is drained by lymphatic and vascular conduits, through which migrating cells can traffic.

In both human and mice, two main lineages of skin-resident DCs are known: pDCs and the tissue-resident mDC. Steady-state human skin contains four phenotypically and functionally distinct subsets of DCs. Within the epidermis CD1a^{high} LCs can be found,

whereas the CD1a⁺, CD14⁺, and CD141⁺ DCs (also known as BDCA3⁺ DCs) are present within the dermis (Klechevsky et al., 2008, 2009; Haniffa et al., 2012; Segura et al., 2012). The latter subset has recently been described in the dermis of human skin as a rather efficient DC in the cross-presentation of antigen (Haniffa et al., 2012). In addition, high expression levels of CCR7 coincided with superior migratory behavior of these DC (van de Ven et al., 2011; Haniffa et al., 2012). Furthermore, LCs have also been described as very efficient in the priming and cross-priming of CD8⁺ T cells, whereas CD14⁺ dermal DCs (dDCs) are able to induce the generation of follicular Th cells (Klechevsky et al., 2008; Banchereau et al., 2012). The precise function of the CD1a⁺ dDCs is still poorly defined, although it has been shown that they are capable of stimulating CD4⁺ T cell proliferation (Klechevsky et al., 2009). It has also been shown that LCs are less responsive to bacteria due to a lack of TLR2, TLR4, and TLR5 expression, but are known to express TLR1, TLR3, TLR6, and TLR10, making them suitable to respond to viruses (Angel et al., 2007; van der Aar et al., 2007; Klechevsky et al., 2009). CD14⁺ dDCs express most of the 10 human TLRs, while CD1a⁺ dDCs seem to express all TLRs with exception of TLR9 and TLR10 (Angel et al., 2007; van der Aar et al., 2007). In contrast, the CD141⁺ DC expresses high levels of TLR3 and TLR10 and moderate expression of TLR1, TLR2, and TLR6 (Hémont et al., 2013). In response to polyI:C, this subset produces inflammatory cytokines such as TNF- α and IL-1 β (Haniffa et al., 2012).

The four skin resident DC subsets can also be separated based on their expression pattern of CLRs: the CD14⁺ dDCs typically express DC-SIGN, DEC-205, DCIR, Dectin-1, and MR (Klechevsky et al., 2009), whereas the CD1a⁺ dDCs can be distinguished based on the expression of MGL and low levels of MR, DEC-205, and DC-SIGN (Unger and van Kooyk, 2011) (**Figure 1**). In humans, LCs are the only cells that express Langerin. Additionally, LCs also express DEC-205 at intermediate levels. In contrast, the CD141⁺ DC express the CLRs DEC-205 and CLEC9A, which is described to be involved in the uptake of dead cells (Poulin et al., 2010; Meixlsperger et al., 2013). Therefore, LCs could be exclusively targeted via Langerin, whereas specific targeting of CD14⁺ dDCs should be possible via DC-SIGN.

TARGETING ANTIGENS TO DC SUBSETS THROUGH SPECIFIC C-TYPE LECTINS

C-type lectins such as DEC-205 and CLEC9A have shown their capacity to internalize antigen for presentation to CD4⁺ T cells and cross-presentation for the induction of antigen specific CD8⁺ T cells. Often antibodies specific for these receptors have been used for targeting purposes (Bonifaz et al., 2004; Caminschi et al., 2008). In contrast, Langerin, MR, and DC-SIGN can be either targeted using specific mAbs or using their natural ligands, since for these receptors the glycan-binding profile has been determined (Holla and Skerra, 2011; Lee et al., 2011; Unger et al., 2012). The use of glycans reports several advantages, such as the relatively easy production of glycans in large scale and their lower immunogenicity as compared to mAbs. The potential use of glycan-based DC-targeting vaccines applied via the skin has hardly been investigated. Often the cross-presenting capacity of skin-resident DC subsets has been investigated, by analyzing the

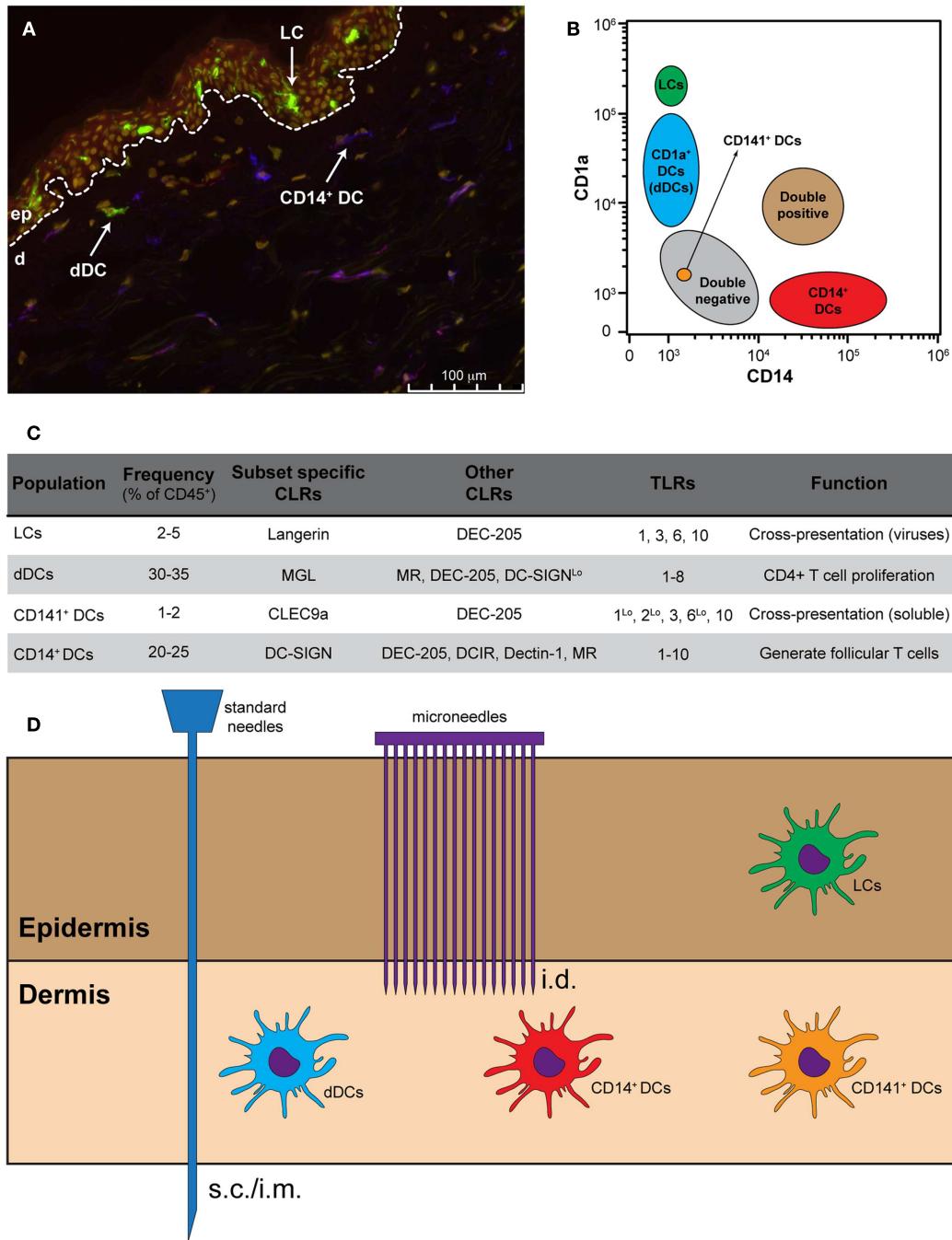


FIGURE 1 | Targeting skin APCs. (A) Representative immunofluorescence staining of human skin using monoclonal antibodies against CD1a (green), CD14 (red), and DC-SIGN (blue). Nuclei are stained with the nuclear dye Hoechst (yellow). **(B)** Distribution of skin APCs according to their CD1a and

CD14 expression levels. **(C)** Frequency, CLR and TLR expression and function of the four main skin APC subsets. **(D)** Standard needles do not allow skin APC targeting, while different models of microneedles allow for the specific targeting of dermal APCs and, in some cases, also LCs.

potency of DC subset to cross-present soluble antigen. In these studies the CD141⁺ DC subsets have shown to have a superior cross-presenting capacity, whereas LCs, CD14⁺, and CD1a⁺ DCs do not have this potential. Conjugation of antigen to anti-Langerin antibodies resulted in increased cross-priming of CD8⁺

T cells by LCs (Klechovsky et al., 2008). We have shown that conjugation of the DC-SIGN binding Lewis-type blood antigens (Le^b or Le^X) or an antibody recognizing DC-SIGN to liposomes resulted in an enhanced uptake by DC-SIGN⁺ cells *in vitro* using monocyte-derived DCs (Unger et al., 2012). Of note, there was no

difference in uptake between the glycan-modified and the anti-DC-SIGN modified liposomes. These findings demonstrate that not all CLRs share the same preference for glycan-modified antigen, and that they can be presented on multivalent carriers or as small, single glycan-modified peptides. In addition, the distinct expression of CLRs by various DC subsets allows specific targeting to the desired DC type using glycan- or antibody-modified vaccine components.

FUNCTIONAL CONSEQUENCES OF TLR ACTIVATION WITHIN THE HUMAN SKIN

Although the targeting of antigens through DC-SIGN and Langerin has already been shown to result in enhanced CD4⁺ and CD8⁺ T cell responses, early work derived from the group of Steinman provided evidence that only in the presence of a potent adjuvant, CLR-mediated DC targeting induced strong cellular immunity *in vivo* instead of generating tolerance (Bonifaz et al., 2002).

During the past years, research has focused on the identification of suitable adjuvants to combine with intradermal skin vaccination strategies (Alving et al., 2012; Schneider et al., 2012; Oosterhoff et al., 2013). We, and others, have shown that, in general, intradermal administration of soluble TLR ligands does not have major effects on DC migration, maturation, and T cell stimulatory capacity, especially not in relation to the effects that TLR ligands show on *in vitro* generated monocyte-derived DCs (Schneider et al., 2012; Oosterhoff et al., 2013). The discrepancy found on DC maturation after TLR stimulation *in vitro* and *in situ* might be caused by specific, local suppression within the skin microenvironment. Consequently, to overcome this suppression, a strong adjuvant should be administered simultaneous with the DC-targeting vaccine. Surprisingly, the effects of Aldara, a FDA-approved immune response modifier skin cream containing 5% of the TLR7 agonist imiquimod, has shown its potential to improve CD8⁺ T cell responses in mice and patients when topically applied on the skin (Zuber et al., 2004; Fenoglio et al., 2013). It will be interesting to determine the maturation effects of the Aldara cream on the population of APC in human skin, and the induction of CD8 T cell responses in comparison to intradermal injection of soluble R838 (imiquimod; TLR7). Selection of the appropriate adjuvant in combination with an anti-tumor vaccine is therefore essential to induce immunity and avoid tolerance. A more detailed knowledge of the different immune responses induced by CLRs and their interplay with TLRs is needed for the improvement of vaccination strategies using CLR ligands. Separate from TLR ligands allowing DC maturation and migration to the draining lymph nodes, also injection of cytokines in the skin as immunostimulators, such as GM-CSF has been investigated (van den Eertwegh et al., 2012; Grotz et al., 2013).

THE IMPORTANCE OF THE ROUTE OF VACCINE ADMINISTRATION

The specific route of administration is often determined by the type of adjuvant present in the vaccine. In humans, vaccines containing aluminum-based or oil-in-water adjuvants are administered intramuscular (i.m.) or subcutaneously (s.c.) (Figure 1). Intradermal (i.d.) administration of these vaccines likely causes

local irritation, induration, skin inflammation, and granuloma formation. The development of novel adjuvants such as synthetic TLR ligands or cytokines facilitates the use of the i.d. route. Vaccination i.d. has shown significant advantages with respect to dose-sparing and immunogenicity in comparison to other routes (e.g., s.c., i.m., i.v.) (Kenney et al., 2004). This is likely due the presence of multiple DC subsets in the skin. Additionally, skin DCs are generally more prone to become immunogenic than, for example, mucosal DCs. However, i.d. vaccination using standard needle and syringes is technically challenging and inaccurate administration of vaccines can even result in adverse side effects. Moreover, vaccination using standard needle and syringe will deliver the vaccine at one spot. It has not been thoroughly investigated whether simultaneous delivery at different/multiple spots leads to superior responses. Simultaneous delivery could be facilitated by the use of microneedle arrays. Microneedle arrays can go into the skin at very low insertion forces and controlled depth, facilitating effective delivery of vaccines.

Indeed, using solid metal microneedles that were coated with an antigen-containing solution it was shown that within 2 h, 50% of DCs that had emigrated out of murine ear explants were antigen positive (del Pilar Martin et al., 2012). Moreover, compared with s.c. vaccination, a single vaccination with influenza-vaccine coated solid metal microneedles induced potent long-lived immunity and improved protection against influenza virus (Koutsonanos et al., 2011). A new generation of microneedles are “reservoir-integrated skin interface devices” that allow microneedle-guided transport of the vaccine while remaining inserted in the skin (van der Maaden et al., 2012). Recently, the microneedle delivery techniques have been broadened by the generation of nanoporous out-of-plane microneedle arrays from ceramic material (Bystrova and Luttge, 2011). The ceramic nanoporous microneedles allow the investigation of a range of parameters related to delivery performance (e.g., cargo loading capacity, amount and arrangement of microneedles, microneedle tip shape). The use of so-called out-of-plane microneedle arrays allows a standardized and regulated delivery of the vaccine to dermal DCs. The intrusion depth into the skin is self-defined by the microneedle length. Notably, this approach also facilitates targeting of the LCs, which is more difficult when using i.d. injection using classic syringe/needles. Moreover, by varying the amount and arrangement of microneedles on the array as well as the microneedle tip shape, different skin DC subset(s) may be triggered. The impact of such microneedle arrays on the efficacy of skin DCs targeting and/or the induction of T-cell mediated immunity has not been fully investigated yet.

As an alternative, polymeric dissolvable microneedle arrays are being explored that release the vaccine into the skin but dissolve within minutes leaving no residual sharps waste. Using these polymeric dissolvable microneedles, 23% of the DCs in draining lymph nodes were loaded with microneedle-applied antigen 72 h later. Unfortunately, this study did not address whether this method was superior in vaccine delivery to dermal DCs than conventional immunization strategies (Zaric et al., 2013). However, Sullivan et al. (2010) showed that vaccination of mice against influenza using these dissolvable microneedles induced enhanced protection compared with conventional i.m. vaccination. Also more recently it was demonstrated that microneedle

array skin delivery systems of live adenovirus vaccines in mice resulted in potent CD8⁺ T cell priming through stimulating Langerin⁻ DCs, indicating that, in this study, LCs were not the best in inducing cross-presentation, but dermal DCs (Bachy et al., 2013).

THE SKIN MICROMILIEU: AN IMMUNE SUPPRESSIVE ENVIRONMENT?

The main constituent of the skin is keratinocytes. Similar to gut epithelial cells, keratinocytes can sense pathogens and mediate immune responses to discriminate between harmless commensal organisms and harmful pathogens. Epidermal keratinocytes express several TLRs, located either on the cell surface (TLR1, TLR2, TLR4, TLR5, and TLR6) or in endosomes (TLR3 and TLR9) (Begon et al., 2007; Lebre et al., 2007). In addition, TLR7 expression is induced through the triggering of TLR3 by double-stranded RNA, which makes keratinocytes responsive to TLR7 agonists. TLR expression by keratinocytes might be crucial for promoting skin immune responses, as activation of these receptors on human keratinocytes leads to a predominant Th1-type immune response and to the production of type I interferons. In addition to antimicrobial peptides, keratinocytes constitutively secrete, or induced to release, numerous cytokines, including IL-1, IL-6, IL-10, IL-18, and TNF α . Of particular interest with regard to the skin in health and disease is the production of IL-1 by keratinocytes. In healthy skin, keratinocytes constitutively synthesize both pro-IL-1 α and pro-IL-1 β but cannot process them or secrete them in their active forms. Following exposure to stimuli such as UV irradiation, keratinocytes process and release IL-1 β through the activation of the inflammasome. Keratinocytes are also an important source of chemokines and express chemokine receptors, and therefore can modulate an immune response by attracting different cell types into the skin. By expressing CC-chemokine ligand 20 (CCL20), CXC-chemokine ligand 9 (CXCL9), CXCL10, and CXCL11 activated keratinocytes selectively attract effector T cells to the skin during diseases or recruit neutrophils or regulate the trafficking of Langerhans cell precursors to the epithelium.

In contrast, TGF β is a cytokine with anti-inflammatory properties that is produced by different cell types in the skin, such as LC and keratinocytes. LC-produced TGF- β_1 has been shown to act in a autocrine/paracrine fashion and to maintain the LC in the epidermis, as inferred from emigration of LC from the skin upon abrogating TGF- β_1 signaling (Bobr et al., 2012). These data suggest

that blocking TGF- β_1 signaling (via anti-TGF β -RI Abs or pharmacological inhibitors) might be beneficial to include in vaccines that aim to induce antigen-specific CD8⁺ effector T cells by targeting LC, such as in cancer. The other main producers of TGF- β_1 in the skin are keratinocytes. It has been shown that TGF- β_1 levels rise upon wounding of the skin or chronic psoriasis (Flisiak et al., 2002, 2003; Wang et al., 2006), but also premalignant keratinocytes express elevated levels of TGF- β_1 . The increased levels of TGF- β_1 were associated with enhanced LC migration and significantly affected dermal DC composition: increased numbers of skin DCs and pDCs had emigrated and were detected in skin-draining LN, while the influx of blood-derived pDC and DC precursors into the skin was highly increased.

FUTURE DIRECTIONS

A lot of knowledge has been gathered on the presence of different human skin-resident APCs, at distinct locations. The expression of innate receptors such as C-type lectins and TLR has been well characterized, and four subsets have been identified individually on their efficacy to mature, migrate, and cross-present antigen for the induction of CD8⁺ T cells. Based on the expression of different set of TLR and CLR it has been speculated that these APC subsets (pDC and mDC) have a division of labor. Some subsets are crucial in the recognition of soluble antigens, while others play a major role in particulate recognition of glycosylated bacterial or viral products. Although we are beginning to understand the function of these APC subsets individually, we hardly have any knowledge available on the function of these APC *in situ* in the human skin. In particular how the suppressive network of keratinocytes may imprint local APC as well as be involved in the inflammatory activation and trigger APC to mature and migrate to draining lymph node for T cell priming. Future vaccination strategies are therefore important to reveal how we can optimally instruct this skin-resident repertoire of APC *in situ* to overcome the suppressive skin micromilieu, and activate them simultaneously (both mDC and pDC) to induce robust antigen T cells responses.

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APPENDIX**KEY CONCEPTS*****In vivo DC targeting***

An interesting alternative to vaccination strategies based on *ex vivo* culturing of DCs. Through *in vivo* DC targeting approaches, vaccines are prepared to specifically reach DCs in their natural micromilieu, and hypothetically benefit from the accumulation of antigens in the most effective APCs, with high migratory capacity and while ensuring proper cross-presentation and DC maturation.

The four skin resident APC subsets differ in the expression pattern of CLRs

While CD14⁺ dDCs express DC-SIGN, DEC-205, DCIR, Dectin-1, and MR, CD1a⁺ dDCs can be distinguished based on the expression of MGL and low levels of MR, DEC-205, and DC-SIGN. On the other hand, LCs are the only APCs that express Langerin. LCs also express DEC-205. In contrast, CD141⁺ DCs express DEC-205 and CLEC9A.

Advantages on the use of glycans to target CLRs on skin APCs

In contrast to other APC-specific receptors used in *in vivo* DC targeting vaccination strategies, the natural ligands of CLRs are low immunogenic, can be mass-produced by chemical methods, and can be easily conjugated to the antigen of choice.

Choice of adjuvant

Several CLRs are able to elicit a signaling response that down-modulates the activatory effect of TLRs. However, when higher doses of adjuvants are provided, the net result of this finely regulated signaling system is activatory. Thus, the selection of the appropriate adjuvant is essential for the success of a CLR-targeting strategy.

Choice of administration route

The availability of novel TLR-specific powerful adjuvants and the advances in needle engineering have allowed the development of intradermal vaccination devices that allow lower antigen dosage and higher immunogenicity compared to classic vaccination administration routes.



Rheumatic heart disease: molecules involved in valve tissue inflammation leading to the autoimmune process and anti-*S. pyogenes* vaccine

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The major events leading to both rheumatic fever (RF) and rheumatic heart disease (RHD) are reviewed. Several genes are involved in the development of RF and RHD. The inflammatory process that results from *S. pyogenes* infection involves the activation of several molecules such as VCAM and ICAM, which play a role in the migration of leukocytes to the heart, particularly to the valves. Specific chemokines, such as CXCL3/MIP1 α as well as CCL1/I-309 and CXCL9/Mig, attract T cells to the myocardium and valves, respectively. The autoimmune reactions are mediated by both the B- and T-cell responses that begin at the periphery, followed by the migration of T cell clones to the heart and the infiltration of heart lesions in RHD patients. These cells recognize streptococcal antigens and human-tissue proteins. Molecular mimicry between streptococcal M protein and human proteins has been proposed as the triggering factor leading to autoimmunity in RF and RHD. The production of cytokines from peripheral and heart-infiltrating mononuclear cells suggests that T helper 1 and Th17 cytokines are the mediators of RHD heart lesions. The low numbers of IL-4 producing cells in the valvular tissue might contribute to the maintenance and progression of the valve lesions. The identification of a vaccine epitope opens a perspective of development of an effective and safe vaccine to prevent *S. pyogenes* infections, consequently RF and RHD.

Keywords: *S. pyogenes*, genes, adhesion molecules, chemokines, Th1 and Th17 cytokines, T and B cells, valve proteins, anti-*S. pyogenes* vaccine

INTRODUCTION

Rheumatic fever (RF) and its major sequelae rheumatic heart disease (RHD) are autoimmune diseases that arise following infection of the throat by *S. pyogenes* in children and young individuals (3–19 years old) who present genetic components that confer susceptibility to the disease.

The disease still remains a major cause of cardiovascular disability in school children and young individuals, and it represents a high burden for public health in the developing world. The incidence of this disease in the so-called “hotspots” ranges from 20 to 51 per 100,000 habitants, causing ~500,000 deaths each year (1). In Brazil, the number of beta hemolytic streptococcus throat infections is ~10 million cases/year, leading to 30,000 new cases of RF, of which ~15,000 cases develop RHD (2).

The aim of this review is to explore the role of several genes in the control of *S. pyogenes* infection and the associated autoimmune reactions, as well as to depict the molecular mechanisms leading to these autoimmune reactions.

GENETIC BACKGROUND

As RF and RHD are post-infectious diseases that involve an inflammatory reaction in addition to T and B cells, several genes are involved in the predisposition and manifestation of the disease.

Table 1 summarizes the genes involved in RF/RHD development and their role.

GENES RELATED TO THE INNATE IMMUNE RESPONSE

The first line of host defense against a pathogen, *S. pyogenes* in the cases of RF and RHD, involves several molecules that bind to specific pathogen-associated molecular patterns (PAMPs) through specific molecules in the host, defined as pattern recognition receptors (PRRs). These PRRs can be soluble in human serum, or they can be cell-associated, and they are described below.

Toll-like receptors (TLRs) are sensors of foreign microbial products that initiate host defense responses in multicellular organisms. The genotype 753Arg/Gln of *TLR2* gene resulting from the replacement of arginine with glutamine at codon 753 was more frequently present in a Turkish ARF cohort compared with controls (3).

Mannan-binding lectin (MBL) is a phase I inflammatory protein encoded by different variants of the promoter and exon 1 regions of the *MBL2* gene. The A and O alleles code for high and low production of MBL, respectively. Interestingly, RHD patients with mitral stenosis (MS) displayed an association with the A allele, while the majority of RHD patients with aortic regurgitation (AR) presented the O allele. The amount of MBL in the sera of RF and

Table 1 | Genes of genetic susceptibility of RF and RHD.

Genetic markers	Role
MBL; TLR2; FCN2;	Innate immunity
FCγRIIa	Inadequate immune response against <i>S. pyogenes</i>
HLA class II genes	Adaptive immune response
(DR and DQ, several alleles)	T cell antigen presentation and immune response
TNF- α , ILRA, TGF- β , IL-10	Both innate immunity/adaptive immune response Mediators of inflammatory reactions

RHD patients presented high and low serum levels of MBL, respectively (4, 5). These results suggest that the *MBL2* gene could play a role in the development of valvular stenosis or regurgitation.

Ficolins trigger the innate immune response by either binding to collectin cellular receptors or initiating the complement lectin pathway. There have been three ficolin genes identified in humans with different functions, sequences, and specificity. Polymorphisms at -986, -602, and -4 within the promoter region of ficolin 2 (*FCN2*) are associated with the serum levels of this protein. In Brazilian chronic RHD patients, the haplotype G/G/A (-986/-602/-4) was more frequent than in controls and correlated with low levels of this protein, leading to a prolonged time of infection or to repeated streptococcal infections (6).

GENES RELATED TO THE ADAPTIVE IMMUNE RESPONSE

The susceptibility of developing RF/RHD was first associated with alleles of the HLA class II genes (*DRB1*, *DQB*, and *DQA*). Among the *DRB1* alleles, HLA-DR2, DR3, DR4, DR7 were the most frequently associated with the disease, with HLA-DR7 being the most consistently associated HLA allele found in Brazilian, Turkish, Egyptian, and Latvian RF/RHD patients [reviewed in Ref. (7)]. The role of the HLA molecules encoded by these genes is to present antigens to the T cell receptor (TCR), thus activating the adaptive immune response.

GENES RELATED TO BOTH THE INNATE AND THE ADAPTIVE IMMUNE RESPONSE

The *TNF- α* gene has an inflammatory role and is located on the same chromosome as the HLA class II genes. The polymorphism of a SNP at the promoter region of TNFA-308G/A was associated with the susceptibility of patients from Mexico, Turkey, Brazil, and Egypt to RHD (8–11).

IL-1 α and *IL-1 β* are cytokines that have been implicated in the inflammatory reactions and are encoded by *IL-1Ra* gene. The most common alleles are 1 and 2, which encode antagonists of *IL-1 α* and *IL-1 β* . The absence or misrepresentation of both alleles results in a strong inflammatory response. Studies in Brazilian RHD patients with severe carditis showed low frequencies of allele 1, suggesting the absence of inflammatory control (12). Some studies showed that alleles of the *TGF β 1* gene were risk factors for the development of valvular RHD lesions (13, 14) as this gene codes for an inflammatory protein secreted by many cell types including macrophages. Thus inflammatory stimuli that activate macrophages enhance the release of active *TGF- β* .

HEART VALVE CHRONIC INFLAMMATION

The healing process of rheumatic carditis results in varying degrees of fibrosis and valve damage. The Aschoff body is considered the hallmark of the disease and consists of a granulomatous nodule usually located in the connective tissue around small vessels. This structure promotes the inflammatory process as the mediator of rheumatic heart lesions. Several inflammatory cells, such as neutrophils, macrophages, and T and B lymphocytes, infiltrate both the myocardium and the valves. These cells enter through the myocardium and the valves upon the upregulation of expression of the adhesion molecules. Cunningham's group showed that streptococcal heart-tissue cross-reactive antibodies increased the amount of VCAM-1 on the valvular endothelial surface, leading to myocarditis and valvulitis (15, 16). Recently, we verified that ICAM, another integrin, was also upregulated, in addition to P-selectin and several chemokines and their receptors. Among the chemokines, *CCL3/MIP1 α* gene expression was up regulated in the myocardium, while *CCL1/I-309* and *CXCL9/Mig* were highly expressed in the valvular tissue of RHD patients (17). An *in vitro* assay demonstrated that valvular lesions infiltrating T cells migrated mainly toward a *CXCL9/Mig* gradient, suggesting that specific chemokines can mediated both the CD4 $^{+}$ and CD8 $^{+}$ T cell recruitment to the site of inflammation in the heart (17).

Cytokines are important secondary signals following an infection because they trigger effective immune responses in most individuals, and they most likely cause deleterious responses in patients with autoimmune disease. Cytokines generally act locally.

In RHD, in both the myocardium and valvular tissue, a large number of infiltrating mononuclear cells secreting IFN γ and TNF α inflammatory cytokines were found. Interestingly, only small numbers of IL-4 producing cells were found in the valves, while several cells producing IL-10 were observed. These data strongly indicated that the low numbers of IL-4 producing cells may contribute to the progression of valve lesions in RHD (18). Recently, we identified large numbers of IL-17 and IL-23-producing cells in the valves; IL-17 and IL-23 are a Th17 subset cytokines that are also frequently involved in the development of autoimmune diseases (19). All of the events currently known to be involved in inflammation and infiltration of the heart tissue by T cells are summarized in Table 2.

AUTOIMMUNE REACTIVITY

The existence of similar or identical antigens in microbes (virus, bacteria, and other pathogens) and their hosts enable the microbe to evade the host immune response. The mechanism known as "molecular mimicry," by which self antigens are recognized after an infection by cross reactivity, was introduced by Damian (20).

The presence of heart-reactive antibodies was described more than 50 years ago in sera from animals immunized with streptococcal cell wall products and in sera from acute RF and RHD patients.

Using immunofluorescence techniques, Kaplan found immunoglobulins and complement bound to the myocardium of acute RF patients (21). Studies conducted by Zabriskie et al. gave support to the hypothesis that RF has an autoimmune origin by describing the presence of antibodies that were cross reactive with streptococcal membrane antigens in acute RF sera (22).

Many studies have focused on identifying the cross-reactive streptococcal epitope recognized by antibodies in sera from both animals and humans (23). Identification of the amino acid sequences of the N-terminal portion of the M protein in the 1980s led to the discovery of cross-reactive epitopes. The molecular mimicry between group A streptococcal proteins and several human-tissue proteins leads to the autoimmune reactions in the diverse phenotypes of the disease (24). Sydenham Chorea (SC), one of the major manifestations of the disease affects the central nervous system (CNS), in which lysoganglioside GM₁ from neuronal cells are the targets of cross-reactive antibodies against *N*-acetyl- β -D-glucosamine, an antigen that is present in the cell

Table 2 | Molecules involved with heart-tissue cellular infiltration.

Adhesion molecules (VCAM, ICAM) P-selectin and integrins are overexpressed in the heart-tissue and facilitates the cellular infiltration, valve scarring
Specific chemokines such as CCL3/MIP1 α gene expression up regulated in the myocardium and CCL1/I-309 and CXCL9/Mig in the valves recruited auto-reactive T cells
High numbers of TNF- α and IFN- γ , IL-17, and IL-23 secreting mononuclear cells are mediators of myocardium and valvular inflammation and drive the autoimmune response
Low numbers of mononuclear IL-4 secreting cells in the valves probably lead to permanent and progressive valvular damage
Infiltrating T cells are predominantly CD4 $^{+}$ (~80%)
Antigen-driven oligoclonal T cells are expanded in the myocardium and valves and recognize streptococcal M peptides and heart-tissue proteins by cross reactivity
The degeneracy of TCR and the epitope spreading mechanism allowed the recognition of several streptococcal and human proteins with some degree of homology (sequences or conformational or chemical properties)

wall of *S. pyogenes* (25). In RHD, the valves are severely damaged by both humoral and cellular autoimmune reactions.

The role of the cellular arm of the immune response in RF only began to be investigated 25 years after the description of heart-reactive antibodies in the sera of RF patients. The first studies focused on the reactivity of T cells from the peripheral blood of RF and RHD patients against streptococcal M protein (26, 27). These studies were followed by the description of increased numbers of CD4 $^{+}$ cells in the tonsils and peripheral blood of RF patients when compared with healthy subjects (28). The cytotoxic activity of CD8 $^{+}$ T cells from normal peripheral blood toward immortalized human heart cells was also described (29). It is now well established that heart-tissue inflammation starts by pericarditis followed by myocarditis and valvulitis, which cause serious damage to the heart valves due to the infiltration of both auto-reactive antibodies and T lymphocytes, leading to the development of valvular lesions and consequently, RHD.

Notably, CD4 $^{+}$ cells are predominant in the rheumatic lesions of the heart tissue. An analysis of the reactivity of both heart-tissue infiltrating T cells and peripheral cells against both the N-terminal region M protein-derived peptides and heart-tissue proteins noted three immunodominant regions of the M5 protein (residues 1–25, 81–103, and 163–177) that were cross reactive with both myocardium and valve-derived proteins (26, 27). The recognition of these regions was mainly in the context of the HLA-DR7 molecule, which is one of the most frequent HLA class II alleles associated with the susceptibility of the disease, as mentioned above (27). Among the heart-tissue proteins, cardiac myosin, the most abundant protein in the myocardium, is one of the targets of streptococcal cross-reactive antibodies and heart-tissue-infiltrating T cells. The autoimmune reaction against several synthetic peptides of the beta chain of the cardiac myosin light meromyosin (LMM) region were described by Ellis et al. (30) and Fae et al. (31) and are examples of autoreactivity against the myocardium tissue

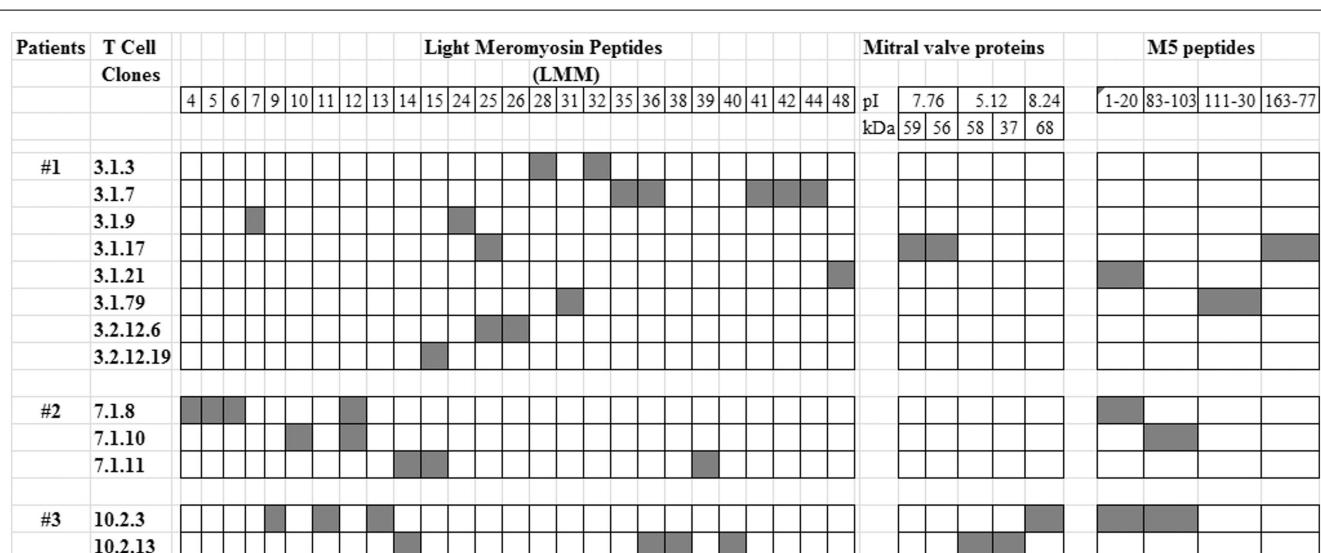


FIGURE 1 | T cell reactivity against streptococcal M5 protein and heart-tissue proteins. Valve-derived intralesional T cell clones from three RHD patients who underwent surgery for valve correction were established *in vitro* as previously described (26) and assayed for their

reactivity against mitral valve-derived proteins and synthetic peptides of light meromyosin (LMM) and the M5 protein (26, 31). Black box: positive reaction in proliferation assays with a stimulation index (SI) >2.5.

mediated by both antibodies and T cells. However, it is interesting to note that the permanent rheumatic lesions occur in the valvular tissue, most likely due to the migration of these auto-reactive T cells from the myocardium to the valvular tissue. In the valves, we found several T cell oligoclonal populations defined by the

analysis of the TCR (32, 33) that recognized M protein peptides from the N-terminal region and human cardiac myosin beta-chain peptides, as mentioned above, as well as valve tissue-derived proteins (31), as summarized in **Figure 1**. Among the valve proteins, we identified vimentin and disulfide isomerase ER-60 precursor

Table 3 | Properties of “StreptInCor” an anti-*S. pyogenes* candidate vaccine.

Characteristics	Properties	Reference
M protein C-terminal portion	55 Amino acids residues long	Guilherme et al. (36, 38)
Structure	Alpha helical and beta-sheet conformation, encompasses both T and B epitopes	Guilherme et al. (37)
Experimental assays	Several animal models (BALB/c, C57BL6, Swiss, and HLA class II transgenic mice)	Guilherme et al. (38), Guerino et al. (39), Postol et al. (40), De Amicis Marafigo et al. (41)
Immunogenicity and safety and survival rate	Specific and high titers of opsonic IgG antibodies Absence of cross reactivity with human heart-tissue proteins Long period of survival after <i>S. pyogenes</i> challenge	Guilherme et al. (38), Guerino et al. (39), Postol et al. (40), De Amicis Marafigo et al. (41)

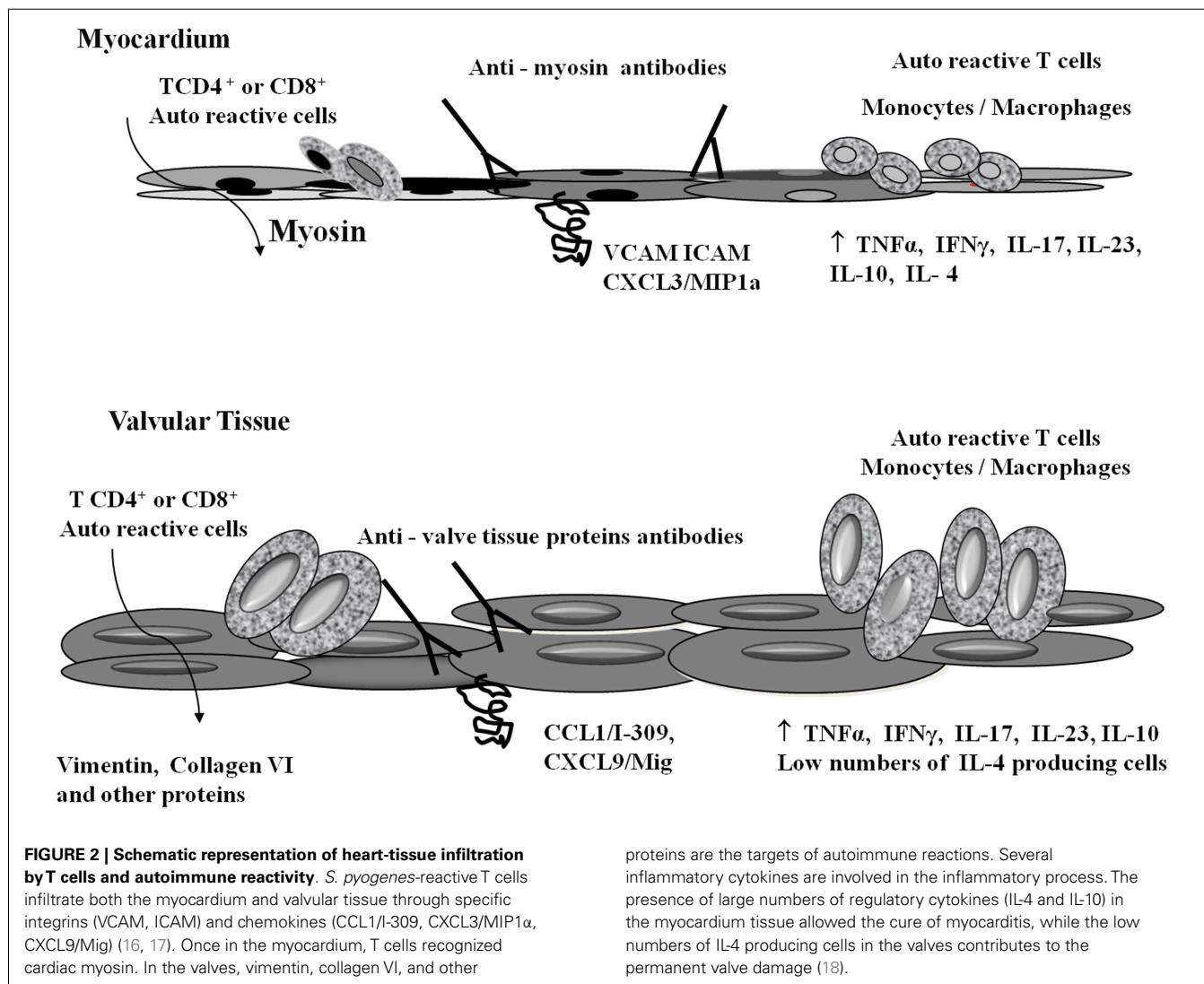


FIGURE 2 | Schematic representation of heart-tissue infiltration by T cells and autoimmune reactivity. *S. pyogenes*-reactive T cells infiltrate both the myocardium and valvular tissue through specific integrins (VCAM, ICAM) and chemokines (CCL1/I-309, CXCL3/MIP1 α , CXCL9/Mig) (16, 17). Once in the myocardium, T cells recognized cardiac myosin. In the valves, vimentin, collagen VI, and other

proteins are the targets of autoimmune reactions. Several inflammatory cytokines are involved in the inflammatory process. The presence of large numbers of regulatory cytokines (IL-4 and IL-10) in the myocardium tissue allowed the cure of myocarditis, while the low numbers of IL-4 producing cells in the valves contributes to the permanent valve damage (18).

(PDIA3) protein and a 78-kDa glucose-regulated protein precursor (HSPA5) as targets of the autoimmune reactions (34). It is interesting to note that apparently the recognition by T cells occurs in a cascade of reactivity from the myocardium to the valves.

In summary several cardiac proteins and streptococcal M peptides are recognized by both antibodies and T cells. The cross-reactivity might occur first through mimicry that results in the recognition of other human proteins, especially valve proteins, and eventually through epitope spreading and degeneracy mechanisms that amplified the number of self antigens that are targets of the autoimmune reactions.

ANTI-S. PYOGENES VACCINE DEVELOPMENT

The epidemiological growth of streptococcal diseases in undeveloped and developing countries has encouraged many groups to study vaccine candidates for preventing Group A Streptococcus (GAS) infections.

There are four anti-GAS vaccine candidates that target the M protein and eight other candidates targeting alternative streptococcal antigens, including group A CHO, C5a peptidase (SCPA), cysteine protease (Spe B), binding proteins similar to fibronectin, opacity factor, lipoproteins, Spes (super antigens), and streptococcal pili (35).

We developed a vaccine epitope (StreptInCor) composed of 55 amino acid residues of the C-terminal portion that is highly conserved among *S. pyogenes*. The StreptInCor epitope is recognized by individuals bearing different HLA class II molecules and could be considered a universal vaccine epitope (36, 37).

Using BALB-c, Swiss, and HLA class II transgenic mice, we evaluated the immune response over an extended period and found that StreptInCor was able to induce a robust immune response in all models (38–40). Vaccinated Swiss mice challenged with a virulent strain of *S. pyogenes* had 87% survival over 30 days. No cross-reaction was observed against cardiac proteins (40). The safety

of the vaccine epitope was evaluated by histopathology and no autoimmune or pathological reactions were observed in the heart or other organs (39). Anti-StreptInCor antibodies were able to neutralize/opsonize *S. pyogenes* strains, thus indicating that immunization with StreptInCor is effective against several *S. pyogenes* strains and can prevent infection and subsequent sequelae without causing deleterious reactions (41). These properties are summarized in Table 3. Taking all results into consideration, StreptInCor could be a safe and effective vaccine against streptococcus-induced disease.

CONCLUSION

The autoimmune process leading to the formation of heart lesions in RHD involves several genes that control both the innate and adaptive immune response. Consequently, several molecules play a role in the different phases of the disease. The molecular mimicry mechanism leads to the recognition of self proteins, mainly heart-tissue proteins, in the case of RHD. The autoimmune reactions are exacerbated by the inflammatory T helper 1 (Th1) and Th17 cytokines. Figure 2 summarizes the events leading to myocarditis and rheumatic valvulitis, and later chronic rheumatic heart disease.

The knowledge acquired by us and others as mentioned through the text, allowed the search of a protective epitope giving a perspective of development of an effective and safe vaccine.

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NKT cells as an ideal anti-tumor immunotherapeutic

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Human natural killer T (NKT) cells are characterized by their expression of an invariant T cell antigen receptor α chain variable region encoded by a $V\alpha 24J\alpha 18$ rearrangement. These NKT cells recognize α -galactosylceramide (α -GalCer) in conjunction with the MHC class I-like CD1d molecule and bridge the innate and acquired immune systems to mediate efficient and augmented immune responses. A prime example of one such function is adjuvant activity: NKT cells augment anti-tumor responses because they can rapidly produce large amounts of IFN- γ , which acts on NK cells to eliminate MHC negative tumors and also on CD8 cytotoxic T cells to kill MHC positive tumors. Thus, upon administration of α -GalCer-pulsed DCs, both MHC negative and positive tumor cells can be effectively eliminated, resulting in complete tumor eradication without tumor recurrence. Clinical trials have been completed in a cohort of 17 patients with advanced non-small cell lung cancers and 10 cases of head and neck tumors. Sixty percent of advanced lung cancer patients with high IFN- γ production had significantly prolonged median survival times of 29.3 months with only the primary treatment. In the case of head and neck tumors, 10 patients who completed the trial all had stable disease or partial responses 5 weeks after the combination therapy of α -GalCer-DCs and activated NKT cells. We now focus on two potential powerful treatment options for the future. One is to establish artificial adjuvant vector cells containing tumor mRNA and α -GalCer/CD1d. This stimulates host NKT cells followed by DC maturation and NK cell activation but also induces tumor-specific long-term memory CD8 killer T cell responses, suppressing tumor metastasis even 1 year after the initial single injection. The other approach is to establish induced pluripotent stem (iPS) cells that can generate unlimited numbers of NKT cells with adjuvant activity. Such iPS-derived NKT cells produce IFN- γ *in vitro* and *in vivo* upon stimulation with α -GalCer/DCs, and mediated adjuvant effects, suppressing tumor growth *in vivo*.

Keywords: NKT cells, adjuvant effects, clinical trial, induced pluripotent stem cells, artificial adjuvant vector cells

DISCOVERY OF NKT CELLS EXPRESSING AN INVARIANT $V\alpha 14J\alpha 18$ ANTIGEN RECEPTOR

Natural killer T (NKT) cells are characterized by the expression of an invariant antigen receptor encoded by $V\alpha 14J\alpha 18$ in mice and $V\alpha 24J\alpha 18$ in humans (1–3). The murine invariant $V\alpha 14J\alpha 18$ NKT cell antigen receptor was identified by cloning of cDNAs encoding T cell antigen receptor (TCR) from 13 independently established hybridomas with regulatory functions (4, 5). Surprisingly at that time, Southern blot analysis of TCR usage by these 13 hybridomas had the same DNA restriction fragment length polymorphism (RFLP) patterns, even when three different enzymes, *Eco*RI, *Bam*HI, and *Hind*III were used. Because of this unusual homogeneous DNA restriction pattern, the TCR cDNAs were cloned and could be classified into four types at the nucleotide level, all of which were composed of $V\alpha 14$ and $J\alpha 18$ with a 1-nt N region. The N region was different in each clone, a C, A, T, or G nucleotide. However, any nucleotide addition in the N region at this position becomes invariant at the amino

acid level, because this N region is the third base of a glycine codon (5).

By RNase protection assays using antisense $V\alpha 14J\alpha 18$ of C57BL/6 (B6) origin as a probe, we detected a single 630 bp band in B6, a single 400 bp band in BALB/c, and 630/400 double bands in DBA/2 mice. Quite remarkably, this band(s) represented 2–4% in the total TCR α expression in these mice (6). The theoretical expression frequency of any one particular TCR α is calculated to be $1/10^6$, because the total TCR α chain repertoire is around 10^8 and there are 100 $V\alpha$ segments in the TCR α loci. Therefore, the $V\alpha 14J\alpha 18$ expression frequency detected in unprimed mice was more than 10^4 times higher than expected, suggesting that $V\alpha 14^+$ NKT cells are clonally expanded under physiological conditions, likely due to their intrinsic autoreactivity. Another interesting finding was that the invariant $V\alpha 14J\alpha 18$ receptor is used only by NKT cells and not by conventional $\alpha\beta$ T cells. This was shown conclusively when the invariant $V\alpha 14J\alpha 18$ together with TCRV $\beta 8.2$ was introduced into RAG-knockout (KO) mice; only NKT cells and

not conventional $\alpha\beta$ T cells or NK cells developed (7). These and other studies confirmed that expression of V α 14J α 18 in mice and V α 24J α 18 in human is a unique NKT cell signature.

DISCOVERY OF THE NKT CELL LIGAND

The ligand for NKT cells was identified as α -galactosylceramide (α -GalCer), which is presented by the MHC class I-like CD1d molecule. However, unlike MHC class I molecule with polymorphic in nature, CD1d is monomorphic among species, indicating that α -GalCer can be used in any potential NKT cell therapy for all humans. The glycolipid nature of the NKT cell ligand was suggested by experiments using mice lacking the transporter associated with antigen processing (TAP), which is essential for translocation of cytoplasmic peptides generated by the ubiquitin-proteasome proteolytic pathway into the endoplasmic reticulum (ER) to make a stable complex with MHC class I molecules. The MHC peptide complex is required to select CD8 T cells, therefore, in TAP-KO mice, CD8 T cells are not generated. However, by RNase protection assays using the invariant V α 14J α 18 as a probe, we could detect significant levels of protected bands in TAP-KO mice but not in β 2M-KO mice, suggesting that the ligand is not a peptide, but likely to be a glycolipid in conjunction with a β 2M-associated MHC-like molecule (8). The MHC-like molecule turned out to be CD1d, which has two large hydrophobic pockets, A' and F', that can bind the two long fatty acid chains of the ceramide portion of α -GalCer (9). Therefore, we screened various synthetic glycolipids and found the essential structure-function relationships critical for the NKT cell recognition, such as: (1) α -linkage between the sugar moiety and the ceramide portion of α -GalCer but not β -GalCer, (2) a 2'-OH configuration on the sugar moiety different from α -ManCer, and (3) a 3'-OH on the sphingosine of α -GalCer (10).

Furthermore, by using alanine substitution to mutagenize CD1d, we also identified important amino acids on CD1d, such as Ser76, Arg79, Asp80, Glu83, and Gln153, for activation of NKT cells in mice (11). In 2007, Borg et al. succeeded in crystallizing the triple complex of α -GalCer/human V α 24J α 18/TCRV β 11/human CD1d (12). Interestingly, the V α 24J α 18 chain docks in parallel with the CD1d cleft without any direct contribution of the TCR β chain to ligand binding. This configuration is quite different from the mode of ligand recognition by the TCR β chain of conventional $\alpha\beta$ T cells, in which only the TCR β but not the TCR α chain recognizes the MHC bound peptide in a diagonal position.

Analysis of the structure also revealed that the first four amino acids (Asp94, Arg95, Gly96, and Ser97) of J α 18, which are conserved in mouse and human, are essential for binding with both CD1d and α -GalCer. The J α 18Asp94 binds with CD1dArg79, J α 18Arg95 with CD1dArg79/Ser76/Asp80 and the 3'-OH on the sphingosine, J α 18Gly96 with the 2'-OH on galactose, and J α 18Ser97 with CD1dGln150. Interestingly, the CD1d amino acid, Glu83, defined as important in functional assays with CD1d mutants, is important for binding with the TCR β chain to make a stable complex with CD1d but has no direct contribution to the ligand binding itself. Moreover, the CD1d amino acids (Ser76, Arg79, and Asp80) important for binding with either α -GalCer or J α 18 are also well conserved among species such as mouse, rat, sheep, and human (10, 13–15). Thus, α -GalCer, identified as

an NKT cell ligand in mice can also be used to activate human NKT cells.

NKT CELL-MEDIATED ADJUVANT EFFECTS ON INNATE AND ADAPTIVE IMMUNITY AGAINST CANCER

In general, tumor cells do not contain any adjuvant materials, so that it is difficult to induce proliferation of specific T cell clones to mount anti-tumor responses in patients. On this particular point, α -GalCer overcomes these problems by its intrinsic adjuvant activity, inducing clonal expansion of tumor-specific T cell cells as well as activating various innate cell types (16). In the initial anti-tumor response after stimulation with α -GalCer/DCs, NKT cells immediately produce large amounts of IFN- γ , which acts on DCs, NK cells, and neutrophils in the innate immune system to eliminate MHC negative tumor target cells and, at the same, also on CD8 cytotoxic T cells and CD4 Th1 cells to kill MHC positive tumor cells, resulting in tumor eradication (Figure 1) (1, 17, 18). Therefore, NKT cell-targeted therapy is expected to overcome the major problem of current anti-cancer immunotherapies – recurrent tumors – due to their targeting of only one type of effector cell (10, 19, 20). For example, in the immunotherapy using tumor peptide CTL or antibodies against PD-1 or CTLA4, the target is the CD8 killer T cell, which kills MHC positive but not negative tumor cells, resulting in tumor recurrence (21). Similarly, in the artificial cells recently developed by the forced expression of Rae1/H60 (NKG2D-L), Mult-1 (NKG2D-L), or CD70 (TNF-L), the target cells are NK cells, which will eliminate MHC negative, but not MHC positive tumor cells (22).

Tumors in general contain both MHC positive and negative cells. Therefore, for an optimal therapy, both MHC types of tumor cells should be eliminated simultaneously by activating both innate and adaptive immune responses (Figure 1A). Since only NKT cells, but not other immune cells, activate NK and CD8 killer T cells at the same time, thus eliminating both MHC positive and negative tumor cells, the NKT cell-targeted therapy is a promising strategy for cancer treatment (Figures 1B,C).

NKT CELL-MEDIATED ADJUVANT EFFECTS ON DC MATURATION

Another important NKT cell function is their ability to interact with immature DCs in the presence of α -GalCer to induce DC maturation (17). Therefore, NKT cell-targeted therapy is also useful for advanced cancer patients, who often suffer from severe immunodeficiency. DCs in these advanced cancer patients are usually immature because of the presence of immune suppressive cytokines, such as IL-10 or TGF β , produced by tumor cells (Figure 1A) (23). The immature DCs are able to capture tumor antigens, but unable to activate specific T cells. However, immature DCs presenting α -GalCer are activated by NKT cells through CD40-CD40L interactions to produce IFN- γ , which induce full DC maturation (24). This leads to a robust interleukin (IL)-12 response to further activate NKT cells, followed by activation of CD8T cells and NK cells (17, 24).

The DC maturation by activated NKT cells is a prominent strategy for the enhancement of protective innate and acquired immune responses. To investigate the mechanisms of bystander potential of α -GalCer-activated NKT cells, an experimental system

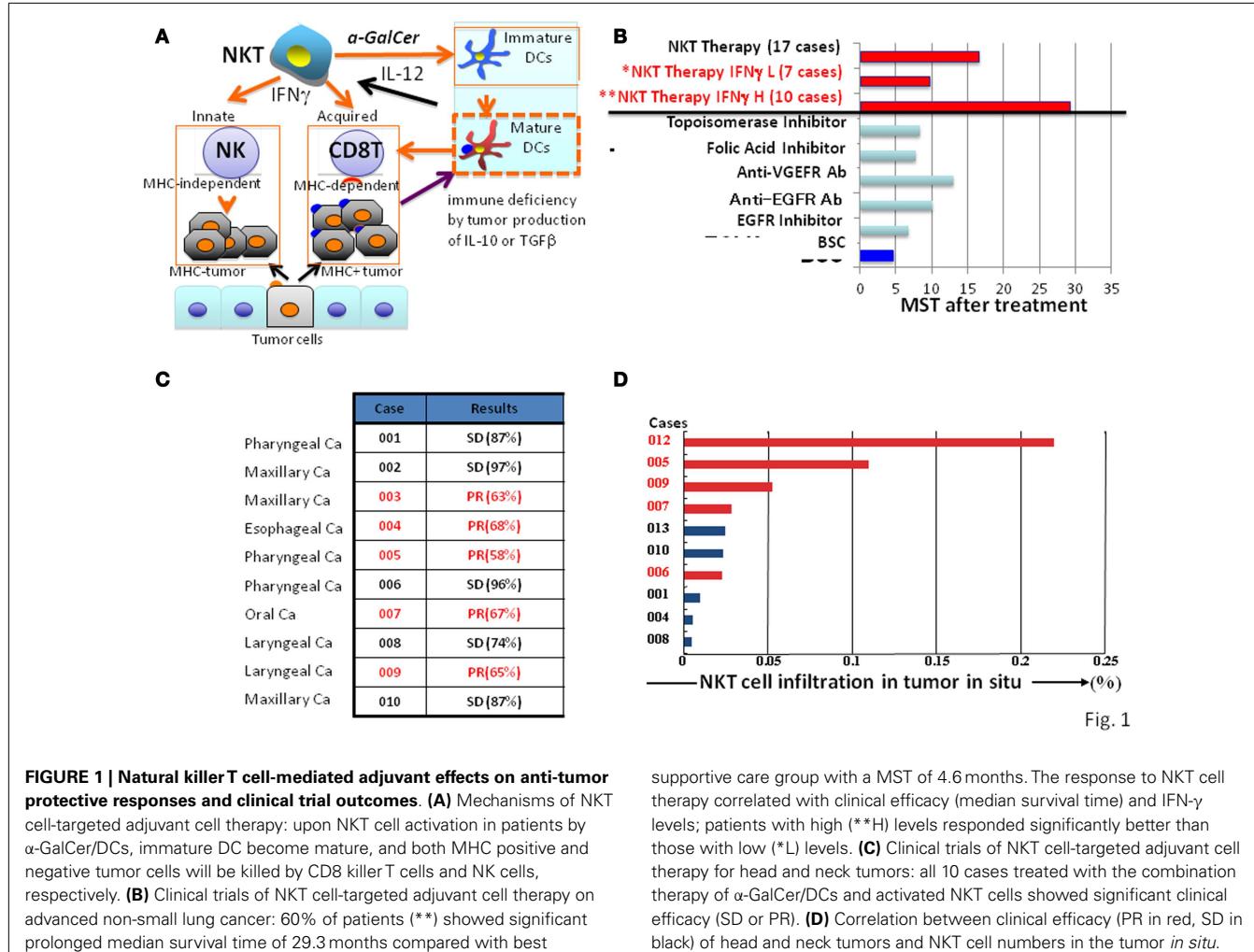


Fig. 1

FIGURE 1 | Natural killer T cell-mediated adjuvant effects on anti-tumor protective responses and clinical trial outcomes. **(A)** Mechanisms of NKT cell-targeted adjuvant cell therapy: upon NKT cell activation in patients by α -GalCer/DCs, immature DC become mature, and both MHC positive and negative tumor cells will be killed by CD8 killer T cells and NK cells, respectively. **(B)** Clinical trials of NKT cell-targeted adjuvant cell therapy on advanced non-small lung cancer: 60% of patients (**) showed significant prolonged median survival time of 29.3 months compared with best

supportive care group with a MST of 4.6 months. The response to NKT cell therapy correlated with clinical efficacy (median survival time) and IFN- γ levels; patients with high (**H) levels responded significantly better than those with low (*L) levels. **(C)** Clinical trials of NKT cell-targeted adjuvant cell therapy for head and neck tumors: all 10 cases treated with the combination therapy of α -GalCer/DCs and activated NKT cells showed significant clinical efficacy (SD or PR). **(D)** Correlation between clinical efficacy (PR in red, SD in black) of head and neck tumors and NKT cell numbers in the tumor *in situ*.

using immunization with OVA-loaded TAP-deficient spleen cells loaded with OVA after permeabilization by osmotic shock was developed. In this system, OVA was used as an artificial tumor antigen to induce OVA-specific CD8 T cells to kill OVA-bearing tumor cells. Only after α -GalCer administration, IFN- γ production by NK and CD8T cells was observed (see Figure 2A). Under these conditions, the clonal expansion of OVA-specific CD8 T cells and strong anti-tumor responses develop in the mice, and the response requires co-administration of α -GalCer (17).

CLINICAL TRIAL OF NKT CELL-TARGETED THERAPY FOR ADVANCED LUNG CANCER AND HEAD AND NECK TUMORS

For effective NKT cell activation, α -GalCer/DC has distinct advantages to induce significant expansion of NKT cells and to inhibit *in vivo* tumor growth in a mouse model of metastatic lung cancer and liver metastasis in melanoma (25, 26). In a preclinical study, we used mouse melanoma cells, which were injected into the spleen to induce liver metastasis. Treatment of tumor-bearing mice by intravenous administration of α -GalCer/DCs (3×10^6) resulted in complete eradication of the liver metastasis within 7 days after treatment (27).

Based on the dramatic effects of α -GalCer/DCs in the pre-clinical studies, a clinical trial of NKT cell-targeted immunotherapy was conducted at Chiba University hospital in patients with advanced non-small cell lung cancer to evaluate the safety, feasibility, immunological responses, and clinical outcomes (28). Seventeen patients with advanced or recurrent non-small cell lung cancer refractory to the standard treatments, including surgery, chemotherapy, and radiation therapy, completed the protocol. The patient's peripheral blood mononuclear cells (PBMCs) obtained by apheresis were cultured with GMP grade GM-CSF and IL-2 for 7 days and then pulsed with α -GalCer (29). The α -GalCer-pulsed PBMCs were then intravenously administered (1×10^9 cells/m 2 /injection) back into autologous patients twice with a 1-week interval followed by two courses with a 1-month interval between the second and third administration.

In the 17 patients who completed the protocol of a phase IIa clinical trial, the treatment was well-tolerated, and no severe adverse events related to the cell therapy were observed (28, 30). To monitor IFN- γ production by NKT cells from the patients, an enzyme-linked immunospot (ELISPOT) assay was performed (31). The results demonstrated that a significant increase in the number of IFN- γ -producing PBMCs was detected in 10 out of

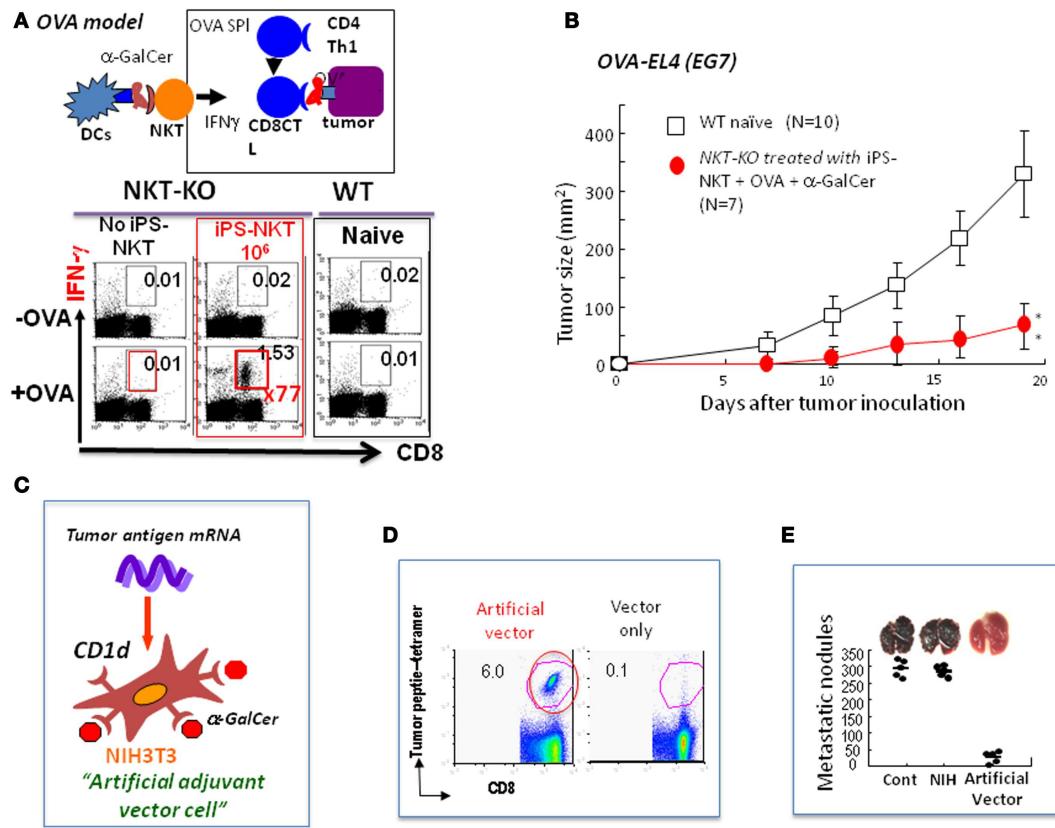


FIGURE 2 | Future directions for NKT cell-mediated cancer therapy.

(A) Experimental model using OVA as an artificial tumor antigen to demonstrate NKT cell-mediated adjuvant activity (OVA model): the NKT-KO mice that had received iPS-derived NKT cells were immunized with OVA-loaded TAP-deficient spleen cells permeabilized by osmotic shock. A week later, the CD8 killer T cells in these immunized mice were analyzed for IFN- γ production after restimulation with OVA antigen *in vitro*. A significant increase in the number of antigen-specific IFN- γ producing CD8 killer T cells was detected in mice transferred with iPS-derived NKT cells. **(B)** Inhibition of the growth of OVA-expressing EL4 (EG7) tumor cells by NKT cell-mediated adjuvant therapy using iPS-derived NKT cells *in vivo* in the OVA model. A significant suppression of tumor growth *in vivo* was detected. **(C)** Generation of allogeneic artificial adjuvant vector cells. Artificial adjuvant vector cells were

loaded with α -GalCer/CD1d and transfected with tumor mRNA. **(D)** Detection of long-term memory antigen-specific CD8 killer T cells even 1 year after a single injection of artificial adjuvant vector cells. Antigen-specific CD8 T cell responses in mice immunized with artificial adjuvant vector cells were analyzed using tetramer staining 1 year later. OVA was used in these experiments. **(E)** Suppression of melanoma lung metastasis after treatment with artificial adjuvant vector cells. Mice were intravenously injected with B16 melanoma cells to induce lung metastasis and, then 3 h later, intravenously with artificial adjuvant vector cells without tumor mRNA. The formation of metastatic nodules analyzed 2 weeks after melanoma cell injection was significantly suppressed according to the mechanisms of the activation of both NKT and NK cells but not that of CD8 killer T cells induced by artificial adjuvant vector cells carrying only α -GalCer/CD1d without tumor mRNA.

17 patients, which was correlated with a significantly prolonged median survival time (MST; 29.3 months) in comparison with the group with no increase compared to the pretreatment status in IFN- γ -producing cells (MST of 9.7 months) (Figure 1B) (32). The α -GalCer-reactive IFN- γ spot forming cells appeared to include both NKT cells and NK cells (31, 33), consistent with the notion that α -GalCer-activated NKT cells subsequently stimulate NK cells to produce IFN- γ (34, 35). We also investigated NKT cell infiltration in the surgically resected tumor samples and found a significant increase (25- to 60-fold) in the number of NKT cells in the tumor *in situ* (36). Because of the clinical correlation between increased IFN- γ production and prolonged overall survival, we conclude that IFN- γ may be a good biological marker for predicting clinical efficacy of this treatment. Although this prediction cannot be made prior to α -GalCer/DCs administration,

the monitoring of IFN- γ production would still be valuable for patients receiving this immunotherapy. Although none of the cases showed significant tumor regression, the overall MST of all 17 patients (18.6 months) was superior to that of patients with best supportive care (4.6 months) or those treated with other types of therapies (average 10 months) in Figure 1B (37–40).

In the case of the head and neck tumors, we used a combination therapy with α -GalCer/DCs (10^8) and activated NKT cells (5×10^7) and completed 10 cases, including patients with pharyngeal, laryngeal, esophageal, maxillary, and oral carcinomas, who had advanced or recurrent disease after standard treatments (41). All treated patients showed either a partial response or achieved a stable disease state, indicating significant clinical efficacy (Figure 1C), which was associated with significant NKT cell infiltration into the tumor *in situ* (Figure 1D). To evaluate

clinical efficacy, a computed tomography (CT) scan was performed a few days before enrollment and also after the treatment. In some cases with partial responses, we observed that the enhanced area decreased in size, and necrosis appeared at the center of the tumor.

These encouraging clinical studies on advance lung cancers and head and neck tumors warrant further evaluation of NKT cell-targeted immunotherapy for survival benefit. In general, the immunotherapy may be more effective in patients with low tumor burden. Currently, we have been conducting α -GalCer/DC therapy for stage IIA to IIIA lung cancer patients with small tumor foci, including remaining micro-metastasis after radical surgery or after receiving the established first-line therapy in collaboration with National Hospital Organization.

FUTURE DIRECTIONS FOR NKT CELL-MEDIATED CANCER THERAPY USING iPS-DERIVED NKT CELLS

Although an NKT cell-targeted therapy has been shown to have significant clinical efficacy, only one third of patients are eligible in the case of advanced non-small lung cancer patients; the frequency of NKT cells in the other patients is too low. To overcome this problem, we established *in vitro* methods for generation of unlimited numbers of functional NKT cells, which then can be transferred into the patients whose endogenous NKT cell numbers are limited.

Induced pluripotent stem (iPS) cells were generated from mature NKT cells using *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* genes and then were developed into functional NKT cells *in vitro* in the presence of IL-7 and Flt3L according to the conventional protocol (42–44). The NKT cells generated *in vitro* from iPS-NKT cells were functional in the *in vivo* setting using the experimental model of OVA as an artificial tumor antigen (44). When NKT-KO mice were reconstituted with iPS-derived NKT cells followed by immunization with OVA and α -GalCer, we detected a 70-fold increase in the number of OVA-specific IFN- γ producing CD8 $^{+}$ T cells above that seen in the control mice (Figure 2A). Under these conditions, the growth of the OVA-expressing EL4 (EG7) tumor cells was suppressed (Figure 2B). Thus, the iPS-derived NKT cells are able to function *in vivo*.

Before any clinical application of iPS-derived NKT cells, two immunological issues need to be addressed, one is whether GvHD is induced by NKT cells and the other is whether semi-allogeneic NKT cells will work *in vivo*, because of the clinical use of iPS-derived NKT cells under semi-allogeneic conditions. To address the first question, iPS-derived NKT cells on a B6 background and B6 or BALB/c CD4 T cells were injected into BALB/c RAG-KO mice. The results were very clear: only B6 CD4T cells, but not iPS-derived B6 NKT cells or BALB/c CD4 T cells, induced GvHD characterized by weight loss, diarrhea, skin disease development, or death after cell transfer. Concerning the second issue of the functional potential of semi-allogeneic NKT cells *in vivo* (129xB6) F1 NKT cells derived from cloned ES cells established by nuclear transfer of mature NKT cells into unfertilized eggs were injected into B6 NKT-KO mice and analyzed for their adjuvant activity in the OVA model. Significant proliferation of OVA-specific CD8 killer T cells was detected, even though these cells are eliminated in a few days. The ability to

generate NKT cells using a simple *in vitro* culture system offers a powerful approach for the establishment of optimal NKT cell therapy. Our clinical application of the iPS-derived NKT cell therapy program has now been selected as a Center for Clinical Application Research on Specific Disease/Organ (Type B) in the Research Center Network for Realization of Regenerative Medicine, Japan.

FUTURE DIRECTIONS FOR THE NEXT GENERATION OF NKT CELL-TARGETED THERAPY

For the establishment of the next generation of NKT cell-targeted therapy, we developed artificial adjuvant vector cells to induce both innate and long-term memory CD8T cell responses against cancer. In this system, allogeneic NIH3T3 fibroblasts were used as a vector cell, into which tumor antigen mRNA and CD1d with α -GalCer were introduced. In the model experiment, we used OVA mRNA as an artificial tumor antigen together with α -GalCer/CD1d to induce the NKT cell-mediated adjuvant effects *in vivo in situ* (Figure 2C) (22). The allogeneic artificial vector cells were destroyed by the host immune system soon after inoculation and all materials carried by the cells were taken up by the host DCs, which immediately stimulated host NKT cells followed by induction of DC maturation and also by activation of innate NK cells and adaptive OVA-specific CD8 killer T cells. Surprisingly, long-term memory CD8 T cell responses were induced in an antigen-specific manner and persisted even 1 year after the initial single injection and suppressed OVA-expressing tumor cell metastasis (Figures 2D,E) (45). To test if this method could be generalized, we used TRP-2, tyrosinase related protein-2, which is a weak tumor antigen expressed by both mouse and human melanoma cells as the tumor antigen, and successfully suppressed tumor growth *in vivo*. Therefore, the artificial vector cells should be useful in the future for vaccines against various tumors.

SUMMARY

Natural killer T cells bridge innate and adaptive immunity, which enhances protective immune responses and also establishes long-term memory responses. Therefore, NKT cells have important therapeutic potential. In support of this notion, clinical trials on NKT cell-targeted therapy have demonstrated clinical safety and significant clinical efficacy in terms of prolonged median overall survival time in lung cancer patients and achieved stable disease status or partial responses in head or neck cancer patients.

The powerful treatment options for the future are to establish iPS cells that can generate unlimited numbers of NKT cells with adjuvant activity *in vitro* and suppress tumor growth *in vivo*. The other option is to establish the artificial adjuvant vector cells containing tumor mRNA and α -GalCer/CD1d, which have been shown to induce tumor-specific long-term memory CD8T cell responses and to inhibit tumor growth even 1 year after single injection. Thus, these could be therapeutic candidates for the next generation of NKT cell-targeted therapy.

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Molecular mechanisms of differentiation of murine pro-inflammatory $\gamma\delta$ T cell subsets

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$\gamma\delta$ T cells are unconventional innate-like lymphocytes that actively participate in protective immunity against tumors and infectious organisms including bacteria, viruses, and parasites. However, $\gamma\delta$ T cells are also involved in the development of inflammatory and autoimmune diseases. $\gamma\delta$ T cells are functionally characterized by very rapid production of pro-inflammatory cytokines, while also impacting on (slower but long-lasting) adaptive immune responses. This makes it crucial to understand the molecular mechanisms that regulate $\gamma\delta$ T cell effector functions. Although they share many similarities with $\alpha\beta$ T cells, our knowledge of the molecular pathways that control effector functions in $\gamma\delta$ T cells still lags significantly behind. In this review, we focus on the segregation of interferon- γ versus interleukin-17 production in murine thymic-derived $\gamma\delta$ T cell subsets defined by CD27 and CCR6 expression levels. We summarize the most recent studies that disclose the specific epigenetic and transcriptional mechanisms that govern the stability or plasticity of discrete pro-inflammatory $\gamma\delta$ T cell subsets, whose manipulation may be valuable for regulating (auto)immune responses.

Keywords: $\gamma\delta$ T cells, T cell differentiation, interleukin-17, interferon- γ , transcription factors, cytokines

$\gamma\delta$ T cells, which were discovered three decades ago (1–3), remain a very puzzling population of lymphocytes. Together with $\alpha\beta$ T cells and B cells, they make up the three somatically rearranged lineages that are found in all jawed and also in jawless vertebrates (lampreys and hagfish) (4, 5), thus highlighting a strong evolutionary pressure to keep the three lymphocyte lineages together.

One of the most striking characteristics of $\gamma\delta$ T cells is their inherent ability to very rapidly secrete pro-inflammatory cytokines. This is likely attributable to the functional maturity of discrete $\gamma\delta$ T cell subsets, producing either IFN- γ or IL-17, that readily populate secondary lymphoid organs (as well as peripheral tissues) where they make a key contribution to “lymphoid stress surveillance” (6). We (7) and others (8, 9) have shown that these functional $\gamma\delta$ T cell subsets develop in the murine thymus before migration to peripheral sites (10). This review outlines our current molecular understanding of the development and function of $\gamma\delta$ T cell subsets that influence both innate and acquired immunity.

ROLES OF IFN- γ AND IL-17-PRODUCING $\gamma\delta$ T CELLS IN IMMUNE RESPONSES

By secreting large amounts of IFN- γ , $\gamma\delta$ T cells participate in controlling infection through the activation of macrophages and cytotoxic lymphocytes. IFN- γ producing $\gamma\delta$ T cells have been shown to play major protective roles during murine West Nile, herpes and influenza viral infections (11–13); *Listeria monocytogenes*, *Escherichia coli*, and *Bordetella pertussis* bacterial infections (14–18); and *Plasmodium chabaudi* and *Toxoplasma gondii* parasitic infections (19–22). Moreover, $\gamma\delta$ tumor-infiltrating lymphocytes

constitute a critical early source of IFN- γ that controls tumor development *in vivo* (23, 24).

With respect to the production of IL-17, $\gamma\delta$ T cells are a key component of the defense against infections with *Mycobacterium tuberculosis*, *E. coli*, *L. monocytogenes*, *Staphylococcus aureus*, *Candida albicans*, and *Pneumococci* (18, 25–32). One of the main functions of these IL-17-producing $\gamma\delta$ T cells is to enable extremely fast neutrophil recruitment at the site of infection.

On the other hand, IL-17-producing $\gamma\delta$ T cells have pathogenic roles in various inflammatory and autoimmune disorders (and animal models thereof), including collagen-induced arthritis (CIA) (33), experimental autoimmune encephalomyelitis (EAE) (8, 34–38), chronic granulomatous disease (39), uveitis (40), ischemic brain inflammation (41), colitis (42, 43), and psoriasis (44, 45). Moreover, IL-17 also seems to promote angiogenesis and consequently tumor growth (46) and metastasis (47).

Therefore, from a therapeutic point of view, it is of utmost importance: (i) to define in detail the $\gamma\delta$ T cell subset(s) that perform each given function; (ii) to understand the extracellular clues that regulate the development of each subset; and (iii) to identify the molecular program(s) of differentiation that control the acquisition and maintenance of a specific effector function.

Here we will essentially focus on mouse models, but to emphasize the relevance of studying specific murine effector $\gamma\delta$ T cell subsets we will highlight their human counterparts. For a comprehensive review on the differentiation of human $\gamma\delta$ T cells please refer to Ref. (48). Moreover, although the present review focuses on IFN- γ - and IL-17-secreting $\gamma\delta$ T cells, we note that some $\gamma\delta$ cell subsets produce other cytokines including IL-4, IL-5, IL-13 (49–51), IL-10 (52, 53), and IL-22 (54–56).

PHENOTYPIC DESCRIPTION OF IFN- γ - OR IL-17-PRODUCING $\gamma\delta$ T CELL SUBSETS

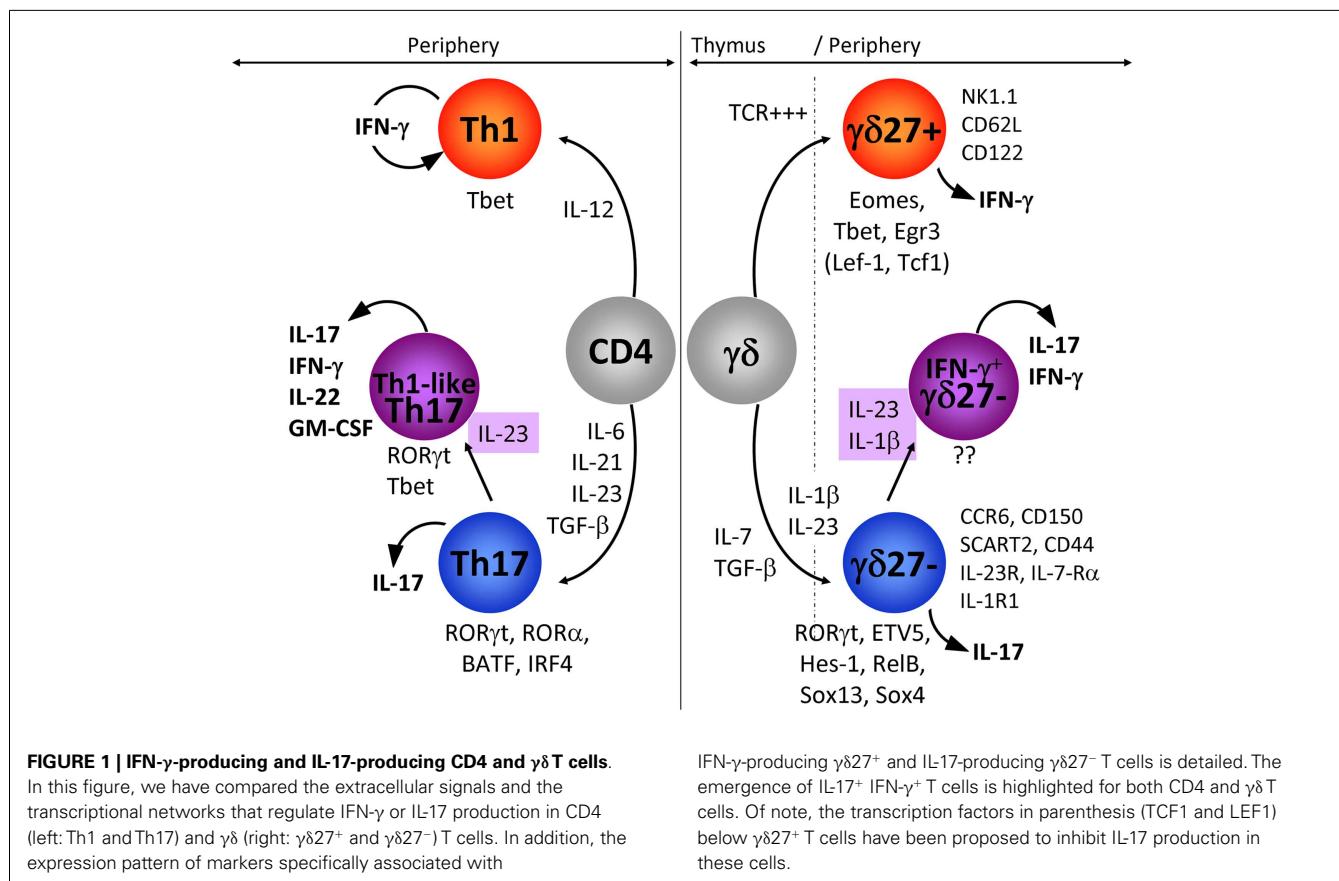
Functional $\gamma\delta$ T cell subsets in the mouse have been traditionally defined by their TCR V γ usage [please note that we use the nomenclature proposed by Heilig and Tonegawa (57)] and preferential tissue distribution. For example, epidermal V γ 5V δ 1 T cells are mainly associated with the production of IFN- γ (58), although they have also been shown to produce IL-17 in response to skin injury (59). V γ 6V δ 1 T cells that are present in the tongue, lungs, and reproductive tracts mainly produce IL-17. Moreover, V γ 1 T cells colonize the liver, spleen, and intestine preferentially secrete IFN- γ , whereas V γ 4 T cells, which recirculate through blood, spleen, and lymph nodes, and are also located in the lungs, favor IL-17 production. However, this dichotomy is not so strict as mouse V γ 4 T cells produce IFN- γ or IL-17 depending on the model studied (7, 60, 61).

Although a genome-wide transcriptional profiling of $\gamma\delta$ thymocytes segregated the expression of some genes associated with IFN- γ or IL-17 production with selective V γ chain usage (62), work from our laboratory (7), together with others (8, 63), has shown that $\gamma\delta$ T cell functions are not mutually exclusive between V γ 1 and V γ 4 T cell subsets. Our collective efforts have identified CD27 and CCR6 as useful markers of discrete pro-inflammatory $\gamma\delta$ T cell subsets: CD27 is expressed on IFN- γ -producing $\gamma\delta$ T cells whereas IL-17-producing $\gamma\delta$ T cells are CD27 $^{(-)}$ but express CCR6 (7, 54, 63) (see Figure 1 for further details). Of note, CD122

and NK1.1 constitute additional markers of IFN- γ -producing $\gamma\delta$ T cells (8, 63). Consequently, we favor categorization of $\gamma\delta$ T cell subsets based on their effector functions rather than on TCR V γ usage (10). The definition of surface phenotypes associated with effector cell functions has greatly facilitated the dissection of the molecular mechanisms that control the differentiation of IFN- γ - or IL-17-producing $\gamma\delta$ T cells.

DIFFERENCES IN CYTOKINE PRODUCTION BETWEEN $\gamma\delta$ AND CD4 T CELLS

One of the main differences between cytokine production by $\gamma\delta$ and CD4 T cells resides in the spontaneous release of cytokine by $\gamma\delta$ T cells, which strikingly contrasts with the delayed response of naïve CD4 T cells. This can be explained by $\gamma\delta$ T cells exiting the thymus already functionally competent to produce either IFN- γ or IL-17 (7–9, 64), whereas CD4 T cells require a long differentiation program in peripheral lymphoid organs that consists of activation, intense proliferation, and induction of transcription factors that selectively control the profile of cytokines produced (65). As CD4 T helper cells have been extensively studied, it is reasonable to question if the programs of differentiation that prevail in CD4 T cells also operate in $\gamma\delta$ T cells. Here we will focus on the molecular mechanisms that govern the differentiation of naïve CD4 T cells into IFN- γ -producing (Th1) and IL-17-producing (Th17) cells, as counterparts to CD27 $^+$ ($\gamma\delta$ 27 $^+$) and CD27 $^-$ CCR6 $^+$ ($\gamma\delta$ 27 $^-$) $\gamma\delta$ T cell subsets, respectively.



IFN- γ -producing $\gamma\delta$ 27 $^+$ and IL-17-producing $\gamma\delta$ 27 $^-$ T cells is detailed. The emergence of IL-17 $^+$ IFN- γ $^+$ T cells is highlighted for both CD4 and $\gamma\delta$ T cells. Of note, the transcription factors in parenthesis (TCF1 and LEF1) below $\gamma\delta$ 27 $^+$ T cells have been proposed to inhibit IL-17 production in these cells.

ENVIRONMENTAL CUES THAT GOVERN THE ACQUISITION OF TYPES 1 OR 17 EFFECTOR FUNCTIONS

Upon peripheral activation, naïve CD4 T cells are polarized toward the Th1 fate in the presence of IL-12 (66). As yet, there is no precise information as to the role of IL-12 in the development of $\gamma\delta^{27+}$ T cells although IL-12 (in synergy with IL-18) induces the production of IFN- γ by $\gamma\delta^{27+}$ T cells expressing NK1.1 (63). Our unpublished data suggest that IL-15 and, to a lesser extent IL-2, strongly promote IFN- γ production by $\gamma\delta^{27+}$ T cells (Barros-Martins et al., manuscript in preparation).

Th17 polarization entails TGF- β , IL-6, and IL-1 β , whereas IL-23 is required for maintenance and expansion (67–69). Although still controversial, the development of IL-17-producing $\gamma\delta$ T cells in the thymus (and their maintenance in the periphery) appears to be dependent on TGF- β but mostly independent of IL-6 (9, 70–73). Unexpectedly, IL-7 induced rapid and substantial expansion of IL-17-producing $\gamma\delta^{27-}$ T cells (74). Furthermore, they require IL-23 and IL-1 β for peripheral expansion and local induction of IL-17 (30, 75, 76). This is clearly evidenced by the significant reduction in IL-17-secreting $\gamma\delta$ T cell numbers following *L. monocytogenes* infection in IL-23 $^{-/-}$ and IL-23R $^{-/-}$ mice (72, 77) or in IL-1R1 $^{-/-}$ mice upon EAE induction (36). It was also shown that IL-18 synergizes with IL-23 to promote IL-17 production by $\gamma\delta$ T cells (78). IL-17 production by $\gamma\delta$ T cells can be triggered independently of TCR signaling (36, 54, 76), but it is worth noting that a small subset of CD44 $^+$ CD62L $^+$ $\gamma\delta$ T cells (a phenotype associated with $\gamma\delta^{27+}$ cells; see Figure 1) selectively recognized phycoerythrin via the TCR and became CD44 $^{+++}$ CD62L $^-$ cells that produced IL-17 (79). In this system too, propagation of the IL-17-response by PE-specific $\gamma\delta$ T cells relies on IL-23. Finally, it has been shown that IL-17 derived from CD4 T cells is a negative regulator of IL-17 $^+$ $\gamma\delta$ T cell development in adult thymus (64), underlying the potential danger that large numbers of these pro-inflammatory cells likely represent to the host.

TRANSCRIPTIONAL REGULATION OF CYTOKINE PRODUCTION IN $\gamma\delta$ AND CD4 T CELLS

During Th1 polarization of naïve CD4 T cells, IL-12 activates STAT4 (80), but it is unclear if this IL-12/STAT4 axis plays any role in IFN- γ production by $\gamma\delta^{27+}$ T cells. The “master” transcription factor that regulates the production of IFN- γ in CD4 T cells is T-bet (81, 82). Whereas Th1 differentiation is fully abrogated in the absence of T-bet, $\gamma\delta^{27+}$ T cells only partially require T-bet to produce IFN- γ (83–85). Other transcription factors that have been proposed to play major roles in $\gamma\delta$ T cells include Eomes and Egr3 (58, 84), although the potential cooperation between these three transcription factors within specific $\gamma\delta$ T cell subsets still needs to be clarified.

Th17 differentiation relies on cytokines that target STAT3 and lead to the expression of the master transcription factor retinoic-related orphan receptor γ t (ROR γ t) (86) that synergizes with ROR α (87), together with IRF4 (88) and BATF (89) to propagate IL-17 production. *In vivo* Th17 cell differentiation also involves the aryl hydrocarbon receptor (AhR) (90, 91). All together this led to the concept that a specific transcriptional network is operating during initiation and stabilization of the Th17 phenotype (92).

IL-17 production by $\gamma\delta^{27-}$ T cells is also strictly dependent on ROR γ t (70, 85, 86, 93). However, the similarities between the Type 17 program of $\gamma\delta$ and CD4 T cells end with this transcription factor, since STAT3 and IRF4 have been shown to be dispensable for the differentiation of IL-17 $^+$ $\gamma\delta$ T cells (93, 94). Of note, detection of IL-17 $^+$ $\gamma\delta$ T cells in STAT3-deficient mice further suggests that IL-6, IL-21, and IL-23 are unlikely to play major roles for their development, although they may be involved in peripheral reactivation of these $\gamma\delta$ cells. AhR has also been shown to be dispensable for IL-17 but required for IL-22 production by $\gamma\delta$ T cells (54). Finally, our unpublished data show that IL-17-producing $\gamma\delta$ T cells are generated in the absence of ROR α or BATF (Barros-Martins et al., manuscript in preparation). Thus, many transcription factors that are essential for Th17 development are not required for the differentiation of their IL-17 $^+$ $\gamma\delta$ T cell counterparts.

In fact, $\gamma\delta^{27-}$ T cells appear to rely on distinct molecular pathways to regulate their production of IL-17. Namely, several transcription factors such as Sox13 and Sox4 (95, 96), Hes-1 (93), RelB (97), ETV5 (98) along with the kinase Blk (99), selectively participate in IL-17 production by $\gamma\delta$ T cells. On the other hand, TCF1 and LEF1 are negative regulators of IL-17 expression in $\gamma\delta$ T cells (96).

These data clearly highlight that distinct mechanisms govern the production of IFN- γ and IL-17 in CD4 and $\gamma\delta$ T cells (Figure 1). Further studies are warranted to precisely delineate the molecular components of the Types 1 and 17 programs of $\gamma\delta$ T cells.

STABILITY VERSUS PLASTICITY OF $\gamma\delta$ T CELL SUBSETS

Initially studies suggested that the segregation between IL-17 and IFN- γ production that emerged in the thymus appeared to be stable in the two $\gamma\delta$ T cell subsets, including in peripheral lymphoid organs and upon challenge with infectious agents *in vivo* (7, 76). Furthermore, incubating the $\gamma\delta^{27+}$ cells in Th17 conditioning milieu, or the $\gamma\delta^{27-}$ cells in Th1 conditioning milieu, failed to “convert” their cytokine production profile (63, 85). It was therefore assumed that, due to thymic “functional pre-commitment,” murine $\gamma\delta$ T cells harbored little plasticity, in stark contrast with CD4 T cells (100).

To get further insight into the molecular mechanisms of stable commitment of the $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ T cell subsets to their respective effector functions, we undertook the first genome-wide comparison of the chromatin landscape of these two $\gamma\delta$ T cell subsets. We analyzed the distribution of methylation marks on histone H3 (H3). Methylation of lysine 4 (H3K4me2/3) signs actively transcribed loci, whereas methylation of lysine 27 (H3K27me3) represses the accessibility for the transcriptional machinery (101, 102). As expected, we found that gene loci associated with IL-17 production harbored active histone modifications only in $\gamma\delta^{27-}$ T cells. By contrast, and to our surprise, gene loci associated with IFN- γ showed active H3K4me2 profiles in both $\gamma\delta$ T cell subsets. Furthermore, whereas *Il17* and related genes were exclusively transcribed in $\gamma\delta^{27-}$ cells, *Ifng* and genes that control its expression were transcribed in both $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ T cells (although to a lesser extent in the latter subset). Thus, *Ifng* and “Type 1” factors are epigenetically and transcriptionally primed for expression in both $\gamma\delta^{27+}$ and $\gamma\delta^{27-}$ T cells, which led us to hypothesize

that $\gamma\delta^{27-}$ T cells could acquire IFN- γ expression under specific conditions.

IDENTIFICATION OF $\gamma\delta$ IL-17 $^+$ IFN- γ $^+$ DOUBLE PRODUCERS

By performing a series of *in vitro* experiments, we found that IL-1 β strongly synergizes with IL-23 to induce IFN- γ expression specifically in IL-17-producing $\gamma\delta^{27-}$ cells (Figure 1). Importantly, epigenetic and transcriptional polarization of IL-1R1 and IL-23R predicted the responsiveness of $\gamma\delta^{27-}$ cells, but not $\gamma\delta^{27+}$ cells, to these two inflammatory cytokines.

This plastic behavior of $\gamma\delta^{27-}$ T cells was also observed *in vivo*, as IL-17 $^+$ IFN- γ $^+$ $\gamma\delta^{27-}$ cells could be found in the peritoneal cavity of mice bearing ovarian tumors (85). Moreover, these cells have been detected in the brain of mice suffering from early stages of EAE (103); and in the mesenteric lymph nodes of mice infected with *L. monocytogenes* (104).

Double producing IL-17 $^+$ IFN- γ $^+$ $\gamma\delta$ T cells have also been characterized in humans. Thus, while a fraction of neonatal and adult V γ 9V δ 2 T cells incubated with IL-6, IL-1 β , and TGF- β in the presence of TCR agonists produced IL-17A, the addition of IL-23 resulted in IFN- γ co-production (105). Moreover, IL-17 $^+$ IFN- γ $^+$ cells of both V81 and V82 subtypes were found in the circulation of HIV $^+$ patients (106).

Thus, although their precise physiological relevance is still to be established, IL-17 $^+$ IFN- γ $^+$ double producers can clearly be a distinct component of the $\gamma\delta$ T cell response in scenarios of infection, cancer, and autoimmunity.

CD4 IL-17 $^+$ IFN- γ $^+$ DOUBLE PRODUCERS AND THEIR BIOLOGICAL RELEVANCE

IL-17 $^+$ IFN- γ $^+$ double producers have been well characterized in the CD4 T cell compartment (Figure 1). In particular, both murine (107, 108) and human (109–111) Th17 cells often show plasticity in acquiring IFN- γ production. Strikingly, these IFN- γ $^+$ (Th1-like) Th17 cells have been strongly associated with pathogenicity in murine (107, 112, 113) and human (114) autoimmune syndromes. The molecular determinants of pathogenicity of Th1-like Th17 cells are still controversial, with studies either implicating T-bet and IFN- γ (108, 112, 115) or not (116–118). Nonetheless, it is clear that IL-23 is a major driver of Th1-like Th17 cell pathogenicity (108, 112, 117).

Similar studies on *in vivo* models should now explore the potential pathogenic role of $\gamma\delta$ IL-17 $^+$ IFN- γ $^+$ double producers. This notwithstanding, it has been proposed that, in response to *L. monocytogenes*, IL-17 $^+$ /IFN- γ $^+$ producing $\gamma\delta^{27-}$ cells become memory cells capable of providing enhanced protection against recall infection (104). Thus, $\gamma\delta$ IL-17 $^+$ IFN- γ $^+$ double producers may potentially play host-protective versus pathogenic roles in distinct disease models, which will be an interesting topic for future research.

CONCLUDING REMARKS

As a population, $\gamma\delta$ T cells perform a wide variety of functions, but discrete subsets have more restricted effector properties. Although thymic development endows a significant fraction of murine $\gamma\delta$ T cells with a “pre-determined” effector function, recent data provide strong evidence for functional plasticity in the periphery (particularly for $\gamma\delta^{27-}$ T cells).

Several fundamental questions remain unanswered. Is functional plasticity restricted to $\gamma\delta$ T cells located in secondary lymphoid organs or does it extend to subsets that populate epithelial tissues/mucosas? Why did $\gamma\delta$ T cells and CD4 T cells evolve different transcriptional networks to regulate the production of the same pro-inflammatory cytokines? What are the specific roles of $\gamma\delta$ IL-17 $^+$ IFN- γ $^+$ double producers in models of infection, cancer, and autoimmunity? More globally, it will be important to dissect the physiological stimuli that drive the activation of effector $\gamma\delta$ T cells. It is particularly puzzling that we still know so little about the role of the TCR $\gamma\delta$, and the identity of its ligands, in the differentiation and activation of functional $\gamma\delta$ T cell subsets. Answering these questions will improve our understanding of $\gamma\delta$ T cell physiology and likely provide new avenues for the design of immunotherapeutic approaches.

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Sensing microbial RNA in the cytosol

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The innate immune system faces the difficult task of keeping a fine balance between sensitive detection of microbial presence and avoidance of autoimmunity. To this aim, key mechanisms of innate responses rely on isolation of pathogens in specialized subcellular compartments, or sensing of specific microbial patterns absent from the host. Efficient detection of foreign RNA in the cytosol requires an additional layer of complexity from the immune system. In this particular case, innate sensors should be able to distinguish self and non-self molecules that share several similar properties. In this review, we discuss this interplay between cytosolic pattern recognition receptors and the microbial RNA they detect. We describe how microbial RNAs gain access to the cytosol, which receptors they activate and counter-strategies developed by microorganisms to avoid this response.

Keywords: pattern recognition receptors, RIG-I-like receptors, pathogen-associated molecular patterns, RNA helicases, cytosol, DExD/H-box helicases, innate immune escape

INTRODUCTION

When Janeway formulated the theory of pattern recognition in 1989, he proposed that host cells could sense microbial infection owing to receptors able to recognize invariant molecular structures defined as pathogen-associated molecular patterns (PAMPs). These patterns would be present in groups of pathogens, but absent in the host (1). Years later, Janeway and Medzhitov described the activity of the first mammalian member of the Toll-like receptor (TLR) family, Toll-like receptor 4 (2). TLRs comprise a family of transmembrane proteins able to recognize conserved microbial features and activate the immune response (3). Once activated, TLRs and others pattern recognition receptors (PRRs) initiate several intracellular pathways, including those mediated by nuclear factor- κ B (NF- κ B), mitogen-activated protein kinases (MAPKs), and interferon regulatory factors (IRFs). Another outcome of activation of distinct members of cytosolic PRRs is their oligomerization into multimeric cytosolic structures called inflammasomes, which activate the cysteine protease caspase-1, subsequently leading to the production of biologically active forms of pro-inflammatory cytokines (4).

Initially thought to detect exclusively microbial derived ligands, PRRs were later shown to recognize host derived danger signals, which are released in response to stress conditions such as cellular damage or tissue injury (3). Under normal physiological conditions, these ligands are not accessible to their respective PRRs and do not activate the immune system. Conversely, it was first suggested that self-DNA artificially introduced into the cytoplasm by transfection could activate NF- κ B and the MAPK pathway (5). Evidence that any DNA, regardless of its origin, can engage innate immune receptors when localized outside of the nucleus was further confirmed by the identification of several endosomal and cytosolic DNA sensors [reviewed in Ref. (6)].

In contrast to cytosolic DNA, RNA sensing in the cytoplasm raises many questions on the mechanisms used by the innate

system to specifically distinguish non-self-RNA from self-RNA. During infection, microbial RNAs share the cytosolic cellular compartment with several host RNA species, including messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), microRNA, and other small regulatory RNAs. As a consequence, cytosolic sensors must display a high affinity for specific microbial features to avoid activation by host molecules that would otherwise elicit autoimmune responses. Despite this apparent challenge, efficient detection of foreign RNA in the cytosol is essential for innate immunity. During certain viral infections, RNA may be the only microbial PAMP produced throughout most of the replication cycle. Additionally, our laboratory previously showed that recognition of bacterial mRNA in the cytosol was critical to elicit a robust innate response against bacterial infection (7). Finally, cytosolic sensing of pathogen invasion by non-immune infected cells provides the very first steps of innate response against infection, before phagocytosis-competent immune cells are recruited to the site of infection.

In this review, we summarize the current understanding of cytosolic RNA sensing. We describe instances in which microbial RNAs gain access to the cytosol, the PRRs they activate, their corresponding ligands and strategies developed by microorganisms to conceal their RNAs.

RNA ACCESS TO THE CYTOSOL

RNA entry into host cells generally takes place during the first steps of a microbial infection. We distinguish four processes leading to the presence of microbial RNA in the cytosol of eukaryotic cells, where it can engage host PRRs (Figure 1).

RELEASE OF THE VIRAL GENOME AND TRANSCRIPTION OF REPLICATION INTERMEDIATES

A first process, observed during RNA virus infection, consists of viral genome release into the cytosol during the cell entry

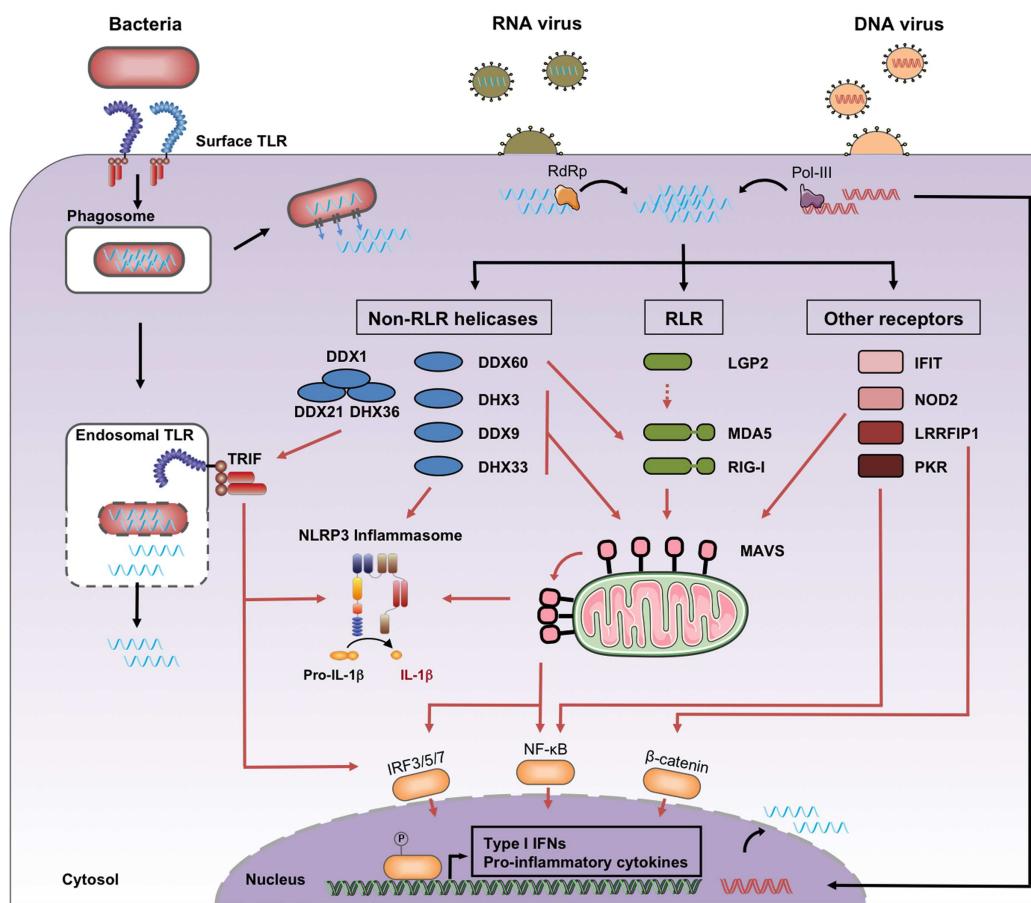


FIGURE 1 | Cytosolic recognition of microbial RNA. Genomic RNA from RNA viruses access the cytosol immediately after the cell entry step of the replication cycle, where it may be amplified by viral RNA-dependent RNA polymerase (RdRp). Genomic DNA from DNA viruses is transcribed by viral or cellular RNA polymerase, including the cytosolic RNA polymerase III. Bacterial RNA can access the cytosol

through the activity of auxiliary secretion systems or during passive leakage of phagosomal products. Once in the cytosol, microbial RNA binds different families of PRRs classified as RLRs, non-RLR helicases, and other receptors. Downstream signaling pathways include activation of MAVS, TRIF, and the NLRP3 inflammasome. Black arrows, RNA entry; red arrows, signaling pathways.

step of the replication cycle. Viruses can directly release their genome at the plasma membrane after binding to a receptor. Alternatively, they can be first internalized through endocytosis or macropinocytosis. Endocytosed virus particles will typically traffic through endosomal vesicles by actin-dependent and/or microtubule-dependent transport (8). Specific environmental triggers like endosomal pH acidification induce either fusion of enveloped virus with the endosome, or membrane penetration of viral proteins, allowing viral genetic material to be released into the cytoplasm (8). Alternatively, viruses can spread by direct cell–cell contact (9). Cell-to-cell transmission of viral material can activate cytoplasmic innate pathways, as exemplified with hepatitis C virus (10), lymphocytic choriomeningitis virus (11), or human immunodeficiency virus transmission (12).

Other viral RNA PAMPs can be produced during viral replication. David Baltimore has defined a classification of viruses based on the mechanism of mRNA production (13). Viruses are clustered in seven groups depending on their genomes (DNA,

RNA), strandedness (single or double), sense or antisense, and method of replication. The type of RNA ligands produced during viral replication will depend on the type of viral genome and the strategy used to generate mRNA. RNA ligands can be generated by DNA viruses and retroviruses via genome transcription, or by synthesis of mRNA and replication intermediates by RNA-dependent RNA polymerases (RdRps) of RNA viruses (8).

PHAGOSOMAL LEAKAGE OF MICROBIAL LIGANDS

It has been shown that ligands generated in phagolysosomes after phagocytosis of bacteria by immune cells can engage cytosolic innate immune receptors (14). Similarly, we showed that RNA from *Escherichia coli* could activate receptors in the cytosol after phagocytosis by macrophages (7). We demonstrated that phagosomes carrying *E. coli* exhibit intrinsic leakiness, suggesting a mechanism by which bacterial RNA, irrespective of the activity of virulence factors, can gain access to the cytoplasm (7).

ACTIVE TRANSLOCATION OF BACTERIAL RNA TO THE CYTOPLASM

Alternatively, bacteria express secretion systems to translocate products outside of the bacterial cell wall. In the case of intracellular bacteria, auxiliary secretion systems like SecA2 in *Listeria monocytogenes* have been shown to actively translocate *Listeria* RNA into the cytoplasm, resulting in activation of cytosolic sensors (15, 16). Similarly, another study proved that cytosolic RNA sensors participate in the type 1 interferon (IFN-I) response to *Legionella pneumophila*. Although the authors did not demonstrate the translocation of *Legionella* RNA into the cytosol of infected cells, they discuss their data through a model where it would be the case (17). Future studies looking for additional secreted RNA will likely provide additional insights on their interaction with the innate immune system.

ACTIVITY OF RNA POLYMERASE III

Two independent groups have demonstrated that cytoplasmic dsDNA triggers IFN-I production via RNA polymerase III, which transcribes DNA into 5'-triphosphate (5'-ppp) RNA, subsequently recognized by cytosolic RNA receptors (18, 19). This pathway has been involved in the sensing of DNA viruses, like Epstein–Barr virus, or intracellular bacteria, like *L. pneumophila* (18, 19). Although the RNA intermediate produced is not *sensu stricto* microbial, its generation is due to the activity of a microbial invader.

KNOWN CYTOSOLIC RNA SENSORS AND THEIR LIGANDS

The best-studied cytosolic RNA sensors are the three members of RIG-I-like Receptors (RLRs), a subfamily of the DExD/H-box family of helicases. They consist of retinoic acid-inducible gene I (RIG-I), melanoma differentiation factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). They share a similar organization with three distinct domains: (i) a C-terminal repressor domain (RD) embedded within the C-terminal domain (CTD); (ii) a central ATPase containing DExD/H-box helicase domain able to bind RNA; and (iii) a N-terminal tandem CARD domain that mediates downstream signaling, and which is present in RIG-I and MDA5 but absent in LGP2. Upon activation by RNA ligands, RIG-I and MDA5 are subsequently recruited to the adaptor protein Mitochondrial Antiviral Signaling (MAVS) via a CARD–CARD interaction and lead to activation of NF-κB and IRFs (20–23). In contrast to TLR expression that is predominantly expressed in specialized immune cells such as macrophages and dendritic cells (DCs), RLRs are found in the cytosol of most cell types and are strongly induced in response to IFN-I (24, 25).

RETINOIC ACID-INDUCIBLE GENE

The RIG-I ligand has been characterized as an RNA molecule with two distinct features: (i) a 5'-ppp moiety (26, 27) and (ii) blunt-end base pair at the 5'-end (28, 29). Blunt-end base pairs can be found in double-stranded RNA (dsRNA) and secondary RNA structures such as hairpin or panhandle conformations (28, 29). Recent structural studies have contributed toward a better understanding of ligand binding and activation of RIG-I. Specificity of 5'-ppp binding is conferred by the CTD, and the helicase domain binds the double-stranded part of the RNA. RIG-I is normally held in an auto-repressed conformation, and ligand binding results in

a conformational change, releasing the CARD domain which can subsequently initiate signaling by association with MAVS (30–32). Despite the increasing amount of high-resolution crystal data, the consensus definition of RIG-I ligand remains controversial. Other RIG-I ligands have been indeed described in the literature including long (33) or short dsRNA (34–36) lacking the 5'-ppp. However, thermodynamic analysis have shown that the full-length human RIG-I protein binds 5'-ppp dsRNA with 126-fold higher affinity than 5'-OH dsRNA, and dsRNA with a 361-fold higher affinity than short single stranded RNA (ssRNA) lacking secondary structure (37).

Many viral families display blunt-end base-paired RNA with a 5'-ppp, directly in their genomic RNA or in replication intermediates. Consistent with this notion, RIG-I has been shown to be involved in the recognition of many viruses, either antisense (−)ssRNA viruses (38, 39) or sense (+)ssRNA/dsRNA viruses (40, 41). Notably RIG-I can detect panhandle structures found in LaCrosse viral particles (39) or in influenza genomic RNA (28, 38). Sendai Virus (SeV) and other *Mononegavirales* produce defective interfering (DI) viral genomes containing panhandle structures that activate RIG-I in infected cells (42).

Retinoic acid-inducible gene I recognition has not been limited to RNA virus since RIG-I is involved in recognition of DNA viruses, such as Epstein–Barr virus or adenovirus through the RNA polymerase III pathway (18, 43, 44). Moreover, RIG-I is also able to detect bacterial infections. Bacterial mRNA are not capped and it has been estimated that approximately 40% of RNA oligonucleotides in *E. coli* have a 5'-ppp (45). Reports in the literature describe sensing of *L. monocytogenes* secreted RNA (15, 16) or purified *Legionella* (17) and *Helicobacter pylori* RNA (46) by RIG-I. Finally, RIG-I can also sense *Shigella flexneri* infection in macrophages through the RNA polymerase III pathway (47).

MELANOMA DIFFERENTIATION FACTOR 5

Melanoma differentiation factor 5 ligand is less characterized than RIG-I. Using poly(I:C) as a synthetic dsRNA mimic, studies have shown that MDA5 binds long, but not short dsRNA (35, 40, 48). Structural analyses have demonstrated that MDA5 specifically recognizes the internal duplex structure of dsRNA and uses it as a platform to stack along dsRNA in a head-to-tail arrangement. This mechanism promotes stochastic assembly of the tandem CARD oligomers that activates the signaling adaptor MAVS (49).

Melanoma differentiation factor 5 detects infection by viral families known to produce long dsRNA structures during their replication cycle, including (+)ssRNA viruses like picornaviruses, dsRNA viruses like reoviruses, or DNA viruses like poxviruses (35, 50–53). In the case of (+)ssRNA virus infection, fluorescent imaging studies have confirmed that MDA5 recognizes preferentially the dsRNA generated during the replication of these viruses, but not the genomic ssRNA (54).

Prior to the structural study mentioned above, multiple observations raised the possibility that there may exist additional MDA5 ligands, different from the consensus long dsRNA. Thus, a study has shown that MDA5 cooperates with the ribonuclease RNase L to induce IFN-I in response to a viral mRNA from parainfluenza 5 virus (55). Interestingly, RNase L converts RNA into small RNA products, with shorter length than the current MDA5

ligand definition (56). Another work published the same year has shown that mRNA lacking 2'-O-methylation at their 5' cap structure induces production of IFN-I through MDA5 activation (57). However, the data published, which focus on coronavirus infection, did not elucidate whether the absence of methylation was directly recognized by MDA5 or via another intermediate (57).

MAVS MEDIATES SIGNALING DOWNSTREAM OF RIG-I AND MDA5

After binding to their specific ligands, both RIG-I and MDA5 activate MAVS to trigger a common signaling pathway. The majority of MAVS is localized on the mitochondrial membrane and its engagement by RLRs causes a conformational change that propagates to adjacent un-activated MAVS in a prion-like behavior (58). The formation of these very large MAVS aggregates results in a large-scale amplification of the signaling cascade. This cascade involves the recruitment of cytosolic adaptor molecules, followed by the activation of the canonical IKKs, IKK- α , IKK- β , and IKK- γ , the MAPK and the non-canonical IKK-related kinase, TBK1 and IKK-i ϵ . Ultimately, specific transcription factors, such as IRF3, NF- κ B, and depending on the cell type IRF5 and IRF7, are translocated to the nucleus where they promote the expression of IFN-I genes and pro-inflammatory cytokines [reviewed in Ref. (59)].

Finally, MAVS has been recently shown to interact with NOD-like receptor family, pyrin domain containing 3 (NLRP3) and promote its recruitment to the mitochondria. The authors emphasize the central role of MAVS in innate immune signaling events by showing its importance in the functioning of NLRP3 inflammasome and the production of IL-1 β (60). Of note, MAVS independent activation of the NLRP3 inflammasome by RIG-I has also been reported (61, 62).

LABORATORY OF GENETICS AND PHYSIOLOGY 2

The third member of RLRs, LGP2, is able to bind dsRNA (63, 64), however, its role in immune activation is poorly understood. LGP2 was proposed to be a modulator of RLR signaling. Studies showed that LGP2 was required for RIG-I and MDA5 activity, in particular during picornaviral infection (65–67). Another work proposed that LGP2 would inhibit RIG-I through competition with its ligand (64). It is however unclear whether LGP2 binds microbial RNA in an infectious context, and what specific features of the RNA it would recognize. Further studies will be required to clarify the precise role of LGP2.

NON-RLR HELICASES

Apart from RLR, several recent studies have highlighted the importance of other DExD/H-box helicases in microbial RNA sensing. RNA helicases of the DEAD box family are involved in various different steps of RNA metabolism [reviewed in Ref. (68)]. They share eight conserved motifs that are involved in ATP binding, ATP hydrolysis, nucleic acid binding, and RNA unwinding activity. Additionally, most DExD/H-box helicases contain auxiliary N- and C-terminal domains that confer on them functional specificities, such as an ability to induce downstream signaling or to bind specific RNA targets (69).

DDX3

DDX3 (DDX3X) can bind poly(I:C) or vesicular stomatitis virus (VSV) RNA and was shown to enhance the IFN-I response to

VSV infection by interaction with the RLR-MAVS complex. Over-expression assays suggest that DDX3 precipitates with RIG-I and MDA5 (70). Since DDX3 is easily detected in resting cells, the authors propose a sentinel role for this helicase, the activity of which would be required during the initial steps of viral infection. Another study showed that upon SeV infection, DDX3 interacts with IKK ϵ , an essential component of the IRF3 signaling pathway, increasing the induction of the IFN- β promoter (71). Moreover, DDX3 is targeted by vaccinia virus protein K7 (71), an inhibitor of IFN- β production, and by HCV core protein, which can disrupt its interaction with MAVS (72). These observations highlight the importance of DDX3 in efficient viral sensing.

DHX9

Using overexpression and knock-down experiments, DHX9 was shown to be required for the production of IFN-I and pro-inflammatory cytokines in response to poly(I:C), influenza virus, and reovirus by a murine splenic DC line and bone-marrow derived DCs. DHX9 can bind dsRNA via its dsRNA-binding motif and interact with MAVS through both its helicase C-terminal domain and HA2-DUF (73).

DDX1, DDX21, AND DHX36

Myeloid DCs have also been shown to express a complex composed of DDX1, DDX21, and DHX36 that triggers an antiviral program in response to poly(I:C), in a pathway dependent of the adapter molecule TIR-domain containing adapter-inducing interferon- β (TRIF). DDX1 binds to poly(I:C) via its helicase A domain, while DHX36 and DDX21 bind the TIR domain of TRIF via their HA2-DUF and PRK domains, respectively. This complex seems to be required for the innate response against influenza or reovirus infection (74). Notably, a separate study also characterized DHX36 and DHX9 as a sensor for the dsDNA species CpG-A and -B, respectively. In this case, both DHX36 and DHX9 activate the cytosolic adapter protein myeloid differentiation primary response gene 88 (MyD88) by binding to its TIR domain (75).

DHX33

Another recent study by Yong-Jun Liu's group identified another helicase, DHX33, as a cytosolic RNA receptor able to activate the NLRP3 inflammasome (76). DHX33 is involved in inflammasome activation after sensing cytosolic RNA such as poly(I:C) or reoviral RNA when directly delivered by lipofection to the cytoplasm of a macrophage cell line or human monocyte-derived macrophages. Additional experiments suggested that DHX33 could also possibly be involved in detection of cytosolic bacterial RNA. The authors showed that DHX33 can bind to dsRNA through its helicase C domain and to NLRP3 through its DEAD domain (76). A few months later, another study performed on myeloid DCs confirmed the role of DHX33 in the sensing of cytosolic poly(I:C) and reoviral RNA. Surprisingly, in this case, poly(I:C)-induced activation of MAPK, NF- κ B, and IRF3 was mediated by MAVS, which binds the helicase C domain of DHX33 (77).

DDX60

DDX60 has also been shown to enhance the IFN-I response to RNA and DNA stimulation through formation of complexes with

RIG-I, MDA5, and LGP2 but not with MAVS. This complex formation has been deciphered with overexpression assays in the case of MDA5 and LGP2, and with endogenous RIG-I during VSV infection. DDX60 expression is induced by viral infection and its helicase domain can bind ds- or ss-VSV RNA generated *in vitro*, independently of the 5'-ppp (78). Interestingly, DDX60 can also bind dsDNA, and was shown to play role in IFN-I expression after infection with Herpes Simplex Virus-1, a DNA virus. This ability to bind both dsRNA and DNA raises the question of the feature DDX60 recognizes. It should be finally noted that the role of DDX60 in the IFN-I pathway has been questioned (79).

OTHER RNA RECEPTORS

Several other cytoplasmic receptors have been shown to play a role in microbial RNA recognition. This is the case for the cytoplasmic protein kinase R (PKR), which is important for antiviral activity. PKR is activated by dsRNA from viruses and is a component of MAPK and NF-κB signaling pathways [reviewed in Ref. (80)]. Activation of PKR can also be mediated by short 5'-ppp RNAs containing limited secondary structures (81).

Proteins from the Interferon-induced protein with tetratrico peptide repeats (IFITs) family, such as IFIT1 and 5, bind 5'-ppp of viral RNA (82). Using short *in vitro* transcribed oligonucleotides, crystal structure studies have demonstrated that IFIT proteins contain a positively charged cavity designed to engage, without any particular sequence specificity, ssRNA with a 5'-ppp end. Contrary to RIG-I, IFIT proteins cannot bind blunt-ended 5'-ppp dsRNA, and owing to the limitations imposed by their RNA-binding pockets, IFIT1 and IFIT5 require 5'-overhangs of at least 5 or 3 nt, respectively (83).

Using a 2'-O-methyltransferase mutant of Japanese encephalitis virus, another study showed that IFIT1 preferentially binds to 5' capped 2'-O-unmethylated mRNA (84), confirming previous findings showing that 2'-O-methylation of viral mRNA caps promotes IFIT1 evasion (85, 86). The mechanism of IFIT1 antiviral action is not completely understood, and it has been proposed that IFIT might sequester viral RNAs (82) or inhibit viral mRNA translation (84). The crystal structure of IFIT2 (known as ISG54) was also described. IFIT2 specifically binds adenylate uridylate (AU)-rich RNAs *in vitro*, independently of the presence of a 5'-ppp (87). The authors showed that RNA-binding capacity of this protein mediates its antiviral properties, using a model of HEK293T cells infected by Newcastle disease virus or SeV (87).

Nucleotide-binding oligomerization domain containing protein 2 (NOD2) is a member of the NOD1/Apaf-1 family and encodes a protein with two CARD domains and six leucine-rich repeats (LRRs). NOD2 is primarily known for its ability to recognize bacterial peptidoglycan, but it also plays a role in the antiviral response. NOD2 has been shown to activate MAVS after stimulation with viral ssRNA or human respiratory syncytial virus infection (88). NLRP3 is involved in cytosolic RNA sensing. Caspase-1 cleavage triggered by influenza virus, SeV, or bacterial mRNA is dependent on NLRP3 inflammasome activation (7, 89, 90). However, direct binding of NOD2 or NLRP3 to microbial RNA has not been established.

Leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) contributes to the production of IFN-β induced by VSV and

L. monocytogenes in macrophages (91). Mostly located in the cytosol, LRRFIP1 can also be found in RNA-containing lysosomes (92). LRRFIP1 can bind both dsRNA and dsDNA and subsequently induce IFN-I expression through β-catenin phosphorylation. Activated β-catenin is translocated to the nucleus and increases IFN-β expression by binding to the C-terminal domain of the transcription factor IRF3 and promoting the recruitment of the acetyltransferase p300 to the *Ifnb1* promoter.

IMMUNOSTIMULATORY FEATURES AND OTHER PUTATIVE RNA PAMPs

Several other microbial RNA features have been suspected or proposed to act as potential signals for cytosolic sensing, suggesting the existence of receptors detecting these characteristics. A computational analysis identified CpG motifs in an AU-rich RNA as an immunostimulatory feature. This sequence motif is underrepresented in both ssRNA viruses and host innate immune gene mRNA, and its frequency in influenza virus genomes has decreased throughout evolution (93). Since this evolutionary pressure seems to also be applied on host mRNA, the implication of a cytosolic receptor is possible, although experimental studies identified endosomal TLR7 as a potential PRR (94). Another study identified the nucleotide bias of A-rich HIV-1 genome as a strong inducer of IFN-I and potent mediator of lentiviral pathogenicity. The authors showed that the ability of RNA sequences derived from the HIV-1 genome to induce an interferon response correlated with their nucleotide bias and that codon-optimized sequences lost their stimulatory activity (95). The experimental procedure used in this study consisted of direct delivery via lipofection of *in vitro* transcribed RNA sequences into the cytosol of a reporter cell line, suggesting a potential role for a cytoplasmic RNA sensor (95). Recently, our group identified bacterial mRNAs as an activator of the NLRP3 inflammasome. Polyadenylation of these RNAs abrogated their immunostimulatory activities, suggesting that features at the 3' end of mRNA, rather than the 5' end, could engage cytoplasmic cellular sensors (7).

Philip Bevilacqua's group has shown that different nucleoside modifications on RNA, such as base or sugar internal modifications, suppress their intrinsic ability to activate immune sensors, notably PKR. The authors propose that self-RNA editing could be a mechanism used by the innate immune system to discriminate self-transcripts from "unmodified" microbial RNAs (96, 97). Conversely, microbial RNA editing by cellular deaminase enzymes such as dsRNA-specific adenosine deaminase (ADAR) have been shown to enhance its recognition by cytosolic sensors (98).

Other host transcript specificities, like association to cellular components that prevent PRR binding, or specific tertiary structure such as the eukaryotic mRNA closed loop conformation (99), could be determinants for the differentiation of host mRNAs from microbial RNAs. Identification of receptors able to recognize such features are lacking so far.

MICROBIAL ESCAPE STRATEGIES

Infectious microorganisms have developed several strategies to evade cytosolic sensing. One of these strategies, which we only mention briefly here, is the direct targeting by microbial proteins

Table 1 | Cytosolic RNA sensors and their ligands.

RNA sensor	Proposed RNA ligand	Families of reported recognized pathogens
RIG-I	5'-ppp with blunt-end base pairing ssRNA; dsRNA	(-)ssRNA viruses, (+)ssRNA viruses, dsRNA viruses, bacteria, DNA viruses, and bacterial DNA through polymerase III pathway
MDA5	Long dsRNA	(-)ssRNA viruses, (+)ssRNA viruses, dsRNA viruses, DNA viruses
LGP2	dsRNA	(+)ssRNA viruses
DDX3	Viral RNA; poly(I:C)	(-)ssRNA viruses
DHX9	Viral RNA; poly(I:C)	(-)ssRNA viruses, dsRNA viruses
DDX1, DDX21, and DHX36	Viral RNA; poly(I:C)	(-)ssRNA viruses, dsRNA viruses
DHX33	Viral RNA; poly(I:C)	dsRNA viruses, bacteria
DDX60	In vitro transcribed ssRNA and dsRNA	(-)ssRNA viruses, DNA viruses
PKR	dsRNA; short 5'-ppp RNA	(-)ssRNA viruses, (+)ssRNA viruses, dsRNA viruses, DNA viruses
IFIT1 and IFIT5	5'-ppp ssRNA; 5' capped 2'-O-unmethylated RNA	(-)ssRNA viruses
NOD2	Viral ssRNA	(-)ssRNA viruses
NLRP3	dsRNA, bacterial RNA	(-)ssRNA viruses
LRRKIP1	dsRNA	(-)ssRNA viruses, bacteria

of host PRRs and molecules involved in downstream signaling pathways. Thus, many pathogens code for proteins that lower cellular levels of PRRs and signaling molecules or directly disrupt their antimicrobial activities [reviewed in Ref. (79, 100)]. Other strategies are discussed below.

RNA EDITING

Some (−)ssRNA viruses edit the 5'-ppp moieties in their genomes as well as replication intermediates into 5' mono-phosphates to avoid recognition by RLRs (101). Arenaviruses produce RNA panhandle structures with a 5'-ppp containing a GTP overhanging nucleotide. This viral structure is suggested to act as a RIG-I ligand decoy, by trapping RIG-I but not activating it (102). We are beginning to understand how eukaryotic cells use nucleoside modifications in order to protect self-RNAs from innate sensing. For example, higher eukaryotes have acquired the ability to 2'-O-methylate their mRNAs, allowing cellular receptors to distinguish self from unmethylated non-self mRNA through specific types of antiviral sensors such as MDA5 and IFITs (57, 85). Consistent with the red queen hypothesis (103), which postulates that parasites have to constantly evolve in order to adapt to their host species, the same immune escape strategy has been mimicked by several pathogens, like flaviviruses (84, 86). Similarly, 2'-O-methylation of G18 (Gm18) on bacterial tRNA suppresses activation of the immune response in plasmacytoid DCs (104, 105).

COMPARTMENTALIZATION IN THE CYTOPLASM

Flaviviruses and other viruses are also known to induce cellular membrane reorganization that allows them to replicate in subcellular compartments, creating new replication-dependent organelles (106). Thus, tick-borne encephalitis virus or Japanese encephalitis virus have been shown to rearrange endoplasmic reticulum membranes to provide a compartment where viral

dsRNA is concealed from PRR recognition. This hijacking of internal cell membrane induces a delayed cytosolic exposure of viral RNA to innate receptors and accordingly, IFN-I responses are only measured late in the replication cycle (107–109).

PROTECTING OR DEGRADING LIGANDS

The NS1 protein from influenza virus can prevent RNA sensing through the formation of a chain of NS1 molecules along the influenza dsRNA backbone (110). Picornaviruses mask their 5'-ppp with a viral encoded protein, VPg, which functions as a 5' cap and as a primer during RNA synthesis. Interestingly, studies have shown that VPg could be used to evade RIG-I recognition (111). Similarly, Ebola virus VP35 assembles into dimmers to cap the ends of viral dsRNA and hide the specific RIG-I recognition site (112). While one VP35 monomer binds the terminus and backbone of dsRNA, the other VP35 monomer binds only the phosphate backbone of the dsRNA, displaying a unique mode of dsRNA concealing from PRR (112). Another hemorrhagic fever virus, Lassa fever virus, uses the 3'-5' exonuclease activity of its nucleoprotein (NP) to degrade stimulatory dsRNA (113). This activity seems to be shared by other arenaviruses (114). Finally, the protein C from human parainfluenza virus type 1 (HPIV1), a paramyxoviridae, has been shown to limit the accumulation of dsRNA. Cell infection by a virus mutant defective for the C protein displays higher accumulation of several viral RNAs, including viral genome, antigenome, and mRNA, eventually leading to the accumulation of dsRNA. Thus, by limiting intracytosolic quantities of viral dsRNA, the C protein of HPIV1 avoids dsRNA triggering of MDA5 and PKR in infected cells (115).

CONCLUDING REMARKS

The multiplicity of PRR pathways is an essential determinant of the immune system's ability to sense with precision the level of

microbial threat and to respond accordingly (4). However, as far as cytosolic RNA sensors are concerned, it is striking to observe the contrast between the high number of PRRs that have been isolated and the similarities of the PAMPs they recognize (**Table 1**). While 5'-ppp and dsRNA are undoubtedly powerful triggers of the innate immunity, they cannot account for the diversity of responses that the organism is able to elicit against a wide range of pathogens. Our understanding of how the immune system distinguishes between foreign and self-nucleic acids will continue to improve over time. This will help us better define the precise role played by cytosolic RNA sensors in the global immune response against pathogens.

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Atheroprotective vaccination with MHC-II restricted peptides from ApoB-100

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Background: Subsets of CD4⁺ T-cells have been proposed to serve differential roles in the development of atherosclerosis. Some T-cell types are atherogenic (T-helper type 1), while others are thought to be protective (regulatory T-cells). Lineage commitment toward one type of helper T-cell versus another is strongly influenced by the inflammatory context in which antigens are recognized. Immunization of atherosclerosis-prone mice with low-density lipoprotein (LDL) or its oxidized derivative (ox-LDL) is known to be atheroprotective. However, the antigen specificity of the T-cells induced by vaccination and the mechanism of protection are not known.

Methods: Identification of two peptide fragments (ApoB_{3501–3516} and ApoB_{978–993}) from murine ApoB-100 was facilitated using I-Ab prediction models, and their binding to I-Ab determined. Utilizing a vaccination scheme based on complete and incomplete Freund's adjuvant (CFA and IFA) [1 × CFA + 4 × IFA], we immunized Apoe^{−/−} mice with ApoB_{3501–3516} or ApoB_{978–993} emulsified in CFA once and subsequently boosted in IFA four times over 15 weeks. Spleens, lymph nodes, and aortas were harvested and evaluated by flow cytometry and real time RT-PCR. Total atherosclerotic plaque burden was determined by aortic pinning and by aortic root histology.

Results: Mice immunized with ApoB_{3501–3516} or ApoB_{978–993} demonstrated 40% reduction in overall plaque burden when compared to adjuvant-only control mice. Aortic root frozen sections from ApoB_{3501–3516} immunized mice showed a >60% reduction in aortic sinus plaque development. Aortas from both ApoB_{3501–3516} and ApoB_{978–993} immunized mice contained significantly more mRNA for IL-10. Both antigen-specific IgG1 and IgG2c titers were elevated in ApoB_{3501–3516} or ApoB_{978–993} immunized mice, suggesting helper T-cell immune activity after immunization.

Conclusion: Our data show that MHC Class II restricted ApoB-100 peptides can be atheroprotective, potentially through a mechanism involving elevated IL-10.

Keywords: T-cell, atherosclerosis, vaccination, inflammation

INTRODUCTION

Improvements in the treatment and prevention of cardiovascular disease (CVD) resulted in a 30% reduction in its mortality rate between 1998 and 2008. Despite this, a recent summary statement from the American Heart Association estimates that each year 785,000 Americans will have a new heart attack, and that someone dies from a coronary event every 60 s (1). These statistics underscore the importance of research that seeks to develop novel therapies in the fight against atherosclerosis.

Recent discoveries suggest that local arterial tissue inflammation is likely a major instigator in the development of atherosclerosis (2–6). This inflammation is mediated, in part, by local immunologic processes at the site of plaque lesions which

involve both innate and adaptive immunity (2, 6–10). The antigens in atherosclerosis driving both pro-atherosclerotic and atheroprotective immune responses are not known with certainty. Oxidized low-density lipoprotein (LDL) (ox-LDL), and the lipoprotein portion of LDL (ApoB-100) have been proposed as candidate antigens (10–13). Recent evidence suggests that autoantigens are presented to antigen-experienced CD4⁺ T-cells by antigen presenting cells (APCs) residing in the atherosclerotic arteries (14). All T-cell subsets (CD4⁺, CD8⁺, TCRγδ⁺, NKT-cells) have been found in mouse and human plaques (2, 5, 15, 16). In particular, the presence of CD4⁺FoxP3⁺ regulatory T-cells (T_{REGS}) in these plaques (17–19) indicates that inflammation within the plaque is a regulated process, offering hope

that therapeutics can be designed targeting T-cell function and differentiation.

Regulatory T-cells are the focus of many studies in atherosclerosis because of their atheroprotective potential. T_{REGS} are reduced in atherosclerotic plaques (18) and in circulating peripheral blood of human subjects with CVD (20) compared to healthy subjects. In murine studies, adoptive transfer of a subset of T_{REGS} (Tr1 cells, CD4⁺FoxP3[±]IL-10⁺) administered to Apoe^{-/-} mice showed a significant decrease in pro-atherosclerotic IFN-γ production, increased IL-10 production, and significant reduction in atherosclerotic lesion size when compared with control mice (21). IL-10, secreted by T_{REGS}, is atheroprotective and protects both from fatty streak formation and atherosclerotic plaque formation (15, 22).

Several studies have reported a protective effect of vaccination with LDL, or ox-LDL, on the development of atherosclerotic plaque (23–25). However, the mechanism by which these immunizations convey atheroprotection is an ongoing area of research. One line of investigation suggests that protective autoantibodies generated during immunization with ox-LDL might be the source of atheroprotection (24, 26, 27). This was initially a promising hypothesis which subsequently led to the search for atheroprotective B-cell (antibody) epitopes. The discovery of such epitopes was first reported by Fredrikson et al. (28) in 2003. These researchers assessed the binding of endogenous antibodies (from pooled human plasma in a case-control cohort of patients with a history of acute coronary heart events) and identified several epitope sequences from human ApoB-100 that bound to endogenous human antibodies. Since then several of these peptides have been used to vaccinate mice, including P2 (TRFKHLRKTYNYEAESSS) (29), P143 (IALDDAKINFNEKSQLQTY) (30), and P210 (KTTKQSFDSLVKAQYKKNKH) (30) each conferring ~40–60% atheroprotection. While the original premise of immunizing with B-cell epitopes was atheroprotection via an increase in peptide-specific antibody levels, this was eventually determined to not be the case (31).

More recently, researchers have sought to describe the changes in cellular (rather than humoral) immunity that may result from immunization with B-cell epitopes from ApoB-100. Several recent papers have reported an increase in FoxP3⁺ expressing T_{REGS} in secondary lymphoid organs [spleens (32) and lymph nodes (33)] after immunization of mice with P210. While these findings are consistent with reports that suggest T_{REGS} might be protective in atherosclerosis (17–19), it is unclear how T-cells can be directly affected by vaccination with peptide sequences originally recognized as B-cell epitopes. This is especially peculiar in the case of P210, which has been shown not to bind to I-A^b (the MHC Class II allele expressed by both Apoe^{-/-} and LDL-R^{-/-} mice) (34), which is a prerequisite for any direct interaction with T-cell receptors. Subsequent studies have further described the changes to the T-cell compartment after immunization with B-cell epitopes (in particular the CD4⁺CD25⁺FoxP3⁺ T_{REG} population) (35). However, there are currently no T-cell epitopes related to atherosclerosis that have been described. Knowledge of such epitopes would help to better characterize T-cell phenotypes after peptide immunization in the treatment of atherosclerosis.

The existence of atherosclerosis-related CD4⁺ T-cell epitopes is suggested by several experiments. First, human CD4⁺ T-cell clones derived from atherosclerotic plaques responded to incubation with ox-LDL and autologous monocytes (acting as APCs) by proliferation and production of cytokine (IFNγ) in an antigen-specific, HLA-DR-restricted manner (11). More recently, live-cell imaging of explanted aortas from CD11cYFP^{-/-} mice after 12 weeks of western diet (WD) showed that activated CD44^{hi}CD62L^{lo} Apoe^{-/-} CD4⁺ T-cells isolated from Apoe^{-/-} mice, but not wild-type mice, productively interacted with CD11c-YFP⁺ APCs in the aortic wall (14). These T-cells were effector-memory CD4⁺ T-cells, had long interactions with APCs in the vessel wall and slowed migration speeds compared to T-cells isolated from naïve wild-type C57BL/6 mice. Furthermore, the “productiveness” of these interactions was demonstrated by increased T-cell proliferation and induction of the pro-inflammatory T_H1 cytokines (IFNγ and TNF). These data suggest that endogenous T-cell antigens are presented in the aortic wall. It is therefore reasonable to suspect that these interactions may be promoting, or inhibiting, atherosclerosis by driving effector T-cell or regulatory T-cell responses, respectively. Manipulation of either these antigens or the T-cells that respond to these antigens would be of great therapeutic value.

Here, we report the discovery of two novel MHC-II restricted peptides identified in the murine ApoB-100 molecule. These peptides have high affinity for I-A^b (the MHC class II molecule in C57BL/6 mice), and injection of these two peptides into Apoe^{-/-} mice in complete Freund's adjuvant (CFA), followed by four boosts in incomplete Freund's adjuvant (IFA), reduces atherosclerosis. These data suggest that atheroprotective CD4⁺ T-cell vaccines can be developed.

MATERIALS AND METHODS

MICE

Eight-week-old female Apoe^{-/-} mice on C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed in a specific pathogen free environment and fed chow diet until 10 weeks of age. At 10 weeks of age, mice were started on WD (adjusted calories diet with 42% from fat, Harlan Labs Cat #: TD.88137, CA, USA) and remained on WD until sacrifice at 23 weeks old.

PEPTIDES

Using previously described algorithms (36–38) for predicting I-A^b (MHC Class II) peptide binding motifs in a protein molecule, we scanned the entire 4505 amino acid sequence of murine ApoB-100 and identified candidate sequences based solely on matches for I-A^b binding. Six of these were selected based on favorable anchor residue and hydrophobicity profiles. These sequences are listed in **Table 1**. Although the minimal sequence to bind to the MHC Class II peptide groove is a 9-mer, all peptides were custom synthesized (Genemed Synthesis, Inc., San Antonio, TX, USA) as 15-mers to build “ends” on to the peptides for better binding. Peptides were synthesized in 0.1% trifluoroacetic acid (TFA) for sterility.

I-A^b BINDING AFFINITY ASSAY

We measured I-A^b binding affinity using a classical competitive inhibition assay utilizing purified MHC and high affinity

Table 1 | Peptide sequences and I-A^b binding affinity as determined by IC₅₀.

Peptide sequences	Mouse ApoB-100 position	H-2 I-A ^b (IC ₅₀ nM)
NNYALFLSPRAQQAS	4505–4520	1295
SRATLYALSHAVNSY	438–453	1368
SQEYSGSVANEANVY	3501–3516	4.3
YENFAASNKLDVTFS	1578–1593	∞
HLEYVSSELRKSLQV	4054–4069	22176
TGAYSNASSTESASY	978–993	7.3
GWYRSPFSPRVVHLY	MOG _{38–51}	354
ISQAVHAAHAEINE	OVA _{324–337}	400

MOG, *Mus musculus* myelin oligodendrocyte glycoprotein; OVA, *Gallus gallus* ovalbumin. Bold font indicates ApoB_{3501–3516} and ApoB_{978–993}.

radio-labeled I-A^b ligands (39). Their ability to displace the radio-labeled I-A^b ligand was measured, and an inhibitory concentration required to displace 50% of the radio-labeled ligand (IC₅₀) was determined. Under the conditions utilized, where (label) < (MHC) and IC₅₀ ≥ (MHC), the measured IC₅₀ values are reasonable approximations of the true K_d values.

ANTIGEN-SPECIFIC PROLIFERATION

Peptides were emulsified in CFA and mice were immunized with 200 μg of peptide subcutaneously. Ten days later, draining lymph node cells were harvested and single cell suspensions were made. 5 × 10⁵ viable cells per microtiter well were cultured with 100 μg/mL of relevant (or irrelevant) peptide for 4 days. Purified protein derivative (PPD) served as a positive control. Sixteen hours before harvesting, 1 mCi of tritiated thymidine (³H) was added to each well. Cells were harvested and incorporation of ³H was determined in a scintillation counter. Results are expressed as stimulation index using the following formula:

$$\text{Stimulation index (SI)} = \frac{\text{cpm experimental} - \text{cpm media control}}{\text{cpm media control}}$$

ATHEROPROTECTIVE IMMUNIZATION

Previous work (25) has shown that atheroprotection through immunization could be achieved by injecting mice with LDL (or ox-LDL) with a combination of CFA initially, followed by antigen in IFA for booster immunizations. Using the same immunization model, 50 μg of ApoB_{3501–3516} or ApoB_{978–993} (diluted in PBS) was emulsified in equal volumes of CFA (BD Difco, Sparks, MD, USA) and injected into the subcutaneous inguinal area at 8 weeks of age. Repeated boosters with 25 μg of ApoB_{3501–3516} or ApoB_{978–993} emulsified in IFA (BD Difco, Sparks, MD, USA) were administered intraperitoneally at age 12, 16, 20, and 22 weeks. This immunization scheme will be referred to as 1 × CFA + 4 × IFA for the remainder of this manuscript. Mice were sacrificed at age 23 weeks and organs were harvested for analysis. Control immunizations with PBS emulsified in CFA and IFA were also performed. MOG_{35–55} [MEVGWYRSPFSPRVVHLYRNGK, (40)] immunizations were also done under identical conditions with the same adjuvants.

ATHEROSCLEROSIS QUANTIFICATION

Aortic root sections were examined as follows. Hearts were harvested, placed in Optimal Cutting Temperature medium (OCT, Electron Microscopy Sciences, Hatfield, PA, USA), and frozen at –80°C. Beginning at the first appearance of the tri-leaflet aortic valve, successive 5 μm transverse sections were made for a distance of 100 μm. From these, we analyzed every other section, for a total of 10 sections per root. Sections were then stained with Oil Red O and counter-stained with hematoxylin. Extent of atherosclerosis was then determined as the area involved on each section. To measure en face lesion formation, the whole aorta was carefully cleaned *in situ* and then the whole aorta pinned out after paraformaldehyde incubation at RT for at least 2 h. Staining for atherosclerotic plaque was performed by incubating samples in Sudan IV. Quantification was performed using ImagePro software (Media Cybernetics, Rockville, MD, USA).

LIPID ANALYSIS

Mouse whole blood was collected by cardiac heart puncture during organ harvest. No anticoagulant was used. The blood was placed on ice for at least 3–6 h, and then spun at 6300 rpm for 15 min at 4°C. The supernatant was collected and frozen at –80°C until analysis to reduce multiple freeze/thaw cycles. Individual samples were then analyzed by Roche COBAS 8000 Analyzer (Roche Diagnostics, Indianapolis, IN, USA).

MEASUREMENT OF ANTIBODY TITERS TO ApoB_{3501–3516} AND ApoB_{978–993}

Antibody titers in plasma were determined by chemiluminescent enzyme immunoassay as previously described (41). In brief, white "U" bottom plates (Thermo Lab systems, USA) were coated with various antigens at 5 μg/mL in PBS for overnight incubation. Following blocking with 1% BSA-TBS serum was added in increasing dilutions and incubated at RT for 90 min. Bound antibodies levels were detected using appropriate alkaline phosphatase-conjugated secondary antibodies and a 50% aqueous solution of LumiPhos 530 (Lumigen, USA). Data are expressed as relative light units counted per 100 ms (RLU/100 ms).

FLOW CYTOMETRY

Aortas, lymph nodes, and spleens were digested as previously described (42). Aortic cell suspensions, spleens, and lymph nodes were individually pressed through a 70-μm filter and incubated for 30–60 min in complete RPMI to encourage CD4 re-expression. Approximately (1–2) × 10⁶ cells were then placed into 96-well round bottom plates and incubated for 5 min with Fc Block (1:200), and subsequently stained with primary antibody [CD45-PerCP 1:50 (BioLegend, San Diego, CA, USA, Cat#103130), CD4-PE-Cy7 1:50 (eBioscience, San Diego, CA, USA, Cat#25-0041-82), TCRβ-AF700 1:50 (BioLegend, Cat#109224), and Live/Dead Aqua 1:200 (Invitrogen, Grand Island, NY, USA, Cat# L34957)] for 45–60 min. Plates were washed twice and then incubated with Fix/Perm buffer solution (eBioscience, Cat# 00-5523-00) for additional 30 min. Plates were again washed twice with a permeabilization buffer prior to intracellular staining with intracellular transcription factor staining [FoxP3-efluor450 1:50 (eBioscience, Cat# 48-5773-82)]. Intracellular staining was performed for 30–45 min and plates were washed twice with permeabilization buffer

solution. Samples were analyzed by LSR-II (BD Biosciences, San Jose, CA, USA). Data was acquired on FACSDiva software (BD Biosciences) and analyzed by FlowJo (Ashland, OH, USA).

QUANTITATIVE RT-PCR

Spleens, lymph nodes, and aortas were placed in 50 µL of RNALater from Qiagen (Valencia, CA, USA) immediately after harvest. QIAshredder kit (Valencia, CA, USA) was used to homogenize each sample after Trizol treatment. RNA extraction performed using RNeasy Mini kit (Valencia, CA, USA). RNA was converted to cDNA using iScript Reverse Transcription kit for RT-qPCR (BioRad, Hercules, CA, USA). Primers were commercially obtained as part of a Taqman PCR kit (Life Technologies, New York, NY, USA), and included CD4, IFN γ , TNF α , IL-2, IL-4, IL-10, IL-17A, Tbx21 (Tbet), GATA3, ROR γ T, and FoxP3. Housekeeping genes used in the analysis were one of two ribosomal proteins, Rpl32 or Rpl13A.

STATISTICAL ANALYSIS

Between groups analysis was performed by one-way ANOVA. Data are expressed as mean ± SEM. *P*-values <0.05 were considered significant.

RESULTS

IDENTIFYING CANDIDATE EPITOPEs ApoB_{3501–3516} AND ApoB_{978–993}

A peptide can only be recognized by a CD4 $^{+}$ helper T-cell if it is bound to MHC class II. Because *Apoe*^{−/−} mice are on a C57B/6 background, candidate epitopes must be able to bind to the Class II allele, I-A b . Previously, it has been shown that I-A b binding motifs can be predicted using algorithms based on the number of anchor residues and hydrophobicity (36–38). We measured I-A b binding affinity of six candidate peptides predicted by these algorithms. The binding affinity is reflected by the amount of peptide, in nanomoles, needed to inhibit binding of a standardized radio-labeled peptide by 50% (the IC₅₀) (39). Of the six peptides synthesized, only ApoB_{3501–3516} and ApoB_{978–993} bound I-A b with significant affinity (Table 1, ApoB_{3501–3516} IC₅₀ = 4.3 nM, ApoB_{978–993} IC₅₀ = 7.3 nM). Peptides containing only parts of these sequences showed reduced binding affinities (Table 2). By comparison, the known T-cell epitopes OVA_{324–337} and MOG_{38–51} (truncated form of MOG_{35–55}, i.e., myelin oligodendrocyte glycoprotein) have an IC₅₀ of 400 nM (43) and 354 nM (44), respectively (Table 1). Based on these binding affinities, the remainder of this work was performed using ApoB_{3501–3516} and ApoB_{978–993}.

ANTIGEN-SPECIFIC T-CELL PROLIFERATIVE RESPONSES

Two hundred micrograms (total) of either ApoB_{3501–3516} or ApoB_{978–993} (emulsified in CFA) were injected subcutaneously into four sites in the flanks. After 10 days the draining lymph nodes were harvested, and single cell suspensions were incubated for 10 days with 100 µg/mL of either ApoB_{3501–3516} or ApoB_{978–993} (or irrelevant, MOG_{35–55}) peptide for 4 days. PPD served as a positive control. Proliferation was measured by ³H incorporation and expressed as SI. After a single immunization of either ApoB_{3501–3516} or ApoB_{978–993} in CFA, antigen-specific T-cell proliferation was observed when relevant peptide or PPD is added, but not when irrelevant peptide is added (Table S1 in Supplementary Material).

Table 2 | I-A b binding affinity of ApoB_{3501–3516} and ApoB_{978–993}-related peptides.

Peptide	Sequence	Len	Pos	H-2 I-Ab (IC ₅₀ nM)
ApoB_{3501–3516}	SFTGNIKSSFLSQEY	16	3489	1169
	SSFLSQEYSGSVANE	16	3497	6.8
	SQEYSGSVANEANVY	15	3501	4.3
	SGSVANEANVYLNSKG	16	3505	172
	NVYLNLSKGTRSSVRLO	16	3513	907
	LFTGMNYCT TGAYSNA	16	969	655
ApoB_{978–993}	TGAYSNASSTESASY	16	977	17
	TGAYSNASSTESASY	15	978	7.3
	SSTESASYYPLTGDR	16	985	1258

Sequence of ApoB_{3501–3516} and ApoB_{978–993} indicated in boldface.

IMMUNIZATION WITH ApoB_{3501–3516} AND ApoB_{978–993}

ApoB_{3501–3516} or ApoB_{978–993} were each used to vaccinate 10–14 female *Apoe*^{−/−} mice. Fifty micrograms of ApoB_{3501–3516} or ApoB_{978–993} emulsified in CFA were subcutaneously injected above the inguinal LN at 8 weeks of age. A WD was then started at 10 weeks of age. Repeated boosters with 25 µg of ApoB_{3501–3516} or ApoB_{978–993} emulsified in IFA were administered intraperitoneally at age 12, 16, 20, and 22 weeks (Figure 1A). Mice were sacrificed at age 23 weeks of age (13 weeks WD) and organs were harvested for analysis. Control immunizations with adjuvant only (1× CFA + 4× IFA) and an irrelevant peptide (MOG_{35–55}) were done under identical conditions.

CHOLESTEROL LEVELS

Western diet-fed *Apoe*^{−/−} mice exposed to the PBS plus 1× CFA + 4× IFA regimen had significantly decreased total plasma cholesterol from 1292 to 960 mg/dL, HDL from 246 to 168 mg/dL, and non-HDL from 1046 to 797 mg/dL, and triglycerides from 203 to 183 mg/dL compared to WD-fed *Apoe*^{−/−} mice not exposed to adjuvant. Similar observations have been reported in other studies (45). However, none of the mice immunized with ApoB_{3501–3516} or ApoB_{978–993} had plasma lipid levels different than the PBS plus 1× CFA + 4× IFA controls (Table 3). Therefore, all subsequent statistical analyses were performed without the untreated group.

IMMUNIZATION WITH EITHER ApoB_{3501–3516} OR ApoB_{978–993} RESULTS IN LESS ATHEROSCLEROTIC PLAQUE WHEN USED IN AN IMMUNIZATION SCHEME USING BOTH CFA AND IFA

Both ApoB_{3501–3516} and ApoB_{978–993}-treated mice showed ~40% reduction in en face lesion size by Sudan IV staining of whole aortas when compared with PBS and MOG_{35–55} (Figures 1B,C) immunized mice. Aortic root lesions were also examined for plaque burden by oil red O (ORO) staining. ApoB_{3501–3516} immunized mice had >60% reduction ($p < 0.01$) in overall aortic sinus plaque development compared to 1× CFA + 4× IFA (adjuvant-only treated) mice (Figures 1D,E). ApoB_{978–993} immunized mice showed no significant reduction in aortic root plaque burden. MOG_{35–55} immunized mice showed no significant decrease. These data demonstrate that immunization with I-A b restricted peptide

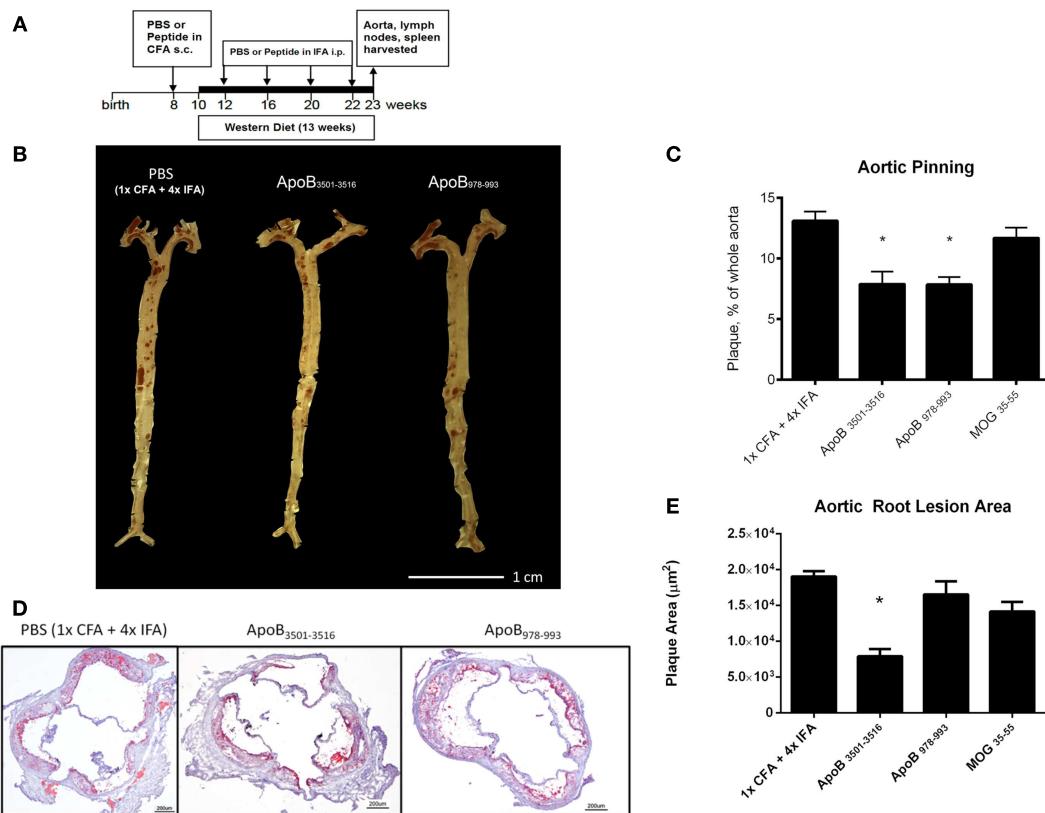


FIGURE 1 | Atherosclerosis is decreased in ApoB₃₅₀₁₋₃₅₁₆ and ApoB₉₇₈₋₉₉₃-treated mice compared to controls. (A) Vaccination schedule: 8-week-old female *Apoe*^{-/-} mice were immunized once with either PBS or peptide in CFA, then boosted four more times with PBS or peptide in IFA. WD was maintained for 13 weeks. Mice were sacrificed and organs harvested at 23 weeks of age. **(B,C)** Results of aortic pinning analysis after Sudan IV staining are shown with

representative photographs. $N = 12-15$ in each group, $*p < 0.05$ when compared to 1x CFA + 4x IFA group. **(D)** Representative aortic root staining sections after ORO staining, counter-stained with hematoxylin. **(E)** Plaque area from aortic roots stained from each group. Lesion sizes from 30 to 40 μm distal to start of the aortic valve were averaged per group. $N = 5$ in each group, $*p < 0.05$ when compared to 1x CFA + 1x IFA control group.

Table 3 | Lipid profile of mice.

	Untreated	PBS	ApoB ₃₅₀₁₋₃₅₁₆	ApoB ₉₇₈₋₉₉₃	MOG ₃₅₋₅₅
TC (mg/dL)	$1292.0 \pm 145.9^*$	960.3 ± 100.5	870.4 ± 103.6	835.2 ± 108.3	1014.7 ± 76.4
HDL (mg/dL)	$245.7 \pm 90.9^*$	167.7 ± 34.6	202.1 ± 23.8	170.3 ± 6.9	213 ± 7.5
Non-HDL (mg/dL)	$1046.3 \pm 105.8^*$	796.6 ± 119.3	663.7 ± 124.7	592.2 ± 187.1	801.7 ± 72.4
TG (mg/dL)	$203.3 \pm 21.0^*$	182.6 ± 21.9	158.1 ± 32.5	144.7 ± 37.3	142.3 ± 33.2
Weight (g)	26.8 ± 2.2	25.4 ± 1.9	26.3 ± 1.8	25.9 ± 2.1	25.3 ± 1.1

$N = 6-7$ per group, presented as mean \pm SD.

$*p < 0.05$ when untreated group is compared to groups exposed to adjuvant.

fragments from murine ApoB-100 can reduce plaque burden in *Apoe*^{-/-} mice.

IMMUNIZATION WITH EITHER ApoB₃₅₀₁₋₃₅₁₆ OR ApoB₉₇₈₋₉₉₃ RESULTS IN PEPTIDE-SPECIFIC IgG TITERS

One possible mechanism of atheroprotection is the development of protective antibodies (24). The production of IgG requires antigen-specific T-cell help and gives insight into antigen-specific

T-cell activation and lineage bias. IgG1 is a marker of T-helper type 2 ($\text{T}_{\text{H}}2$) activity and IgG2c of T-helper type 1 ($\text{T}_{\text{H}}1$) activity in C57BL/6 mice, which do not express IgG2a (25). Pooled plasma from each group was analyzed for immunoglobulin titers by formal antibody dilution curves using chemiluminescent ELISA (Figure S1 in Supplementary Material). As shown in Figures 2A,B for the 1:250 dilution, as expected IgG responses to ApoB₃₅₀₁₋₃₅₁₆ and ApoB₉₇₈₋₉₉₃ peptides were detected in

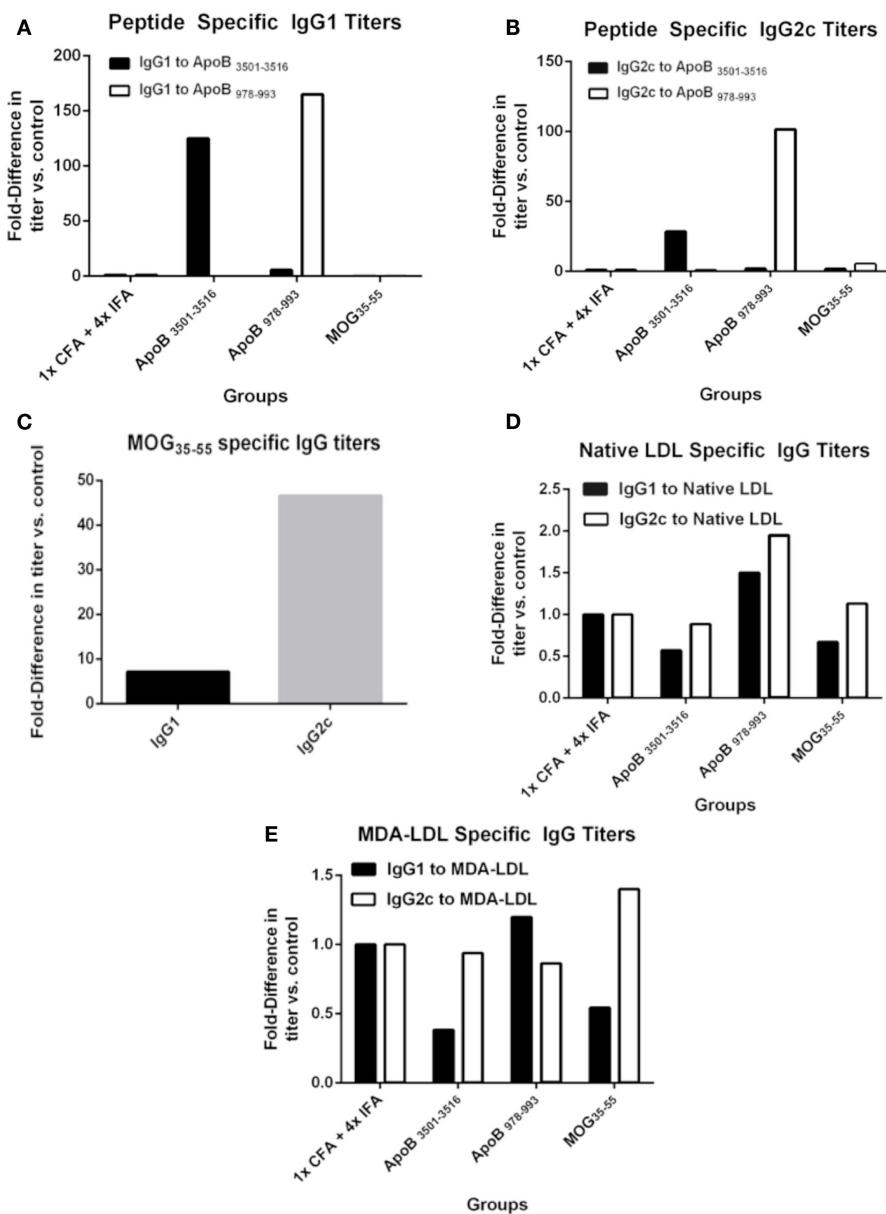


FIGURE 2 | Specific antibody titers after immunization. Sera from 9 to 10 animals per group were pooled and formal antibody dilution curves were measured by chemiluminescent ELISA against each of the antigens. Shown here are comparative binding data at serum dilutions of 1:250 and in each case, values shown are the fold increase compared to values found with

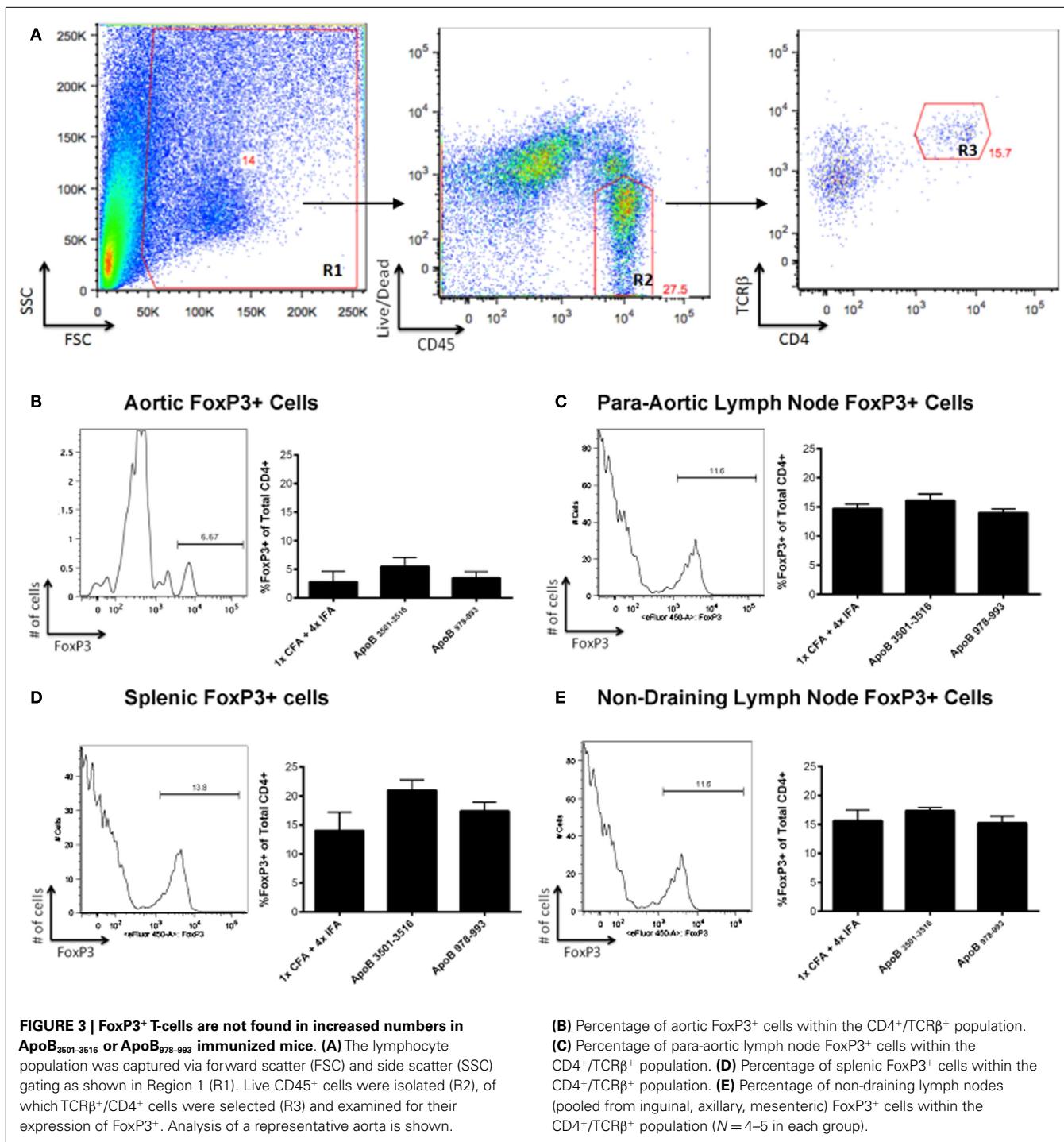
pooled sera from 1x CFA + 4x IFA group. **(A)** IgG1 titers against ApoB₃₅₀₁₋₃₅₁₆ and ApoB₉₇₈₋₉₉₃. **(B)** IgG2c titers against ApoB₃₅₀₁₋₃₅₁₆ and ApoB₉₇₈₋₉₉₃. **(C)** IgG1 and IgG2c titers against MOG35–55 in MOG₃₅₋₅₅ immunized mice. IgG1 and IgG2c titers against **(D)** native (unmodified) LDL and **(E)** MDA (oxidized)-LDL.

ApoB₃₅₀₁₋₃₅₁₆ and ApoB₉₇₈₋₉₉₃ immunized mice, respectively. Their responses showed complete peptide specificity, with strong responses in both the Th1 and Th2 helper T-cell compartments. Total IgG1 and IgG2c antibody levels (not antigen-specific) were similar across all groups (data not shown). MOG₃₅₋₅₅ immunization produced a predominantly IgG2c response to MOG₃₅₋₅₅ (Figure 2C), but no antibody titers to ApoB₃₅₀₁₋₃₅₁₆ or ApoB₉₇₈₋₉₉₃ were detected (Figures 2A,B). 1x CFA + 4x IFA immunized mice did not have detectable levels of IgG1 or IgG2c

against ApoB₃₅₀₁₋₃₅₁₆ or ApoB₉₇₈₋₉₉₃ (Figures 2A,B). None of the immunized mice had elevated IgG titers against native LDL or MDA-LDL compared to adjuvant only (Figures 2D,E).

REDUCED ATHEROSCLEROTIC PLAQUE BURDEN DOES NOT CORRELATE WITH AN INCREASE IN THE NUMBER OF FoxP3-EXPRESSING CELLS

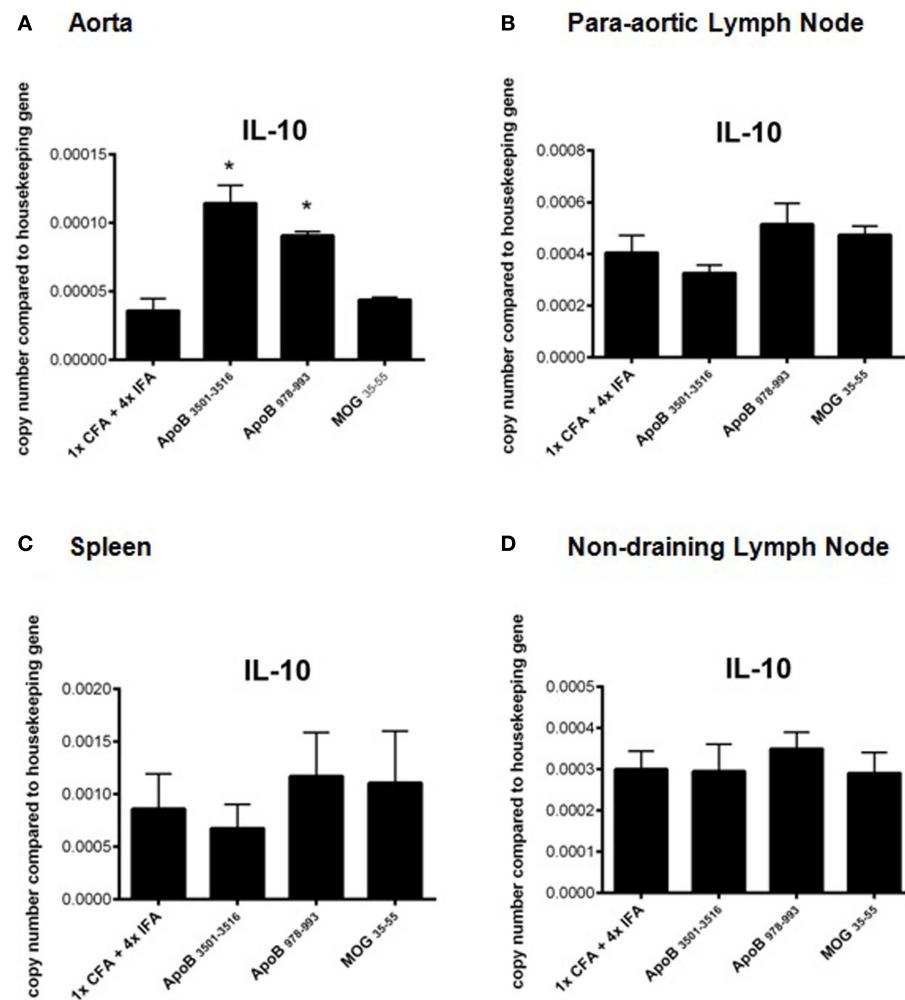
Another possible mechanism of atheroprotection conveyed by immunization with ApoB₃₅₀₁₋₃₅₁₆ or ApoB₉₇₈₋₉₉₃ could be related to increased numbers of FoxP3-expressing regulatory T-cells.



Whole aortas, along with spleens and lymph nodes (para-aortic, inguinal, axillary, mesenteric) were harvested from immunized mice at the time of sacrifice. There were no significant differences in FoxP3 $^{+}$ cells within the CD4 $^{+}$ /TCR β $^{+}$ cell population in the aorta (Figures 3A,B), para-aortic lymph nodes (Figure 3C), spleens (Figure 3D), or non-draining lymph nodes (inguinal, axillary, mesenteric; Figure 3E) when ApoB₃₅₀₁₋₃₅₁₆ and ApoB₉₇₈₋₉₉₃ were compared to the 1x CFA + 4x IFA control group.

IMMUNIZATION WITH ApoB₃₅₀₁₋₃₅₁₆ OR ApoB₉₇₈₋₉₉₃ INCREASES mRNA EXPRESSION OF THE ATHEROPROTECTIVE CYTOKINE, IL-10

RT-PCR analysis of spleens, lymph nodes, and aortas of mice from each group were analyzed for mRNA expression levels of the T_H1 cytokines IFN γ , TNF α , and the T_H1 transcription factor Tbx21 (Tbet), the T_H2 cytokines IL-4, IL-10, and the T_H2 transcription factor GATA3, the T_H17 cytokine IL-17A, and the T_H17 transcription factor ROR γ T, and the regulatory T-cell transcription factor

**FIGURE 4 | Real time RT-PCR analysis of IL-10 mRNA expression.**

Organs were harvested and immediately placed into RNA stabilization reagent, and frozen at -80°C . IL-10 mRNA expression from (A) aortas,

(B) para-aortic lymph nodes, (C) spleens, and (D) non-draining lymph nodes are shown. $*p < 0.05$ compared to 1x CFA + 4x IFA. $N = 3-5$ in each group.

FoxP3. No significant differences were found except in aortas of ApoB₃₅₀₁₋₃₅₁₆ and ApoB₉₇₈₋₉₉₃ immunized mice where a significant increase in IL-10 mRNA expression was noted ($p < 0.05$) compared to 1x CFA + 4x IFA treated and MOG₃₅₋₅₅ immunized control mice (Figures 4A–D). There was no significant difference in mRNA expression of any other cytokine (IFN γ , TNF α , IL-4, or IL-17A) or transcription factor (Tbet, GATA3, ROR γ T, or FoxP3) examined between groups in any organ (data not shown).

DISCUSSION

Our results show that MHC Class II restricted CD4 $^{+}$ T-cell peptides from the ApoB-100 are effective at reducing atherosclerotic plaque burden in *Apoe*^{-/-} mice. ApoB₃₅₀₁₋₃₅₁₆ or ApoB₉₇₈₋₉₉₃ are high affinity binders for MHC Class II (I-A b). This is the first publication to identify such peptides within an atherosclerosis-relevant protein (ApoB-100).

T-cell responses to vaccination with either (ox)LDL or peptide epitopes from ApoB-100 have become an area of great interest

because they may enable the development of a vaccine for clinical use. Recent reports have suggested that immunization with an epitope from ApoB-100 that binds to serum antibodies, P210, results in atheroprotection via an increase in T_{REGS} (32, 33), but the mechanism by which a B-cell epitope could induce T_{REGS} was not elucidated. It is not known whether these T_{REGS} are causally related to the atheroprotection that is observed. In fact, P210 does not bind to I-A b (34), suggesting that any actions immunizing with P210 might have are highly unlikely to be related to CD4 $^{+}$ helper T-cells. One publication has instead suggested that the observed atheroprotection is a result of enhanced CD8 $^{+}$ cytotoxic T-cell activity against dendritic cells leading to a decreased number of CD11c $^{+}$ cells within the aorta, and thus reduced atherosclerosis (46). These researchers demonstrated that adoptive transfer of P210 primed CD8 $^{+}$ T-cells recapitulated the atheroprotective response in naïve mice. These contrasting views of how immunization may result in atheroprotection highlight the need for systematic studies into MHC-restricted peptide vaccinations.

Mice immunized with ApoB_{3501–3516} or ApoB_{978–993} in the context of CFA and IFA show increased IL-10 mRNA expression levels in the aortas, significantly above control mice. Since this increased expression is not associated with an increased in the percent of aortic FoxP3⁺ regulatory T-cells, we speculate that the IL-10 mRNA may be derived from FoxP3⁻ Tr1 cells (47, 48) (i.e., one subset of inducible TREGS) or from myeloid cells (49). While both IFNγ and IL-17A have been implicated as pro-atherosclerotic cytokines, no changes in mRNA expression of either cytokine was observed in the aortas, lymph nodes, or spleens of immunized mice compared to controls.

We did not detect IgG1 or IgG2c antibody titers to ApoB_{3501–3516} or ApoB_{978–993} in non-immunized mice. This is despite highly elevated plasma cholesterol levels in *Apoe*^{-/-} mice (50). One possibility is that ApoB_{3501–3516} and ApoB_{978–993} may not be naturally processed products of APCs. Another possibility is that the endogenous forms of ApoB_{3501–3516} and ApoB_{978–993} are not presented efficiently or in high enough quantities by APCs. Further investigations into the mechanism of atheroprotection will provide new targets for therapy and prevention of atherosclerosis. It is our hope that these two peptides, and future peptides that can be discovered by the described immunologic methods, will lead to a new frontier in atherosclerosis research and ultimately provide a treatment for this worldwide epidemic.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://www.frontiersin.org/Journal/10.3389/fimmu.2013.00493/abstract>

Figure S1 | Specific antibody dilution curves. Antibody specific titers were measured by formal antibody dilution curves using chemiluminescent ELISA. Dilutions of 1:50 (when possible), 1:250, 1:2500 and 1:6250 were performed. *Baseline* group (blue line) represents pooled serum from two female *Apoe*^{-/-} mice at 8 weeks of age on chow diet, without immunization. The *untreated* group (green line) represents 9–10 female *Apoe*^{-/-} mice fed western diet for 13 weeks, starting at 10 weeks of age but without any immunizations. Data are expressed as relative light units counted per 100 ms (RLU/100 ms). **(A)** IgG1 titers to ApoB_{3501–3516}, ApoB_{978–993}, and MOG_{35–55}. **(B)** IgG2c titers to ApoB_{3501–3516}, ApoB_{978–993}, and MOG_{35–55}. **(C)** IgG1 and IgG2c titers to native (unmodified) LDL. **(D)** IgG1 and IgG2c titers to MDA(oxidized)-LDL.

Table S1 | Antigen-specific T-cell proliferation. Mice were immunized with either ApoB_{3501–3516}, ApoB_{978–993}, PPD (positive control), or MOG_{35–55} (negative control). Draining lymph node cells were harvested 10 days later and incubated with the relevant peptide, PPD or MOG_{35–55}. Proliferative responses were measured by ³H incorporation and expressed as SI. We show here that there are antigen-specific T-cell responses to both ApoB_{3501–3516} and ApoB_{978–993}.

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Phagocytosis of particulate antigens – all roads lead to calcineurin/NFAT signaling pathway

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Antigen-presenting cells (APC) possess multiple cell surface receptors that recognize common microbe-associated antigens as well as immune complexes and inert particles. Upon encountering such antigens these receptors must cooperate to achieve phagocytosis and trigger signaling cascades that initiate the innate immune response. While the stimuli initiating these signaling cascades are diverse, recent data have revealed that their effects in APC and particularly in dendritic cells (DC), all have something in common: downstream activation of nuclear factor of activated T cells (NFAT). NFAT is a family of transcription factors that has emerged as a key mediator of the initiation of immune responses by APCs, and specifically of IL-2 production by DC as reviewed (1). Intriguingly NFAT activation now seems to be the shared endpoint of several signaling pathways that all begin with uptake of particulate antigens.

Notably, the NFAT family members appeared at the origin of vertebrates whereas nearly all the other signaling pathways, including NFκB pathway, are very ancient and present in all invertebrate species. NFAT signaling plays essential roles in vertebrate organogenesis and development but also in the formation of adaptive immunity. In addition, G. R. Crabtree has suggested that “NFAT may have contributed to the evolutionary adaptation of innate immunity: e.g., minimize the costs of inflammation by collaboration with adaptive immunity” (2). It is likely that the ability of DC to link innate with adaptive immunity might be the result of DC’s ability to couple phagocytic functions to NFAT activation, leading to extensive gene reprogramming.

This is the latest in a series of new hypothesis to better understand of the true complexity of the process of pathogen

sensing, uptake, and response in APC and in particular in DC. But as is so often the case, with new hypotheses and knowledge has come new questions: how can such diverse stimuli all converge on similar pathways of immune activation? How do APCs integrate signaling from multiple immune uptake receptors? And how can we explain the difference in APC responses to soluble and particulate antigens?

In this article we will review the recent steps forward in our understanding of the intricate cross-talk between pathways of phagocytosis and immune signaling in APC, and the evidence that NFAT activation is a unique hallmark of this process.

PHAGOCYTIC RECEPTEORS AS MASTER REGULATORS OF UPTAKE

Pattern recognition receptors (PRRs) are expressed abundantly by APCs, both on the cell surface and in intracellular compartments, and are ligated by conserved microbe-associated molecular patterns (MAMPs). Signaling by PRRs is important for innate immune cell activation, maturation, antigen processing, and presentation, and it now seems to be influenced by the process of phagocytosis itself. For example, it has long been known that responses to soluble and particulate forms of the same MAMP can vary enormously, but it is only recently that the interaction of phagocytosis and PRRs has been implicated in the mechanism of this distinction.

Phagocytosis and MAMP detection by APC have often been considered as complementary but separate processes; however, it was noted that during microbe uptake, certain cell surface PRRs were actively recruited to the forming endosomes or phagosomes together with the microbial load. This subset of PRRs, including C-type lectin receptors and CD14, was termed the

“Phagocytic Receptors” (3), and has since been extended to include opsonic receptors such as Fc receptors, which mediate uptake of immune complexes. However, it now seems that Phagocytic Receptors do not only mediate uptake of particulate antigens, but can also determine the recruitment, activation, and intracellular signaling of other PRRs during the process of phagocytosis and antigen/pathogen degradation (4).

PHAGOCYTIC RECEPTEORS CO-ORDINATE COMPLEX IMMUNE RESPONSES TO SIMPLE MAMPs

Lipopolysaccharides (LPS) are highly immunogenic MAMPs on Gram-negative bacteria, and are recognized by several receptors on the APC surface; CD14, for example, is well-established as a non-opsonic Phagocytic Receptor for bacteria (5), but has recently been found to mediate the internalization of another LPS receptor, TLR4, via signaling through the tyrosine kinase Syk and PLC γ 2 (6). What is particularly interesting about TLR4 is the effect that this process of internalization has upon the resulting signaling pathways that are triggered. Ligation of TLR4 by LPS at the plasma membrane activates the adaptor proteins TIRAP and MYD88, which enable pro-inflammatory gene transcription. However, once internalized with its ligand, TLR4 instead promotes TRAM-TRIF signaling, culminating in the production of type I IFNs (7, 8). Thus CD14 controls TLR4 internalization, and specific adaptor localization controls the downstream signaling pathways that result.

Alongside its interaction with TLR4, CD14 is also required for LPS signaling through Src-family kinases and PLC γ 2 activation. As shown in murine DC this leads to Ca $^{2+}$ flux and the activation of

the phosphatase calcineurin, which causes NFAT nuclear translocation and so the release of IL-2 (9–11).

PHAGOCYTIC RECEPTORS FACILITATE DISTINCTION OF SOLUBLE AND PARTICULATE MAMPs

Whole microbes are significantly more potent immune stimulators than the soluble versions of their MAMPs, but understanding how APCs sense this difference has been challenging. Substantial progress was made recently when a study exposing murine DC to either soluble or bead-bound LPS revealed stronger triggering of Syk/PLC γ 2 in response to “particulate” LPS (6), possibly resulting in increased Ca $^{2+}$ influx-dependent NFAT activation and transcription of downstream targets. This hinted at the importance of the NFAT pathway in determining immune outcomes to particulate antigens, however it was an elegant set of studies on the C-type lectin receptor, Dectin-1, that illustrated a molecular mechanism underlying APC detection of MAMP forms (12).

Dectin-1 is a non-opsonic Phagocytic Receptor that recognizes the major component of the outer fungal cell wall, 1,3- β glucan (13–15). Upon ligand binding and receptor dimerization, the hemi-ITAM motif in the cytoplasmic tail of Dectin-1 is phosphorylated by Src-family kinases, which enables recruitment and activation of Syk. In turn, Syk mediates the MAPK response, canonical and non-canonical NF κ B activation and also, importantly, stimulates the Ca $^{2+}$ flux that drives calcineurin dephosphorylation of NFAT, leading to its nuclear translocation (15). This NFAT signaling is clearly linked with the particulate form of 1,3- β glucan and phagocytosis (Figure 1) and inducing different cytokine pattern than soluble form (Figure 1).

What has now emerged is that the differences in this process following ligation by soluble versus particulate ligands enable receptor-driven distinction between the two forms of the same antigen. The soluble β -1,3/ β -1,6-linked glucans isolated from the cell wall of *Saccharomyces* bind single molecules of Dectin-1 and induce hemi-ITAM phosphorylation by Src-family kinases, but downstream signaling events, including calcium flux, are limited and transient. This results from

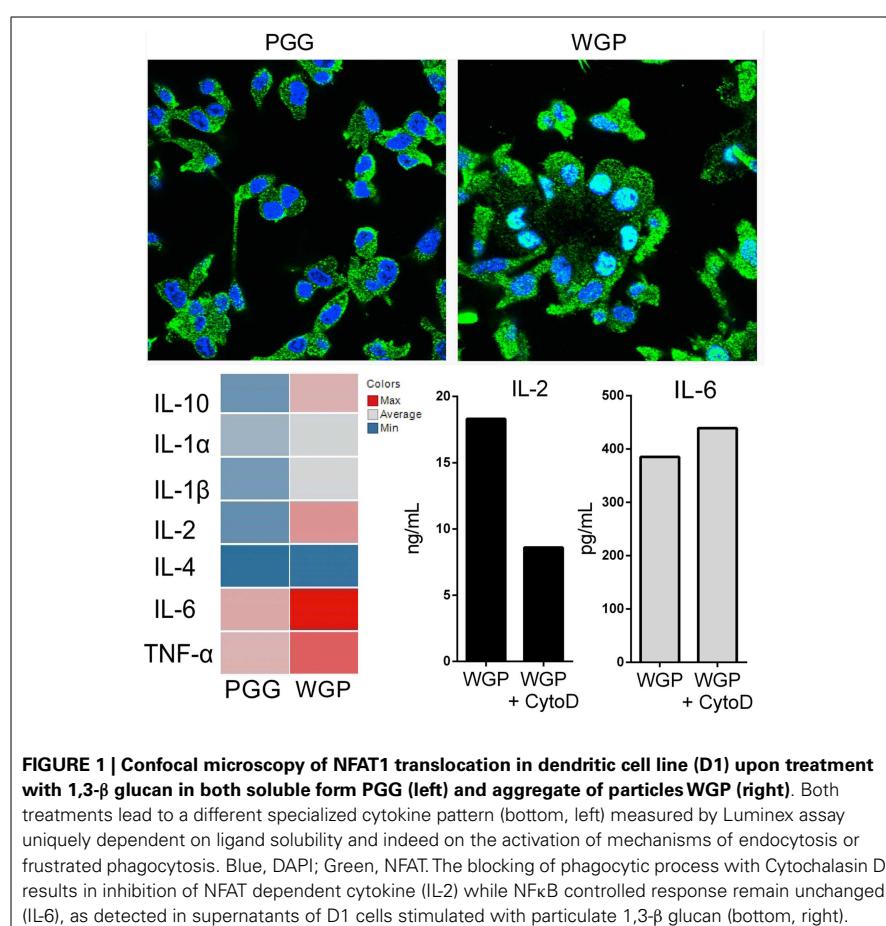


FIGURE 1 | Confocal microscopy of NFAT1 translocation in dendritic cell line (D1) upon treatment with 1,3- β glucan in both soluble form PGG (left) and aggregate of particles WGP (right). Both treatments lead to a different specialized cytokine pattern (bottom, left) measured by Luminescent assay uniquely dependent on ligand solubility and indeed on the activation of mechanisms of endocytosis or frustrated phagocytosis. Blue, DAPI; Green, NFAT. The blocking of phagocytic process with Cytochalasin D results in inhibition of NFAT dependent cytokine (IL2) while NF κ B controlled response remain unchanged (IL6), as detected in supernatants of D1 cells stimulated with particulate 1,3- β glucan (bottom, right).

expression of the membrane phosphatases CD45 and CD148 alongside Dectin-1, which serve to de-phosphorylate the Src kinases thereby impeding effective Dectin-1 signaling (12). In contrast, the aggregation of the β -1,3 glucan ligand on the surface of a yeast enables a parallel aggregation of Dectin-1 on the APC surface which increases receptor avidity and results in the formation of a “phagocytic synapse.” The close physical arrangement of ligands and receptors in the synapse excludes CD45 and CD148, releasing the brakes on sustained Src activation, and enabling induction of the cytoskeletal rearrangements required for phagocytosis, as well as the sustained calcium flux that is linked to NFAT activation and initiation of transcription of NFAT dependent cytokines (Figure 1).

PHAGOCYTIC RECEPTORS DELIVER EXTRACELLULAR MAMPs TO INTRACELLULAR PRRs

The macrophage receptor with collagenous structure (MARCO) (16) is a non-opsonic

multi-ligand Phagocytic Receptor whose function remained an enigma for many years; while MARCO is implicated in the pathogenesis of many inflammatory diseases, it is completely unable to initiate pro-inflammatory signaling itself (17). Several studies now indicate that it is MARCO’s interactions with other PRRs that define its role in shaping responses to particulate antigens. For example, strong and sustained signaling through the intracellular PRR TLR3 in response to the ligand PolyIC, required MARCO for rapid delivery and concentration of the ligand in the phagosome (18). In the case of the mycobacterial cell wall glycolipid trehalose 6,69-dimycolate, MARCO serves to enhance signaling through the TLR2/CD14 complex (Bowdish PlosOne, 2009), where both molecules are known to be able to induce NFAT activation (10, 15). Indeed, MARCO-mediated phagocytosis of sterile particulates such as silica is the first step in a signaling cascade ending in NFAT activation and TNF transcription

(19, 20). Cytoskeleton rearrangement has been observed as a result of MARCO clustering in activated DC (21).

PHAGOCYTIC RECEPTORS LINK ANTIGEN UPTAKE TO PRR ACTIVATION

Fc γ Rs are members of the immunoglobulin superfamily and are opsonic Phagocytic Receptors that recognize the Fc portion of antibodies bound to their cognate antigen. This process is central to the clearance of pathogens during infection, but has also been well studied for its role in autoimmune disease: in systemic lupus erythematosus (SLE) large self-DNA-containing immune complexes (DNA-ICs) are internalized by Fc γ Rs on the surface of DC that then produce pro-inflammatory cytokines which contribute to disease pathology (22, 23). Signaling via the Fc γ R is integral to both these processes; DNA-IC binding to the Fc γ R induces its phagocytosis and also triggers activation of Src-family kinases, which first drive the cytoskeletal rearrangements needed to recruit the DNA-sensing TLR9 to the phagosome, and subsequently mediate phosphorylation of TLR9 (23). TLR9 activation in turn results in the recruitment and activation of the kinase Syk, and also activates the adaptor MYD88 which induces NF κ B-mediated cytokine gene transcription and the secretion of type I IFNs. Whether Fc γ R-mediated uptake and immune activation specifically link with NFAT nuclear translocation has not yet been assessed, though the central role of activated Syk is suggestive (24). However, the related Phagocytic Receptor Fc ϵ RI (25), does trigger substantial calcium flux and NFAT translocation following ligand binding (26–28).

CONCLUDING REMARKS

It is increasingly evident that the processes of immune uptake and immune signaling can no longer be considered as discrete, but rather are highly integrated by APC in order to induce multiple and inter-linked signaling pathways. One aspect of this is the roles of Phagocytic Receptors, some of which have been highlighted here. Despite diverse molecular structures and varied ligands, members of the Phagocytic Receptor family share the ability to direct downstream signaling toward triggering calcium influx, followed by calcineurin activation and NFAT translocation. NFAT signaling

has been studied within myeloid cells (11, 29). Here we propose that the activation of calcineurin-NFAT signaling might be considered a hallmark of successful initiation of early innate responses toward phagocytosed particulate antigens. Interestingly, there is now evidence that NFAT activation may be part of a multi-component signature of the APC response to particulates; a recent study has shown that a common characteristic of the phagosomes formed during uptake by Fc γ Rs, MARCO, and Dectin-1 is the accumulation of the autophagy marker LC3 (30). While the full implication of this observation remains to be investigated, the presence of LC3 implies a degree of cross-talk between pathways of particulate uptake and the non-canonical autophagic response of cells to ligands likely to include DNA-IC, Mycobacteria, and fungi.

These advances in understanding of the Phagocytic Receptors are already beginning to pay dividends; MARCO-mediated uptake of antigen-coated microparticles has successfully been exploited to ameliorate disease in a murine model of autoimmune encephalomyelitis (EAE) (31), while particles coated in the Dectin-1 ligands β -1,3-linked and β -1,6-linked glucans have been used as a potent vaccine delivery system in mice (32). Further understanding of the fine-tuned mechanisms underlying particulate uptake and immune signaling in APC, and the ensuing innate outcomes, e.g., the production of regulatory cytokines by DC such as IL-2 and IL-23 (1) has significant potential to improve our ability to design effective vaccines against infectious diseases and for the treatment of autoimmune conditions.

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Designing vaccines for the twenty-first century society

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The history of vaccination clearly demonstrates that vaccines have been highly successful in preventing infectious diseases, reducing significantly the incidence of childhood diseases and mortality. However, many infections are still not preventable with the currently available vaccines and they represent a major cause of mortality worldwide. In the twenty-first century, the innovation brought by novel technologies in antigen discovery and formulation together with a deeper knowledge of the human immune responses are paving the way for the development of new vaccines. Final goal will be to rationally design effective vaccines where conventional approaches have failed.

Keywords: vaccination, glyco-conjugate, technologies, vaccine design, structural vaccinology

INTRODUCTION

In the last century, vaccines demonstrated to be a successful and effective medical intervention representing one of the most important applications of immunology to prevent infectious diseases. A landmark in the history of immunology is the experiment conducted by Edward Jenner in 1796, when he demonstrated that inoculation with pus from cowpox lesions was conferring protection against smallpox infection (1) providing an innovative contribution to immunization and the ultimate eradication of smallpox (2). Smallpox was one of the most severe human diseases, responsible only in Europe for the death of more than 400,000 people per year. In 1979, smallpox was eradicated through a global vaccine administration campaign. Jenner's work was further refined by Louis Pasteur, who artificially attenuated viruses for use in vaccines and in 1885 developed the first rabies human vaccine. He brought a breakthrough in the prevention and treatment of infectious diseases by establishing the basis of vaccinology, meaning the principle of isolation, inactivation, and administration of disease causing pathogens. The Pasteur's principles have allowed the development of "first generation" vaccines based on whole microorganism killed or live-attenuated (e.g., *Bacillus Calmette Guerin* BCG, plague, pertussis, and smallpox) (3,4).

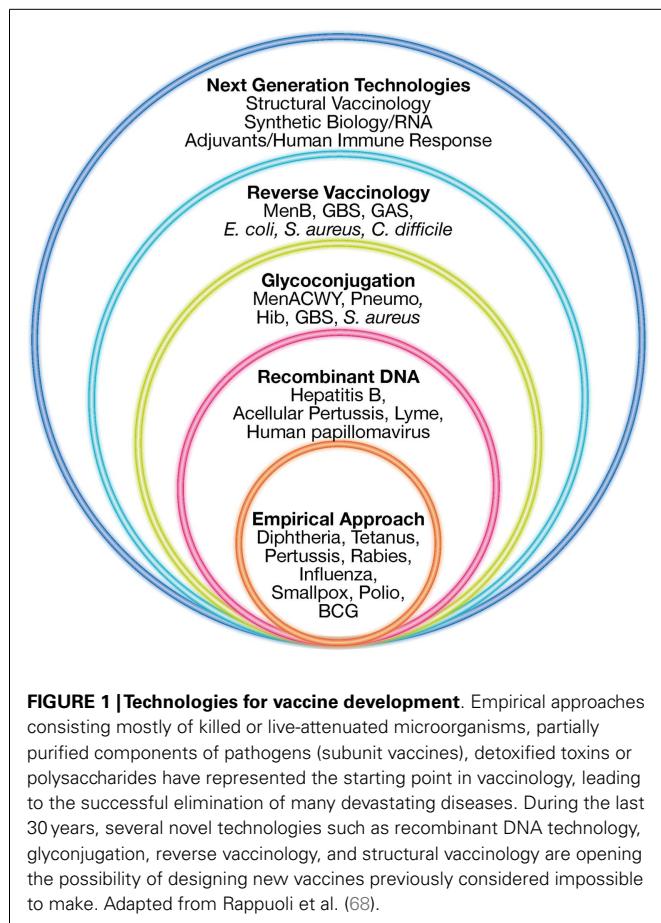
In the second half of twentieth century improvements and innovation in mammalian cell culture technology led to the growth of viruses and development of live attenuated "second generation" vaccines such as polio (Sabin oral), measles, rubella, mumps, and varicella. More recently, the use of inactivated polio vaccine (Salk type) together with oral vaccine has almost eradicated polio from the world thanks to global vaccination (5). In developed countries national immunization programs have drastically reduced most of the viral and bacterial infections that traditionally affected children. In May 2012, the 194 Member States of the World Health Organization Assembly endorsed the global vaccine action plan (GVAP) with the vision of delivering universal access to immunization, with at least 2–3 million lives saved per year worldwide (http://www.who.int/immunization/global_vaccine_action_plan/). Although traditionally developed vaccines

have been in the last century of unquestionable value, saving more than 700 million cases of disease and more than 150 million deaths, the conventional methods of vaccine design have some limitations. For example, they could not be used to develop vaccines against microbes that do not grow *in vitro* (e.g., *Mycobacterium leprae*, papilloma virus type 16 and 18). They do not provide broadly protective vaccines against pathogens with antigenic hypervariability (e.g., serogroup B meningococcus, HIV, HCV) or against pathogens with an intracellular phase, causing infections that are predominantly controlled by T cells, such as tuberculosis and malaria (6). Finally, traditional approaches of vaccine development can be very slow and time consuming, not allowing a rapid response to the need of a new vaccine, as in case of an influenza pandemic.

To overcome all these limitations, during the last 30 years new technologies have been applied to vaccine development. Recombinant DNA, polysaccharide chemistry, and more recently reverse vaccinology (RV), structural vaccinology, and synthetic RNA vaccines are opening up the view for the designing and development of "third generation" vaccines, previously defined as impossible to make (Figure 1).

POLYSACCHARIDE CHEMISTRY AND GLYCO-CONJUGATE VACCINES

One of the major immunological problems faced in the development of polysaccharide vaccines has been their low immunogenicity especially in children below 2 years of age, who represent the main target population of vaccination (7, 8). Bacterial polysaccharides are made of repeated monosaccharides linked together by glycosidic linkages. Their multiple identical antigenic epitopes cross-link multiple membrane immunoglobulins on a B cell to allow activation without the help of T cells (9). Polysaccharides cannot be processed and presented to T-helper cells and because of the lack of T-cell help, there is no germinal center reaction and the associated isotype switching, avidity maturation of the B cell receptors and induction of memory B cells.



Vaccines composed of plain bacterial polysaccharides have been introduced since the 1970s to control diseases caused by *Neisseria meningitidis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* type b (Hib) (10–12). However, these vaccines were poorly immunogenic in infants >2 years of age and did not appear to provide herd immunity, which is now recognized as a key element to prevent invasive bacterial infections in children. To improve immunogenicity of plain polysaccharides, they were chemically conjugated to protein carriers such as tetanus toxoid (TT), diphtheria toxoid (DT), and a non-toxic cross-reacting mutant of DT (CRM197) (13). Glyco-conjugate vaccines activate B cells via engagement of the B cell receptor following polysaccharide binding, processing of the protein carrier by polysaccharide-specific B cells, and presentation of the resulting peptides or glycopeptides in association with MHC class II molecules to T-helper cells. The MHC class II-restricted cognate interaction between B and T cells provides the costimulatory signals to B cells to start the germinal center reaction with somatic hypermutation and class-switch recombination generating B cells that will secrete high-avidity IgG antibody against the polysaccharide antigen (14, 15). As a consequence, vaccination with protein-polysaccharide conjugate vaccines is able to induce a long last immune response, with high affinity IgG antibodies and with the capacity to be boosted by subsequent immunizations (14, 16, 17). Protein-polysaccharide conjugate vaccines were introduced in the 1980s against *H. influenzae*

type b (Hib) (18–20) inducing a better and persistent antibody response in all age groups. Today, different strategies to prepare conjugate vaccines can be used and effective glyco-conjugate vaccines are available for *S. pneumoniae* and the strains A, C, W, and Y of *N. meningitidis* (meningococcal meningitis) (21, 22). These vaccines are highly immunogenic and brought a huge reduction of bacterial infections in those countries that have introduced them into their immunization schedules (23–25). Although the progress made in the technology of glyco-conjugate vaccines made possible the successful control of different bacterial infections, this approach could not be applied to develop *N. meningitidis* type B (MenB) vaccine. MenB is a major cause worldwide of meningitis and sepsis, two devastating diseases that can kill children and young adults within hours (26). It is a gram-negative bacterium part of the commensal flora that colonizes the upper respiratory tract of healthy individuals. In a small proportion of cases, the bacterium can invade the host bloodstream and, after crossing the blood-brain barrier, causes meningitis (27, 28). The unsuccessful attempt of developing a MenB vaccine based on its capsular polysaccharide was largely due to the fact that it is identical to the polysialic acid present in human glycoproteins such as N-CAM. Many efforts were directed toward the development of a protein-based vaccine, all frustrated by the inconsistency of the protection data probably due to the extreme variability of the known surface proteins tested as vaccine antigens.

REVERSE VACCINOLOGY

A major revolution in vaccine discovery is linked to the advent of genome sequencing technologies that have changed the landscape in the slowly evolving field of vaccinology. Turning point was the publication in 1995 of the genome sequence of the first living organism (29). By sequencing the genome and by determining the whole antigenic repertoire of the infectious organism, several candidate protective targets could be identified and tested for their suitability as vaccine. The method, named Reverse Vaccinology (RV), has provided a change in the perspective of vaccine design. The idea of the RV was originated to overcome the problems faced to develop an efficacious vaccine against MenB. The genome sequencing of the MenB virulent strain MC58 (30) allowed to select from the genomic data potential vaccine targets (31). The principle at the basis of the RV approach was that successful vaccine targets were proteins either exposed on the surface of the pathogen or secreted into the extracellular milieu. Starting from 2,158 encoded proteins bioinformatics analysis predicted that over 600 were either surface exposed or secreted. Of these, 350 were cloned in *Escherichia coli*, expressed and used to immunize mice. The sera of immunized animals were screened in a bactericidal assay that is known to correlate with protection. At each step candidates not satisfying quality criteria were discarded; the process led to the identification of previously unknown vaccine candidates. Through this process three protective antigens that are common to multiple MenB strains have been identified (fHbp, NadA, and NHBA) and combined with a MenB outer membrane vesicle (OMV) resulting in the first universal vaccine against MenB (32). This is the first vaccine based on RV that has recently received a positive opinion from the European Medicines Agency and has been approved with the commercial name of Bexsero®. Following

the success of the MenB project, the RV approach has been applied to a variety of other important pathogens, such as *S. pneumoniae* (33, 34), *Streptococcus pyogenes* (35), *Chlamydia pneumonia* (36), *Chlamydia trachomatis* (37), *Streptococcus agalactiae* (38), *E. coli* (39), and *Leishmania major* (40). Thus, the genome-based RV strategy can provide innovative solutions for the design of vaccines difficult or even impossible to develop using conventional methods (41).

NEXT GENERATION TECHNOLOGIES FOR VACCINE DESIGN

Novel technologies currently under investigation represent the most valuable tools to be applied in vaccinology and could be used today for addressing the medical needs of the twenty-first century. Despite decades of efforts and investigation, satisfactory vaccines have not yet been developed against several of the most life-threatening infections, including tuberculosis, malaria, and HIV, which claim the lives of more than 4 million people worldwide each year. The high levels of variability of their antigenic proteins and the required induction of both humoral and cellular immune responses have not allowed us to use conventional vaccinology methods as successful strategies. The advent of a new approach, named structural vaccinology, could represent today a valid revolutionary alternative leading in the next years to an efficacious vaccine design. Through the combination of human immunology, structural biology, and bioinformatics knowledge, antigenic epitopes are identified based on the protein amino acid sequences and the resulting secondary and tertiary structures. The principle is based on the observation that an efficacious immune response does not require the recognition of the entire antigenic protein, but the recognition of multiple selected epitopes might be sufficient to induce protective immunity (42). Progresses in technologies aimed at interrogating the human B cell repertoire are providing for the first time the possibility of isolating broadly neutralizing antibodies targeting relevant conserved epitopes (43–45). A deeper characterization of the crystal structure of an antigen in complex with protective antibodies represents the launching point for immunogen design to select relevant epitopes from a vaccine standpoint. Once identified they can be expressed in a recombinant form and in an immunodominant fashion to be used as potent immunogens (Figure 2). Recently, the group of Kwong et al. (46) using a structure-based approach designed an immunogen for respiratory syncytial virus (RSV) that elicits higher protective responses than the postfusion form of the fusion glycoprotein, which is one of the current leading RSV vaccine candidates entering clinical trials. Importantly, highly protective responses were elicited in both mice and macaques. Structural vaccinology combined with human immunology are therefore rapidly emerging as a powerful alternative strategy for the rational design of engineered vaccines bearing multiple antigenic epitopes offering the opportunity of developing broadly effective immunity (47–49).

Another challenge in vaccinology is due to the limited capacity of the immune system to develop potent and sustained antibody responses at the extremes of age. Several studies in the last decades have shown that antigen exposure in early life results in blunted, delayed, or undetectable antibody responses to infections and immunizations (50, 51). Effective IgG responses in infants require several doses of vaccine, and to avoid a rapid wane of the titers

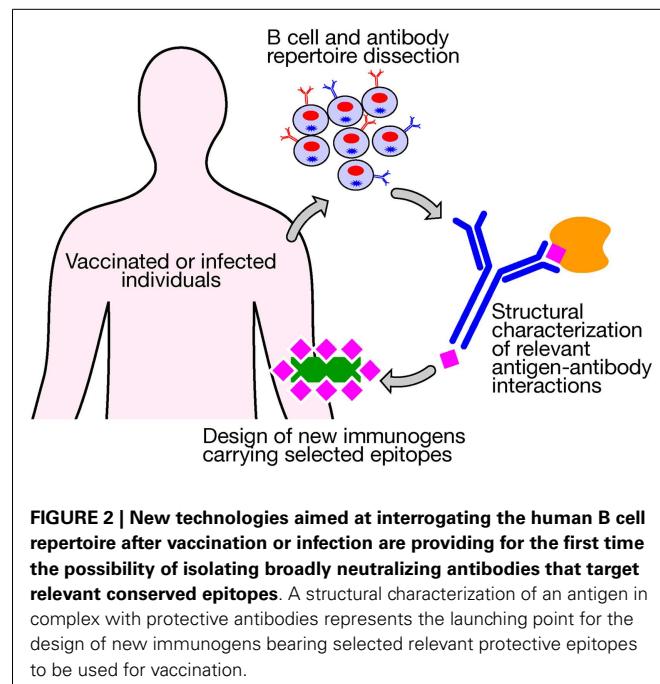


FIGURE 2 | New technologies aimed at interrogating the human B cell repertoire after vaccination or infection are providing for the first time the possibility of isolating broadly neutralizing antibodies that target relevant conserved epitopes. A structural characterization of an antigen in complex with protective antibodies represents the launching point for the design of new immunogens bearing selected relevant protective epitopes to be used for vaccination.

booster doses might be required after the first year of life. At the other extreme of age, a significant decline in the capacity to induce protective antibody titers is observed in individuals of 65 years or older. The discovery of new adjuvants that help in eliciting the appropriate sustained antibody response represents a valuable strategy to overcome age-related hypo-responsiveness to vaccination. MF59 is an oil-in-water emulsion potent vaccine adjuvant licensed in more than 20 countries for more than 13 years for use in an influenza vaccine focused on elderly subjects. Recently, MF59 has been shown to be safe also in a seasonal influenza vaccine given to infants and children, providing an increase in vaccine efficacy from 43 to 89% (52, 53). However, in the last years increased knowledge of the molecular mechanisms underlying the immune response is leading to the discovery of molecules that can trigger the immune system modulating antigen-specific immunity. Monophosphoryl lipid A (MPL) is known to be a highly specific agonist of the toll-like receptor 4 (54). MPL adsorbed onto aluminum salts has been included in the formulation of the AS04-adjuvanted HPV-vaccine, the first example of a toll-like receptor agonist to be licensed as part of a vaccine for human use (55). As MPL other newly discovered innate immune receptor agonists might be ideal molecules to be exploited as adjuvants for next generation vaccines aimed at improving immunogenicity mainly in extreme ages.

Finally, together with the above described technologies used to design new protective antigens and to optimally present them to the immune system, also the new synthetic methods of vaccine production are driving the development of the twenty-first century vaccines. Nucleic acid-based vaccines represent a key advancement in combining the benefits of *in situ* expression of antigens, with the safety of inactivated and subunit vaccines. They might represent a valuable tool to overcome problems encountered when designing vaccines against pathogens that require a protective immunity

mediated not only by antibodies but also by T cells. Up to now the success rate of vaccine development decreases with the decreasing ability of antibodies to confer protective immunity. A large body of literature suggests that cytotoxic T cells are important in protection from infectious diseases, such as tuberculosis, malaria, and AIDS. This would require the creation of vaccines able to induce strong T-cell responses, a weakness for existing vaccine approaches. The evidence that CD8+ T cells can control infections comes mainly from HIV. Given the failure to protect using the antibody-based gp120 vaccines (56, 57) T-cell-based vaccines have been widely tested in non-human primates (58) demonstrating to be promising. Nevertheless an HIV prime/boost vaccine based on adenovirus vector delivering T-cell epitopes failed to protect patients from disease, and had little impact on viral load (59). It is possible that this result may represent a proof-of-concept that pure T-cell vaccines are not a solution for preventive vaccines. However, there remains a need for vaccines to protect against infections for which antibodies are not sufficient or against non-infectious diseases such as cancer or autoimmunity, where also T cell immunity plays a critical role in conferring protection. DNA based vaccines showed to be very promising in animals, but in humans the magnitude of the immune response was lower than that observed with conventional vaccines. To overcome these limitations, approaches as DNA delivery by electroporation and stimulation of the immune system via the use of genetic adjuvants (i.e., *in situ* expression of immunologically active molecules encoded by the DNA vaccine) have been used in human clinical trials with encouraging preliminary results (60, 61). RNA vaccines represent a valid alternative over DNA vaccines. They are based on mRNA and self-amplifying RNA replicons that when injected intramuscularly in mice result in local production of an encoded reporter protein (62) and induction of immune responses against the encoded antigen (63). RNA vaccines have several advantages compared to DNA vaccines. RNA would eliminate the issue of possible integration of plasmid DNA into the genome of the immunized host, and it is translated directly in the cytoplasm (64). It has not been clearly elucidated the mechanisms utilized by RNA vaccines to induce an immune response, but it is likely that expression and presentation of encoded antigens follow rules similar to DNA vaccines. The efficiency and stability of RNA-based vaccines have been increased through the use of viral-particle engineered to express a heterologous antigen in place of the viral structural genes. RNA vaccines, particularly self-amplifying replicons, have therefore the potential of capturing the advantages of both DNA vaccines and viral delivery while overcoming the drawbacks of each technology. These favorable observations, supported by preclinical proof-of-concept in animal tumor models, have led mRNA vaccines into human clinical trials as immunotherapeutics in metastatic melanoma and renal cell carcinoma patients (65) showing to be able to elicit antigen-specific immune responses (both antibodies and T cells). Clinical trials have also been performed with RNA replicon vaccines packaged in viral particles encoding for cytomegalovirus (CMV) gB and pp65/IE1 proteins. The vaccine has shown to be well tolerated and immunogenic in healthy CMV seronegative volunteers, with the added value of inducing also CD8+ T-cell responses (66). The future of the RNA vaccines will rely on the formulation with new synthetic delivery

systems to combine the effectiveness of live attenuated vaccines, an equal or better safety profile than plasmid DNA vaccines, and completely synthetic methods of manufacture.

Improvements in the synthetic vaccines research have provided a unique tool to rapidly respond to the need of vaccine availability in case of flu pandemics. Dormitzer et al. have developed a synthetic approach to generate vaccine viruses from sequence data (67). Starting from the available hemagglutinin (HA) and neuraminidase (NA) gene sequences, a cell-free gene assembly technique has allowed rapid, accurate gene synthesis. Viral RNA expression constructs encoding HA and NA and plasmid DNAs encoding viral backbone genes were used to transfect Madin-Darby canine kidney (MDCK) cells, qualified for vaccine manufacture. Viruses for use in vaccines were rescued from MDCK cells with increased yield of the essential vaccine antigen, HA. The implementation of synthetic vaccine seeds has demonstrated the capability of accelerating the response to influenza pandemics reducing the time required for vaccine manufacturing from months to weeks.

CONCLUDING REMARKS

In conclusion, the last 30 years have represented a turning point in vaccinology. New technologies such as recombinant DNA, polysaccharide chemistry, and more recently RV, structural vaccinology, and synthetic RNA vaccines have greatly improved the efficiency of the vaccine-target identification, selection, and development process. Continuous progresses will be made in the twenty-first century to design new vaccines that will become the most efficient life insurance of the modern society, contributing significantly to a disease-free long life.

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Polarized cells, polarized views: asymmetric cell division in hematopoietic cells

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It has long been recognized that alterations in cell shape and polarity play important roles in coordinating lymphocyte functions. In the last decade, a new aspect of lymphocyte polarity has attracted much attention, termed asymmetric cell division (ACD). ACD has previously been shown to dictate or influence many aspects of development in model organisms such as the worm and the fly, and to be disrupted in disease. Recent observations that ACD also occurs in lymphocytes led to exciting speculations that ACD might influence lymphocyte differentiation and function, and leukemia. Dissecting the role that ACD might play in these activities has not been straightforward, and the evidence to date for a functional role in lymphocyte fate determination has been controversial. In this review, we discuss the evidence to date for ACD in lymphocytes, and how it might influence lymphocyte fate. We also discuss current gaps in our knowledge, and suggest approaches to definitively test the physiological role of ACD in lymphocytes.

Keywords: cell polarity, asymmetric cell division, immunological synapse, scribble complex, cell fate

INTRODUCTION

An effective immune response relies on the coordination of signals to control major cell fate checkpoints such as proliferation, differentiation, survival, and death. While many key players, including surface molecules, transcription factors, and cytokines have been identified to be important for immune cell fate control, it is still not clear how these signals are integrated during the differentiation and function of B and T cells. These questions of how signals are orchestrated during cell fate determination have been particularly well addressed in progenitor cells of the developing worm and fly. In these two organisms, cell fate is strongly influenced by the asymmetric distribution of fate determinants into the two daughters of a dividing cell, known as asymmetric cell division (ACD) (1). ACD involves the differential partitioning of protein, mRNA, microRNA, and other cellular constituents into the two daughter cells. Therefore, ACD imparts differential fates such as self-renewal, quiescence, proliferation, differentiation, and apoptosis. The mechanisms and consequences of ACD were initially studied in *Drosophila melanogaster* neuronal precursors, and *C. elegans* zygote formation, but have now been elucidated in many tissues, including those of mammals. In this review, we describe our current understanding of the mechanisms and consequences of ACD in cells of solid tissues, discuss the evidence that similar processes might apply in hematopoietic progenitor cells, B cells, and T cells. We also discuss, what will be required to determine whether there are physiological roles for ACD in lymphocyte development, function, and disease.

THE ROLE OF ACD IN SOLID TISSUES

Homeostasis of stem cells frequently involves ACD, where a parent cell divides to generate a daughter cell identical to itself ("self-renewal"), as well as another daughter that is programmed to proliferate, differentiate, or both (1). In some instances, the different fates of the two daughters can occur through stochastic responses in which each daughter has some probability of either self-renewing or adopting a different fate to maintain an appropriate balance of self-renewing and differentiating progeny on a population level. In other instances, the balance between self-renewal and differentiation is controlled at the single cell level by ACD. An example in which ACD controls the expansion and differentiation of the cells occurs in the developing *Drosophila* central nervous system (2) (Figure 1A). During development of the larval central nervous system, neuroblasts delaminate from the neurepithelium to undergo up to 20 rounds of ACD, each round creating another neuroblast ("self-renewal") and a ganglion mother cell (GMC) that can further proliferate and differentiate to form mature neurons. Neuroblasts become quiescent during pupation but then re-enter the cell cycle and reinitiate ACD for further rounds of proliferation and differentiation (1). The limited set of neuroblasts therefore undergoes controlled ACD that contributes to the thousands of adult neurons and neuronal associated cells of the central nervous system.

ACD plays a dominant role in dictating fate in *C. elegans*, starting at the first zygotic division after fertilization, when the fertilized egg divides asymmetrically to produce an anterior AB cell and a

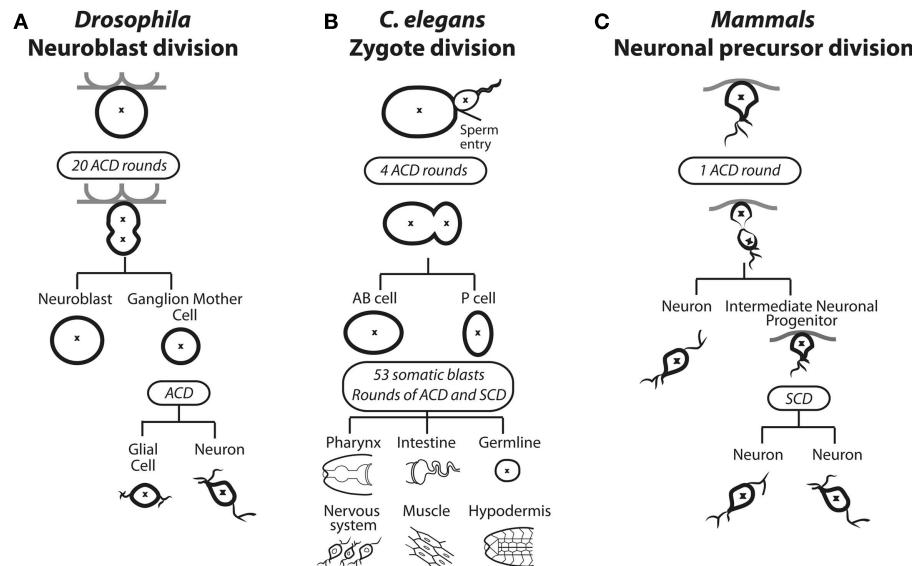


FIGURE 1 | Asymmetric cell division in solid tissues of (A) *Drosophila*, (B) *C. elegans*, and (C) Mammals. (A) In *Drosophila*, selected neuroblasts undergo up to 20 rounds of asymmetric cell division (ACD). The asymmetric distribution of polarity and cell fate determinants causes spindle asymmetry to result in a large self-renewing neuroblast cell and a smaller ganglion mother cell (GMC). The GMC undergoes a subsequent ACD to produce a glial cell and a neuron. (B) ACD during zygotic division in *C. elegans*. The site of sperm entry serves to determine the asymmetric distribution of polarity and

cell fate determining proteins as well as spindle asymmetry. During the embryonic stage four rounds of ACD results in the emerging anterior body (AB) and posterior (P) cells. During the larval stage, 53 somatic blasts undergo bursts of ACD and symmetric cell division (SCD), specifying all future posterior or soma fates in various tissues. (C) Neuronal precursor asymmetric division in mammals. The first asymmetric cell division produces a neuron and an intermediate neuronal precursor (INP), which undergoes a symmetric division to produce two neurons.

posterior P1 cell (Figure 1B). Four rounds of ACD follow; each producing one daughter that contributes only to soma and the other only to the germline. Thus, ACD controls differentiation and influences the expansion of cells from one generation to the next (3). ACD also occurs in mammals during brain and gut development. During brain development, a burst of symmetric cell divisions (SCDs) increases the progenitor pool, then sequential ACD in the neurepithelium balance self-renewal with differentiation of cells committed to the neuronal lineage (Figure 1C) (4). During mammalian gut development, in particular the colonic crypt, there is a high turnover of tissue where up to 10^{10} mature gut cells are replenished using a balance of symmetric and asymmetric divisions (5, 6). Within the folds of epithelium lining the colon, crypt cells continually undergo ACD to self-renew and generate proliferative daughter cells that terminally differentiate and transiently populate the migrating compartment, then die. ACD in mammals has also been observed during the development and differentiation of muscle, mammary glands, and skin (7–12). The mechanisms guiding these decisions in mammals are not well understood, but many molecular players that were identified in *C. elegans* and *Drosophila*, as discussed in the next section, have also been implicated in mammalian ACD.

An interesting aspect of ACD is the varied extent of influence that has been observed in different developmental systems. ACD is absolutely required during zygotic development in *C. elegans*, where the molecular differences between the daughter cells directly specify their different fates (13–18). In contrast, ACD of *Drosophila* nervous system is not (or less) deterministic, as subsequent fate

decisions are subject to influences from the microenvironment [reviewed in Ref. (19)]. In some instances, the primary molecular consequence of ACD is a difference in signaling between the two daughter cells. Rather than specifying the differentiation path for the two daughter cells, this merely ensures that the two daughter cells adopt different fates from each other in response to external influences (20, 21). Context can play another important role by controlling whether a cell divides symmetrically or asymmetrically. In contrast to the prescriptive pattern in *C. elegans*, where the early divisions are uniformly asymmetric, cell divisions in the mammalian developing nervous system can switch from symmetric to asymmetric to selectively expand specific cellular pools, or to generate more differentiated cell types as the need arises (22, 23).

MOLECULAR REGULATION OF ACD

ACD involves three processes: (i) cellular cues to dictate the axis of polarity; (ii) opposing actions of polarity proteins to dictate molecular differences along this axis; and (iii) the alignment of the mitotic spindle with the polarity axis to maintain asymmetry during division (Figure 2). Many of the proteins involved in establishing polarity and aligning the mitotic spindle are evolutionarily conserved, but differences occur in the cues that dictate the orientation of polarity, the composition of the polarity modules, and the fate determinants that dictate the differences in the functional outcome in different cell types. Here, we focus on the *Drosophila* central nervous system to illustrate the principles of mutual antagonism and connectivity with the spindle pole that are required for ACD (Figure 3A).

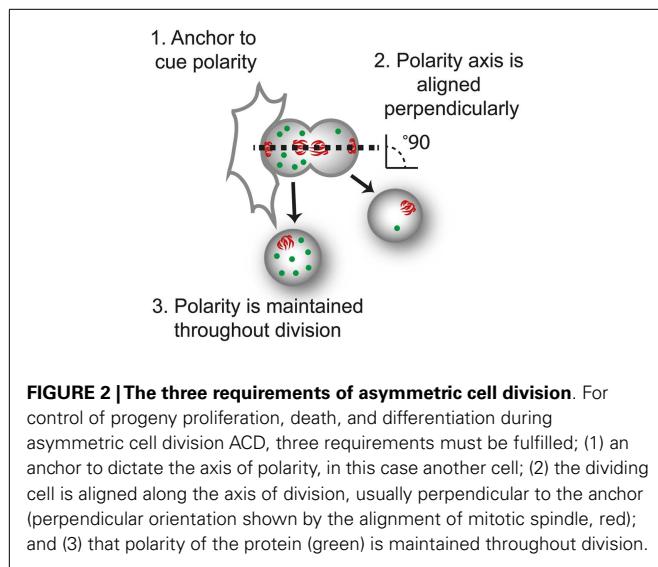


FIGURE 2 | The three requirements of asymmetric cell division. For control of progeny proliferation, death, and differentiation during asymmetric cell division ACD, three requirements must be fulfilled; (1) an anchor to dictate the axis of polarity, in this case another cell; (2) the dividing cell is aligned along the axis of division, usually perpendicular to the anchor (perpendicular orientation shown by the alignment of mitotic spindle, red); and (3) that polarity of the protein (green) is maintained throughout division.

Asymmetric cell division and segregation of cell fate determinants in *Drosophila* neuroblasts is regulated by the interactions between the Scribble and Bazooka (Par3 in mammals) polarity complexes. Through the interaction with the $\text{G}\alpha_i$ complex, the Scribble and Bazooka complexes also coordinate the orientation of the mitotic spindle. During ACD, the Bazooka and $\text{G}\alpha_i$ complexes are linked via an adaptor protein, inscuteable, and polarize to the apical cortex of the dividing cell (24–26). In addition, Dlg (from the Scribble complex) binding to the plus-end directed microtubule motor protein Khc-73 (Kinesin heavy chain 73) and Pins regulates the positioning of the $\text{G}\alpha_i$ complex (27, 28).

The mechanism by which the Par3/Bazooka and Scribble complex delineate the two poles of the cell is not yet clear, but it is thought to involve the regulation of aPKC phosphorylation activity by Lgl. The activity of aPKC is inhibited when it is in a complex with Par6 and Lgl (part of the Scribble complex) (17, 18). During mitosis, Par6 is phosphorylated, which relieves the repression of aPKC activity and allows aPKC to phosphorylate and release Lgl from the complex. This in turn allows the restriction of aPKC localization to one side of the cell cortex, where it is free to phosphorylate and release the cell fate determinants such as Numb from that side of the cortex (29, 30). One key observation is that while proteins of the Par3/Bazooka and Scribble complex localize at the apical side during early neuroblast division, some members of these complexes disperse cortically at telophase (6). Moreover, mutations in *scribble* or *lgl* do not affect Dlg polarization, but Dlg is required for the cortical recruitment and polarization of both Scribble and Lgl (6).

The role of ACD in steering fate determinants preferentially into one daughter cell is illustrated by the phenotypes in *Drosophila* in which the polarity and spindle regulators are mutated. For instance, the loss of *lgl* results in loss of asymmetric recruitment of fate determinants such as Numb in neuroblasts (6, 31, 32). Mutations in *scribble*, *dlg*, or *lgl* lead to mislocalization of multiple basal cell fate determinants and disrupt orientation of the mitotic spindles, which results in perturbed cell size and decreased GMC fate specification. In contrast to *lgl* mutations, mutations in

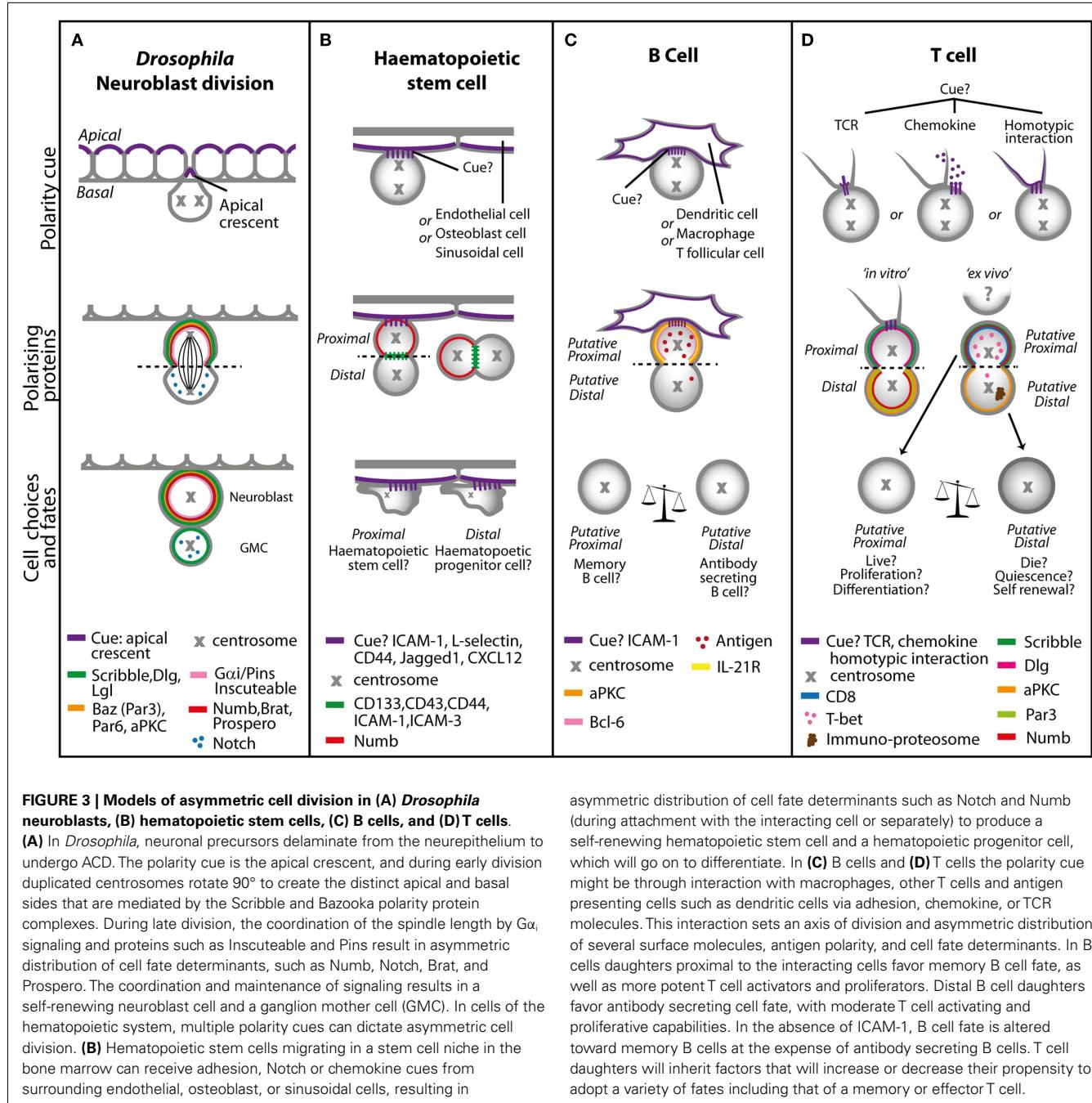
aPKC lead to reduced neuroblast proliferation (33). Interestingly, the neuroblast hyperproliferation and mislocalization of cell fate determinants that is associated with *lgl* mutant flies can be partially rescued when crossed with *aPKC* mutant flies (34), indicating that interactions between aPKC and Lgl specify respective cell fates. Other studies have also identified that Numb is a substrate for aPKC, and that aPKC-mediated phosphorylation is critical for the asymmetric segregation of Numb and the specification of neuroblast fate (29, 30, 35). Other genetic and biochemical assays show that Numb directly binds to the intracellular domain of the cell fate determinant, Notch. It is postulated that Numb acts as a negative regulator by mediating Notch degradation via endocytic pathways mediated by alpha-Adaptin, a component of the clathrin associated endocytosis pathway, which targets proteins for endocytosis (36, 37).

DOES ACD OCCUR IN THE HEMATOPOIETIC SYSTEM?

As with the numerous fate choices governing *Drosophila* neuroblast fate, all cells of the hematopoietic system make fate decisions related to differentiation, proliferation, death, and self-renewal. It would seem reasonable that cells of the hematopoietic system should also adopt the process of ACD as a means of controlling such decisions. Elucidating a possible role for ACD in fate determination of hematopoietic cells, however, has been slower than elucidating the role for ACD in cells of solid tissues. First, the seminal work in model organisms such as *C. elegans* and *Drosophila* is more readily applicable to solid tissues in mammals, where the cue and fate determinants are often conserved across species (Table 1). For instance, elucidation of ACD in mammalian neurons has benefited directly from findings in *Drosophila*, but there is no *Drosophila* parallel to guide studies in the hematopoietic system. Second, most progress has been made in systems, where the entire developmental program can be tracked and correlated with molecular behavior during and subsequent to each cell division. This has been the case with *C. elegans*, where more than three decades of research effort has been invested into exploring the mechanisms by which ACD regulates development of the *C. elegans* embryo by time lapse microscopy (38). Similarly in *Drosophila*, two decades ago direct observations that Numb was recruited into the GMC upon division were reported, and that levels of Numb dictate neuronal differentiation (39).

Frustratingly, longitudinal analyses *in vivo* are still not conceivable for ACD in the hematopoietic system because morphology is less informative (size differences do not indicate subsequent fates) and the cells are highly motile (cannot be tracked *in vivo* over generations). Also, differentiation generally occurs gradually over a longer time period. Instead, we have relied so far on correlative findings, each of which contributes to, but does not definitively prove the notion that ACD in cells of the hematopoietic system, including lymphocytes impacts upon cell fate decisions. Experiments to explore a role for ACD in cells of the hematopoietic system such as HSC, and lymphocytes such as B cells and T cells, so far have involved seeking three lines of evidence:

1. Evidence of a bifurcation in cell fate in the daughter cells of a dividing hematopoietic cell;



- Evidence of asymmetry in dividing hematopoietic cells (defining the polarity cue and the fate determinants that are asymmetrically distributed);
- Evidence of fate alterations upon disruption of the control of ACD.

The burden of proof lies in trying to combine these approaches to demonstrate in the same system that both ACD and fate bifurcation occurs, that ACD is associated with cell fate decisions by the daughter cells, and that both are disrupted by deregulation of a cell polarity regulator.

ACD IN HSC

As with all stem cells, it is well accepted that blood homeostasis involves a bifurcation in HSC fate whereby one daughter of an HSC is a copy of the parent (self-renewal) and the other expands and differentiates to give rise to the many blood lineages. There is growing acceptance that HSC may also undergo ACD to regulate fate choices, and the cues that might regulate ACD have been well established (38, 40). HSCs interact with a niche within the bone marrow, fetal liver, and peripheral blood, which could provide polarity cues to mediate ACD for fate determination (41). For example, the osteoblasts in bone marrow can

Table 1 | Known functions of polarity proteins in lymphocytes.

Polarity protein	Known phenotypes	Lymphocyte phenotype
PAR-1 PAR-1 Par1b/MARK2/EMK	<ul style="list-style-type: none"> Mutation (<i>C. elegans</i>): controls spindle positioning (12) Mutations (<i>Drosophila</i>): failed oocyte polarity. Phenotypes can be rescued by expressing ParN1 isoforms (121) 	<ul style="list-style-type: none"> Dominant negative mutation (T cells): loss of Par1b polarization and TCR-induced MTOC polarization (111) Loss (B and T cells): normal B and T cell development. CD4⁺ T cells exhibit higher TCR activation, B cell T-dependent and T-independent responses are altered, suggesting autoimmunity (112)
PAR-3 Bazooka Par3, ASIP, PARD3	<ul style="list-style-type: none"> Mutation (<i>C. elegans</i>): posterior shift during P0 asymmetric cell division (15) Mutation (<i>Drosophila</i>): loss of apical cue for Inscuteable localization in asymmetrically dividing neuroblasts (25) Loss (<i>Drosophila</i>): disruption of embryo basolateral membrane polarity during mid-gastrulation (122), mislocalization of Numb, and planar symmetry of pl cell during sensory organ precursor ACD (123) failed oocyte ACD (124) Removal/ectopic expression (mammalian neocortex): reduction of ACD and transformation of glial progenitor fate (57) 	<ul style="list-style-type: none"> N/A. Par3 is excluded from the T cell uropod, and may localize transiently to the synapse during immunological synapse formation (78)
PAR-6 Par6 Par6 α , β , and γ /PARD6 A, B, and G	<ul style="list-style-type: none"> Mutation (<i>Drosophila</i>): failure of oocyte differentiation (45) 	<ul style="list-style-type: none"> Overexpression of Par6 N-terminal aPKC interacting domain reduces T cell uropod formation (106)
Pkc-3 Atypical PKC PKC γ /PKC λ and ζ	<ul style="list-style-type: none"> Mutation (<i>C. elegans</i>): posterior shift during P0 division (15) Knockdown (<i>Drosophila</i>): reduced cell proliferation in both neuroblasts and epithelia (34, 125) Loss (<i>Drosophila</i>): loss of AB cell polarity (126), failed oocyte ACD (124) 	<ul style="list-style-type: none"> Overexpression/dominant negative mutation (T cells): randomizes F-actin distribution, impairs uropod formation, motility, defects in T cell scanning (106, 127) Drug inhibition of aPKC/Par6 interaction (T cells): defective Numb localization during T cell ACD (94) Loss: delay in secondary lymphoid organ formation (66), no naive T cell defects, Th2 differentiation but not Th1 differentiation is impaired, inhibition of ovalbumin induced allergic airway disease (108) Combined PKCγ/PKCλ loss (HSC's, B and T cells): normal HSC self-renewal, engraftment, differentiation, interaction with the bone marrow microenvironment, polarization, self-renewal. Normal mature B cells and T cells numbers (56)
Crb-1/Crb-like Crumbs Crumbs1-3	<ul style="list-style-type: none"> Mutations (<i>Drosophila</i>): loss of epithelial cell morphology in the ectoderm (128) Ectopic expression of Crumbs3 (mammalian epithelia): loss of tight junction formation and intracellular polarity (129) 	<ul style="list-style-type: none"> N/A
C01B7.4/tag-117 Stardust Pals1/MPP5	<ul style="list-style-type: none"> Loss (single and combined with Crumbs loss, <i>Drosophila</i>): disruption of the establishment and maintenance of epithelial morphology in the embryo (130), disrupted embryonic basolateral membrane polarity during mid-gastrulation (122) Knockdown (mammals): loss of MDCK cell polarization in confluent cellular monolayers (129) 	<ul style="list-style-type: none"> Knock down (T cells): suboptimal T cell activation and proliferation. Strongly localized to the Golgi apparatus, and is mislocalized upon Bref A (Golgi disrupting) treatment (55)

(Continued)

Table 1 | Continued

Polarity protein	Known phenotypes	Lymphocyte phenotype
Let-413 Scribble Scribble	<ul style="list-style-type: none"> Mutations (<i>Drosophila</i>): reduced neuroblast size, increased ganglion mother cell size, defects in targeting cell fate determinants, and altered spindle asymmetry (6) Loss (<i>Drosophila</i>): defects in junction formation and epithelial organization. Hyperproliferation, formation of solid tumors in imaginal disk and follicular epithelia (121) 	<ul style="list-style-type: none"> Knockdown (T cell line): prevention of TCR receptor polarization in response to antigen presentation, reduction in migration due morphological changes resulting in reduction of uropod formation (78) Knockdown (thymocyte): defects in cell–cell clustering and maturation (77) Loss: (T cells): altered pERK signaling in T cells but responses to influenza infection are intact. (B cells): delayed B cell proliferation, but T dependant and T independent activation are normal (63)
Dlg-1 Disks large Dlg 1–4	<ul style="list-style-type: none"> Mutation (<i>Drosophila</i>): imaginal disk hyperproliferation, tumorogenesis, and transform into solid tumors (131), defects in neuroblast size and mitotic spindle asymmetry (6) 	<ul style="list-style-type: none"> Overexpression (T cells): attenuates basal and Vav1-induced NFAT reporter activation (92) Knockdown (T cells): enhances both CD3- and superantigen-mediated NFAT activation (89, 90, 92). Accumulation of actin at the T cell synapse, altered production of Th1 and Th2 cytokines (68) Loss (thymocytes and T cells): normal T cell development (63, 68). Variable defects in mature CD4⁺ T cell differentiation (68, 92) Normal TCR-induced early phospho-signaling, actin-mediating events, proliferation, (68)
Lgl-1 Lethal Giant Larvae Lgl1, Lgl2	<ul style="list-style-type: none"> Mutation (<i>Drosophila</i>): defects in neuroblast apical cell and spindle pole size resulting in symmetric or inverted neuroblast cell divisions. Loss of polarity in tissues that leads to overproliferation and tumor growth (5, 132) Loss (mammals): neural progenitor cells fail differentiation, fail to exit cell cycle, then over proliferate and result in neural ectodermal tumors. Mislocalization of Numb in the neuroectoderm of the tumors (133) 	<ul style="list-style-type: none"> Loss (HSC): increase in HSC numbers, cycling, increased HSC repopulation capacity and competitive advantage after transplantation (58)

Homologs: *C. elegans*, *Drosophila*, Mammals.

express Notch ligands such as Jagged-1, adhesion molecules such as ICAM-1/LFA, L-selectins, and CD44, and also express chemokines such as CXCL12 (3) (Figure 3B). Initial evidence that ACD might occur in HSC came from Reya and colleagues, who provided preliminary evidence of asymmetric distribution of Numb in HSC treated with nocodazole to block cells in mitosis. This observation is difficult to reconcile with findings that hematopoiesis seems completely normal in Numb and double *numb–numblike* conditional mutants (42, 43) and in mice with deletion of Numb-like combined with hypomorphic alleles of *numb* that produce 5–10% of Numb protein (44–46). Possible explanations for this apparent discrepancy include: incomplete deletion of Numb [recombination at the Numb locus can be context specific (47), and an incomplete deletion of Numb/Numb-like might still leave a few wildtype hematopoietic progenitors to undergo normal lymphoid lineage development, as one or few HSC can repopulate the entire hematopoietic system (48, 49)]; or that the Numb allele under investigation deletes only exons 5 and 6 and so might not act as a complete null in hematopoietic tissues (50). Another explanation might relate to the notion that ACD, rather than impacting

the levels of proteins in individual cells, might create differences in expression levels between neighboring cells to influence fate (51). In this case, mutant alleles that could not segregate asymmetrically might be more informative than mere deletion of the gene. Regardless, an exciting finding from the Reya study was that by using a fluorescent reporter of Notch signaling and time lapse imaging of paired daughters, they showed that HSC can produce daughters with different Notch signaling capacities, and that the proportion of HSC with differential Notch signaling in the daughters differed depending upon the stromal cells with which they were cultured (52).

Work from the Sauvageau laboratory using gain-of-function *in vitro* and *in vivo* assays found a component of the endosomal AP-2 complex, alpha-Adaptin (encoded by the Ap2a2 gene), to endow *in vivo* proliferative advantage and an increase in *in vitro* HSC maintenance (53). Given that alpha-Adaptin is also important for ACD in *Drosophila* neuroblasts and sensory organ precursors, these findings suggest that mechanisms of fate determination through ACD could be evolutionarily conserved in HSC. In support of this notion, time lapse imaging of HSC containing

fluorescently-tagged alpha-Adaptin showed asymmetric inheritance in approximately 50% of HSC divisions. Knockdown of alpha-Adaptin did not affect HSC proliferation, differentiation, homing or apoptosis, despite alpha-Adaptin mRNA expression being fourfold to eightfold higher in long-term HSC than in intermediate term HSC. Interestingly, alpha-Adaptin and Numb were not co-localized in HSC, unlike in *Drosophila* neuronal precursors (54), highlighting possible divergent mechanisms of cell fate control.

Besides these two studies, remarkably little is known of the mechanisms by which ACD of HSC might be regulated. Perhaps because cell division of an HSC is, by definition, an extremely rare event, there has been little imaging to determine what molecules are localized asymmetrically at the time of division. The role of polarity proteins in controlling cell fate in HSCs has not yet given a strong indication of the importance of ACD in hematopoiesis. While RNA interference of the Par3 complex proteins, Par6 and PKC ζ can impair HSC repopulation (55), single and double knockouts of PKC ζ and PKC ι/λ have no effect on HSC function, in primary and secondary engraftment (56). Adding to this confusion is that aPKC phosphorylates and regulates the Scribble complex protein, Lgl, and loss of Lgl1 leads to enhanced engraftment and better HSC repopulation capacity due to increased proliferation (57). The evidence to date is therefore suggestive rather than definitive that ACD might control aspects of HSC self-renewal and differentiation.

ACD IN B CELLS

ARE THERE BIFURCATIONS IN B CELL FATE THAT COULD BE INFLUENCED BY ACD?

B lymphocyte development involves fate choices such as proliferation, self-renewal, and differentiation to result in the formation of memory B cells, and plasma cells that produce antibodies of unique specificity (58). Duffy and colleagues recently produced the most exhaustive study to date to determine whether the daughter cells of a dividing B cell exhibit asymmetric fates (59). Time lapse analysis of differentiation, death, and time to next division imaged from one cell division to the next, showed that daughters from B cell divisions stimulated by interleukin-4 (IL-4) and IL-5 largely undergo symmetrical fates. Interestingly, a small proportion of B cell divisions displayed asymmetric cell fates in which one daughter died and the other survived. The authors determined that the discrepancy in fate observed in this fraction was not a result of asymmetric programming but of the internal competition for fates within each cell. In this model, which is well supported by examples across many species, each cell is programmed for “time to die” and “time to divide,” and these times are reset upon each cell division (60–62). Similarly, in the B cell study, the “time to die” and “time to divide” were set very close together, such that in some instances the two daughter cells from a single B cell had an equal probability of adopting either fate. This study argues against ACD controlling B cell fate. It should be noted that the symmetrical fate observed here was in the context of soluble activating factors, rather than a directional cue, so does not discount a role for ACD in other forms of B cell activation. In line with the Duffy et al. study, our time lapse analysis of B cells stimulated with another soluble agent lipopolysaccharide also argued against ACD (63).

IS THERE EVIDENCE OF POLARITY IN DIVIDING B CELLS?

In support of the notion that B cells could receive instructional cues through engagement with dendritic, macrophage, or T helper cells to dictate downstream fates via ACD, Barnett and colleagues explored polarity in the germinal center (64). Dividing B cells within the germinal center asymmetrically localized the transcription factor Bcl6, the receptor for IL-21, and the polarity protein PKC ζ (64). Asymmetry of these proteins during division required constant signaling through contact with antigen presenting cells, possibly via adhesion through LFA-1/ICAM-1. In ICAM-deficient mice, B cells did not efficiently polarize Bcl6 or PKC ζ , and showed a defect in the number of antibody secreting plasma cells. The evidence of polarity at division, and the correlation of loss of polarity with cell fate differences caused by loss of ICAM-1 (which might have many non-polarity related effects), is compatible with the notion that germinal center B cells undergo ACD to influence cell fate, but further quantification and evidence that the polarity is controlled rather than stochastic, is required to confirm this.

In a separate study, multi-photon microscopy of explanted lymph nodes showed that B cells acquired antigen from macrophages in a polarized manner *in vivo*, and that the acquired antigen could accumulate preferentially in one daughter cell after B cell division (65). Antigen asymmetry persisted for up to three rounds after B cell division, and, statistical modeling predicted that up to 25% of B cells undergo asymmetric inheritance of antigen. There was no evidence for involvement of a polarity cue, or of the molecules involved in polarity, suggesting that the asymmetry of antigen inheritance was more likely a stochastic response than a result of ACD (Figure 3C).

WHAT IS THE PHENOTYPE OF POLARITY-DEFICIENT B CELLS?

As with the fate tracking information above, analysis of polarity-deficient B cells provides evidence both for and against a role for ACD (Table 1). Mice deficient in PKC ζ exhibited subtle delays in B cell development, but these defects were normalized in older mice (66). The B cells from 4- to 6-week-old PKC ζ -deficient mice also show severe defects in *in vitro* proliferation, enhanced ERK signaling in response to B cell receptor cross-linking (but not in response to non-B cell receptor stimuli), could mount a normal T independent humoral response *in vivo*, and showed slight defects in T-dependent humoral responses (67). B cell development is grossly normal in the absence of Dlg1 (63, 68), Lgl1, and Scribble (63), although a recent paper suggests that Dlg1-deficient B cells, like PKC ζ -deficient B cells, exhibit developmental defects in young mice that are rescued in older mice (69). Knockdown of Dlg1 (also called SAP97) in B cells *in vitro* impaired the formation of the immunological synapse and inhibited BCR-dependent responses (70). Scribble-deficient mice have intact *in vitro* and *in vivo* humoral responses to activation and infection respectively, but again show perturbed kinetics of ERK phosphorylation (63) as previously seen in epithelial tissues when Scribble is depleted (71–73). Combined, these data do not provide compelling support for a role for ACD in B cell development or responses. The hints of B cell phenotypes in some knockouts, and the observations that these phenotypes diminish with age, suggest that compensatory mechanisms that might make combined or more acute deletions

necessary to determine the role of ACD in B cell development and function.

ACD IN T CELLS

ACD IN THYMOCYTES

A small number of observations suggest that polarity proteins, and perhaps ACD, might also play a role in developing T cells. Nearly 50 years ago, the proportions and kinetics of proliferation of three types of thymocytes, as distinguished by their size, were assessed using autoradiographic analysis of tritiated thymidine uptake, and the data fit a requirement for ACD (74). This was followed by microscopic evidence of asymmetry at division, as defined by differences in the cytoplasmic or nuclear size in the two daughters in several species including the mouse (75). The involvement of polarity proteins was shown in *in vitro* interactions between thymocytes and dendritic cells, where Dlg1 was rapidly polarized to the synapse following TCR activation (76). Pike and colleagues demonstrated that *in vitro* DN3 thymocyte development was perturbed by knockdown of Scribble, with an accumulation of DN3 thymocytes and inefficient double positive CD4⁺CD8⁺ thymocyte generation (77). Interestingly, depletion of Scribble affects DN3 thymocyte clustering by limiting the polarization of the integrin ICAM-1/LFA-1 (77, 78). In a study by Aguado and colleagues, the transgenic expression of wildtype or dominant negative forms of Numb result in altered DN3 thymocyte pre-TCR signaling, proliferation, and differentiation (79). Asymmetry of Numb was also proposed by this group as a mechanism for these signaling and fate differences, but asymmetry was not rigorously assessed. Taken together, these studies provide hints that polarity and cell fate proteins are important for aspects of T cell development and downstream fate choices. Careful analysis of protein localization at division, and correlation of any asymmetry with alterations in fate, will be required to elucidate a possible role for ACD in thymocyte differentiation.

ARE THERE BIFURCATIONS IN T CELL FATE THAT COULD BE INFLUENCED BY ACD?

Perhaps the most studied and most controversial aspect of lymphocyte ACD is in mature T cells. In part, the controversies are due to the elusive nature of the fate choices that a naïve T cell makes upon stimulation by an antigen presenting cell. CD4⁺ cells can differentiate along many pathways upon stimulation (80), but will not be discussed in detail here as the role of ACD in CD4⁺ differentiation has not been extensively pursued. CD8⁺ naïve T cells give rise to both effector and memory progeny, and many subpopulations within these categories. A bifurcation of fate decisions by the two daughters of a naïve CD8⁺ cell would be an appealing explanation for how one naïve T cell can yield both effector and memory populations (81). Despite the wealth of literature on the subject, it is still not clear exactly when the two lineages arise from a naïve T cell, and for instance whether (and how far) memory cells progress down the effector differentiation pathway before committing to a memory fate (82–84).

Several recent papers provide support for the notion that fate is controlled at many stages during T cell activation, including the time of first division, when ACD could play a role. Three recent studies assess the progeny of individual CD8⁺ clones *in vivo* and

made two important observations (85–87). First, a striking diversity in number of progeny (over 1000-fold) from individual clones was observed, indicating a remarkable degree of variation in the naïve T cell responses. Whether this variation was the result of cell intrinsic programming of the naïve precursor, stochastic responses to activation, or differences in the microenvironment, was not clear. Second, even within individual clones, disparity in fate decisions was observed with some naïve precursors giving rise to uniform progeny, and others giving rise to progeny that had variable effector and memory characteristics. The data from one of these studies assessed 304 possible models for progression between naïve, effector, central memory and effector-memory states, and found only two of the models to fit their data, one of which allowed for ACD in the control of cell fate and the other did not (86). To support the notion that decision making could occur at multiple stages of T cell activation, limiting dilution, and short term progeny analysis demonstrated that T cell fate determination occurs before, during, and after the first T cell division (30).

IS THERE EVIDENCE OF POLARITY IN DIVIDING T CELLS?

It is now well established that in mature T cells, activation of the TCR triggers recruitment of polarity proteins (Scribble, Dlg1–4, PKC ζ) to the immunological synapse (36, 88–93). Chang and colleagues contributed the first of steadily mounting evidence that mature T cells polarized polarity proteins during mitosis (94). Mitotic CD8⁺ T cells undergoing their first division following *Listeria* infection demonstrated asymmetric polarization of several polarity proteins including Scribble and PKC ζ , the cell fate determinant Numb, and surface molecules important for T cell function such as CD8. This asymmetry was dependent upon the adhesion molecule, ICAM-1, and when populations of daughter T cells from the first division were sorted on the basis of differential CD8 expression and injected into *Listeria*-infected mice, mice receiving daughter cells with lower surface CD8 cleared the delayed infection more efficiently (94). This suggested that ACD could control memory differentiation in CD8⁺ T cells, although it has not yet been determined whether the disparate CD8 levels were a direct consequence of ACD. The finding that the transcription factor, T-bet, was asymmetrically partitioned into the daughters preferentially expressing CD8 provided support for the notion that ACD controls key fate determinants for effector memory decisions (95). This study also demonstrated that CD4⁺ cells display polarity at mitosis (95), and further work by the Reiner group showed that CD8 memory T cells can reinitiate ACD after rechallenge (96). Work by Palmer and colleagues also showed ACD of CD8⁺ T cells, and further demonstrated that peptide affinity can determine the extent of asymmetry during effector differentiation, and that the extent of asymmetry correlated with pathology (97).

These *in vivo* studies together provided the first indications that T cells can undergo ACD. The necessity for fixed staining of cells extracted from lymph nodes, however, means that the context of the cell division is not apparent. Without a defined cue, it is not possible to discriminate between ACD and asymmetry due to stochastic distribution of proteins at the time of division. For instance, it has not been possible to observe in these *ex vivo* experiments whether the dividing cell was attached to an antigen presenting cell to directly observe the subsequent behaviors of each

T cell daughter. To address some of these issues, we have established an *in vitro* assay in which divisions can be observed in the context of interactions with the antigen presenting cell (98). In this system, T cell ACD required sustained contact with the antigen presenting cell but not a sustained immunological synapse. ACD of naïve T cells utilized conserved mechanisms, involving the Par3, Scribble, and Pins complexes to orchestrate spindle orientation. The cell fate determinant Numb was also localized asymmetrically, and disruption of mitotic spindle orientation caused mislocalization of Numb as well as altered memory and effector T cell fate ratios. Interestingly, there were several differences in protein asymmetry in this study and the *ex vivo* analyses described above. These include that the TCR and associated proteins were no longer polarized at the time of division in the *in vitro* system, and differences in the pole to which Numb was recruited. These differences might reflect differences in the experiments, such as *ex vivo* versus *in vitro* analysis, and the use of different transgenic systems and/or the use of Cytochalasin in the *ex vivo* experiments.

It would not be at all surprising for T cell ACD to be highly context dependent, with both qualitative and quantitative differences in ACD depending upon the context of T cell activation. This notion is supported by the study by Palmer and colleagues, in which different peptide ligands caused different degrees of asymmetry in the dividing cells (97). To further complicate the picture, *in vivo* imaging has suggested that interactions with the dendritic cell are transient around the time of division (99, 100), and that homotypic adhesions at this time can play a key role in fate determination (101, 102). Perhaps, therefore, some or all of the *ex vivo* dividing cells that exhibited asymmetry (94, 97) were polarized as a result of homotypic adhesions, which also depend upon ICAM-1 (101). A scenario in which ACD of CD8⁺ T cells could be qualitatively or quantitatively altered depending upon interactions with antigen presenting cells or other lymphocytes is compatible with the requirement that naïve CD8⁺ T cells must integrate many signals to orchestrate a robust but fine-tuned response to antigen presentation (103).

WHAT IS THE PHENOTYPE OF POLARITY-DEFICIENT T CELLS?

Initial studies utilizing knockdown approaches to reduce the expression of Scribble complex proteins suggested that they played important roles in the development and function of T cells. T cells with reduced Dlg1 and Scribble showed impaired polarity and signaling in response to antigen presentation (78, 89, 90, 104), and impaired regulatory T cell function (105) as well as the developmental defect described above (77). In contrast, the analysis of T cells from mice with deleted polarity genes has shown either no, or very subtle, phenotypes. Expression of kinase dead forms of aPKC results in a reduction of polarization during migration and scanning (106), yet mice deficient in the atypical PKC isoforms PKC ζ or PKC ι/λ have an intact mature T cell repertoire, and normal responses with the exception of a defect in Th1 responses (56, 107–110). Mice deficient in Par1b exhibit alterations in CD44 expression on CD4 T cells, which might reflect aberrations in memory development, and this correlates with an involvement of Par1b in T cell polarity (111, 112). Three independently generated mice deficient in Dlg1 also exhibited normal T cell development and function, although again a defect in Th1 responses was observed

in one mouse (68). Interestingly, the Th1 defect was observed in acute knockout (gene deletion driven by the CD4 promoter) and knockdown T cells, but not in T cells where the gene had been deleted in HSC, suggesting that compensatory mechanisms can occur during development to mask polarity-deficient phenotypes (68). In another study, Dlg1-deficient mice showed normal development and proliferative response, but a subtle change in the expression of CD44, 10 days after immunization, suggestive of a skewing of central and effector memory responses that was supported by differences in IL-2 production in immunized mice (113). Similarly, T cell development in Scribble and Lgl1 deficient mice was normal, as were the responses of Scribble-deficient mice to an influenza infection (63). Together, these studies indicate that the polarity proteins are not essential for HSC, T or B cell development and function, but that subtle effects can arise under some circumstances (**Table 1**).

A model for T cell ACD fate that links these findings could be as follows (**Figure 3D**). The polarity cue could derive from an interaction between the T cell and the antigen presenting cell or from homotypic interactions. A sustained immunological synapse may not be needed, but other molecules on the antigen presenting cell or the homotypic T cell, or chemokines might provide polarity cues. Quantitative and qualitative aspects of polarity at mitosis could be influenced by several factors such as the affinity of the TCR-MHC interaction, the duration of contact with the antigen presenting cell, the availability of other T cells for homotypic interactions. Partitioning of molecules, such as Numb and T-bet, differentially into one daughter cell would then cooperate with other signals from the microenvironment to fine tune the differentiation response.

FUTURE/CONCLUDING REMARKS

Much more work is needed to reconcile the differences in phenotypes between different studies of polarity-deficient mice, and to determine whether or not immune defects in polarity-deficient mice are due to defects in ACD. The effect of knockout or knockdown of several polarity regulators has now been assessed, and the general picture is that the most striking phenotypes occurred with acute knockout or early in development, with emerging evidence that compensatory mechanisms can occur with time. Furthermore, no publications yet have indicated a correlation between these defects and evidence of asymmetry at mitosis, so it is not possible to definitively ascribe any of the phenotypes to a defect in ACD. In support of a role for ACD in immune cell development and function, some correlations are now emerging in which alterations in ACD are associated with alterations in fate. In this light, the relationship between ACD and pathology discovered by the Palmer group (97) is very encouraging. Similarly, inhibition of aPKC by the drug aurothiomalate (“Gold”) altered both ACD (polarization of Numb in dividing cells) and effector:memory ratios in our *in vitro* study (98). The loss of ICAM-1 also correlates with disruption in ACD and alterations in T cell fate (94), although the multifaceted role of ICAM-1 in effector and memory differentiation (114) complicates interpretation of this observation. Even the phenotypes from direct deletion of a polarity protein must be interpreted with caution, as these effects might be attributed to either ACD or the role of polarity proteins in the formation and

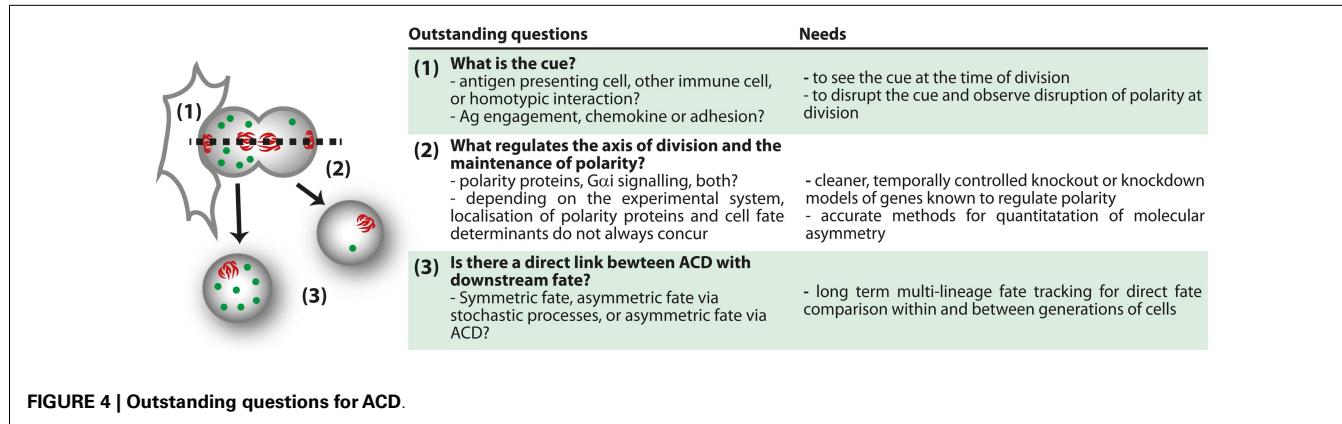


FIGURE 4 | Outstanding questions for ACD.

function of the immunological synapse and downstream signaling (70, 76, 89, 90, 105). More acute knockouts, identification of genes that influence polarity at mitosis but not earlier (perhaps by regulating spindle orientation), and more extensive correlations of asymmetry and fate are required to fill these gaps in our knowledge (Figure 4).

Ultimately, definitive evidence of the impact of ACD on fate determination in lymphocytes will require more extensive integration of the three aspects of ACD, such that bifurcation of fate and polarity at division can be directly correlated. Several factors need to be taken into account in this endeavor. First, evidence to date suggests that ACD of lymphocytes is not uniformly adopted, but seems to arise in a fraction of the cells measured. Disruption of ACD would most likely impact upon some, but not all, the progeny of the population. Second, given the complexity of lymphocyte fate determination, and the many external cues that can influence lymphocyte fate determination, it seems that ACD of lymphocytes would more likely modify than determine fate decisions. Third, fate choices in lymphocytes often emerge incrementally over several generations, so measurements of the fate decisions made by each daughter cell must also be performed over a protracted period of time.

Such studies are now conceivable using *in vitro* approaches. Time lapse imaging of the process of division has been performed (95), and this type of approach can now take advantage of the rapid development in methods of quantification and duration of imaging (115–119). With these tools, it will be possible to directly observe how asymmetry at division can impact upon lymphocyte fate determination. Although it is unlikely that *in vivo* imaging will enable long-term fate tracking in the near future, the ability to observe cells over several hours (99, 100), and to track protein distribution (120) *in vivo*, will yield important information regarding the physiological context in which ACD can be observed. These approaches, combined with others such as the long-term *in vitro* time lapse imaging and the *ex vivo* analysis pioneered by the Reiner group (94), will together enable a comprehensive understanding of the mechanisms and roles of ACD in lymphocytes. The bulk of the research so far has been performed in CD8 $^{+}$ T cells, but many other aspects of lymphocyte differentiation and function might also involve ACD. Tracing the progeny of a single cell *in vivo* using approaches such as cellular barcoding, which have already

provided evidence of fate bifurcation in the response of CD8 $^{+}$ T cells (85–87) are likely to yield important new information regarding the most physiologically relevant situations in which to look for ACD. Interestingly, a recent barcoding study argued against a clear bifurcation of fate in HSC (49). With the creation of more suitable knockout models, such as acute disruption of spindle orientation, new phenotypes might further highlight the systems in which ACD is most likely to play a physiological role (Figure 4). By combining all these approaches, a clearer picture of the mechanisms and consequences of ACD in lymphocytes is probably not too far away.

AUTHOR CONTRIBUTIONS

Kim Pham, Faruk Sacirbegovic, and Sarah M. Russell all wrote the manuscript.

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Reverse translation in tuberculosis: neutrophils provide clues for understanding development of active disease

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Tuberculosis (TB) is a major health issue globally. Although typically the disease can be cured by chemotherapy in all age groups, and prevented in part in newborn by vaccination, general consensus exists that development of novel intervention measures requires better understanding of disease mechanisms. Human TB is characterized by polarity between host resistance as seen in 2 billion individuals with latent TB infection and susceptibility occurring in 9 million individuals who develop active TB disease every year. Experimental animal models often do not reflect this polarity adequately, calling for a reverse translational approach. Gene expression profiling has allowed identification of biomarkers that discriminate between latent infection and active disease. Functional analysis of most relevant markers in experimental animal models can help to better understand mechanisms driving disease progression. We have embarked on in-depth characterization of candidate markers of pathology and protection hereby harnessing mouse mutants with defined gene deficiencies. Analysis of mutants deficient in miR-223 expression and CXCL5 production allowed elucidation of relevant pathogenic mechanisms. Intriguingly, these deficiencies were linked to aberrant neutrophil activities. Our findings point to a detrimental potential of neutrophils in TB. Reciprocally, measures that control neutrophils should be leveraged for amelioration of TB in adjunct to chemotherapy.

Keywords: tuberculosis, biomarker, inflammation, microRNA, interferon, neutrophil, chemokine

INTRODUCTION

Until today, segregation between basic and applied research has not been fully overcome in medical science, including immunology. Principally, basic research is conceived as hypothesis-driven, which benefits from choice of the most suitable experimental approach. Successful studies end with an outlook on medical application. In rare instances when this outlook indeed materializes, the whole endeavor is considered a perfect example of translational medicine. In contrast, applied clinical research is hampered by various layers of complexity, including heterogeneity of human populations and limitations in experimental approaches.

Recent advances in “omics” (i.e., high-throughput, HT, approaches, such as genomics or transcriptomics) have allowed a reciprocal strategy related to data-driven and hypothesis-generating approaches, which have been recognized as valuable complements to the hypothesis-driven path (1). Comparative studies of patients and healthy controls using different omics readouts allow a deeper understanding of mechanisms underlying disease progression, and identification of thus far unknown or insufficiently understood biological functions (2, 3). Hence, new research questions can be formulated, based on findings in the clinical context, which can subsequently be dissected in appropriate experimental models (Figure 1) – in short, from the bed to the bench (4).

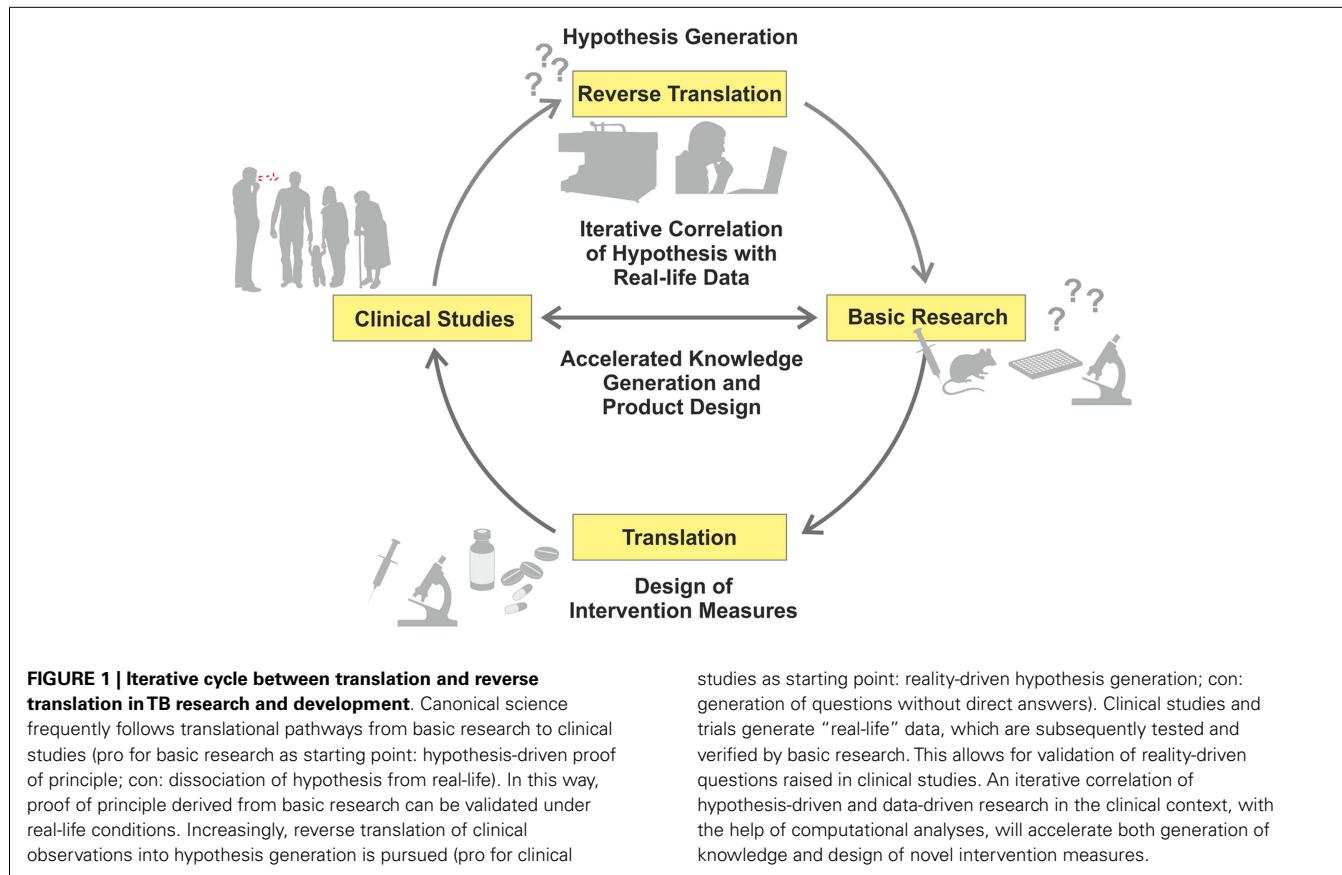
Immunology of tuberculosis (TB) is a case in point: mechanisms underlying pathology and protection in TB are highly intertwined and quantitative rather than qualitative differences

tip the balance toward disease progression (5). We have embarked on analyzing biomarkers that distinguish TB patients from healthy individuals and identified markers, which have fueled our interest to better understand their biological functions in TB.

THE ISSUE: TUBERCULOSIS

With a morbidity of 8.6 million cases and a mortality of 1.3 million deaths annually, TB remains a major health issue, surpassed only by human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) (6, 7). In fact, the two diseases have joined forces to create a perfect storm: TB is the major cause of death of HIV-infected individuals and HIV/AIDS the driving force for TB reemergence (6). Infection with *Mtb* transforms into active disease in only 5–10% resulting in 2 billion individuals with latent TB infection (LTBI), who remain healthy. Immunity is orchestrated by T lymphocytes that activate anti-mycobacterial activities in infected macrophages (5, 8). This is a local event focused on granulomatous lesions. *Mtb* is efficiently contained in solid granulomas where it adopts a dormant stage characterized by low replication rate and minimal metabolic activity (9, 10).

Globally, the ca. 2 billion healthy individuals with LTBI maintain this stage by means of an active immune response (5, 8). Yet, once it deviates, active disease develops. This is preceded by increasing necrosis of the granuloma followed by liquefaction. In the caseous granuloma, *Mtb* is resuscitated and resumes high replicative and metabolic activity (9, 10). Often in TB patients, granulomas of different maturation stages coexist (11). Whilst in



some granulomas the immune response is still capable of controlling *Mtb*, in others, it has already failed and *Mtb* has mastered the battle field (9, 10). Divergent immune activities in different lesions create major obstacles in the analyses of relevant immune responses in the periphery: it is likely that the circulating leukocytes reflect an averaged dominant stage of immune defense, but not unique activities operative in different lesions of varying immune status.

In infants, serious forms of TB can be prevented by vaccination. The vaccine, bacille Calmette–Guérin (BCG), therefore is still part of vaccination programs in countries where TB is endemic (12). Today, BCG is the most widely used vaccine with at least 4 billion total, and ca. 100 million annual, administrations. Yet, an efficacious vaccine against pulmonary TB in all age groups, which is not only the most prevalent form, but also the major source of transmission, is not available. Currently, novel vaccine candidates are being developed of which more than a dozen are undergoing clinical evaluation (13).

Tuberculosis can be cured by chemotherapy. However, successful treatment is long-lasting, requiring three to four drugs given over a period of at least 6 months. Not the least due to the complex and long-lasting treatment regimen, resistant TB is on the rise (14–16). Fifty million individuals are infected with multidrug-resistant (MDR)-*Mtb* strains that cannot be treated adequately with first-line drugs. Therefore, for half a million new MDR-TB cases annually, second-line drugs requiring longer and

more complex treatment schedules with higher side effects must be employed. The ca. 50,000 individuals registered in 92 countries, who have developed extensively drug-resistant (XDR)-TB, are even worse off since this form of TB is almost untreatable (6). In the meantime, totally drug-resistant (TDR)-TB has been notified in countries in Asia, Europe and Africa. No drugs are available to treat this form of TB. As a corollary, cost for TB control is increasing. It has been estimated that the European Union invests more than half a billion € for TB control and that the real cost including loss of human capital is in the order of 6 billion € (17). Globally, somewhere between 20 and 200 billion US\$ are lost due to TB, resulting in 0.5% loss of gross national income.

The epidemiologic facts of 1.3 million deaths and 8.6 million new cases of TB annually, may be viewed as perplexing in the face of 2 billion healthy individuals with LTBI. This conundrum immediately raises the question: what is the difference between individuals who successfully control one of the most devastating pathogens, whilst others succumb? This enigma is difficult to study in experimental animal models, which, aside from non-human primates, do not reflect the dissociation between LTBI and active TB disease adequately. At the same time, modern HT gene expression profiling of peripheral blood leukocytes could allow a gateway toward the elucidation of gene products involved in resistance underlying LTBI and pathology present in active TB. We launched a critical analysis of biomarkers that distinguish patients with active TB from healthy individuals with LTBI and selected

markers of interest for in-depth analyses in experimental mouse models.

BIOSIGNATURES, BIOMARKERS AND REVERSE TRANSLATION

The past decade has witnessed increasing interest in biomarker research in the area of TB. The potential of these biomarkers ranges from differential diagnosis to predictive response to therapy and risk to disease progression (2, 3). Biomarkers will also play a crucial role in future vaccine and drug trial design (18–20). On top of this, omics marker research can provide a wealth of information, which can be further exploited to decipher underlying resistance and disease mechanisms.

Principally, biomarker research is set in a clinical context and seeks to answer questions such as: How to diagnose or predict disease? How to monitor treatment outcome? How to predict vaccine efficacy and safety? Thus, large-scale HT platforms have been launched with the goal of screening thousands of genes or hundreds of metabolites [for a review see Ref. (2)]. From these studies the view emerges that quantitative biosignatures, rather than individual, “on-off” biomarkers are informative predictors of disease progression, treatment outcome or vaccine efficacy/safety. Basically, mere presence or absence of a single gene in a biosignature is insufficient to achieve error rates below 10%. Rather, relative expression levels of a set of genes or abundances of a set of metabolites form a more informative multidimensional biosignature. Metaphorically, instead of a single decision maker (i.e., a single biomarker), we view a biosignature as a house of representatives that agree on a mechanism, state of disease, or outcome of an intervention by casting a majority vote.

The primary goal of attempts to define biosignatures remains in the clinical arena. Yet, added to this, the functional interpretation of biosignatures allows precious insights into the pathophysiology of TB, as well as the generation of specific hypotheses to be tested in an experimental setting (3, 21). Vice versa, identifying relevant biological processes can clearly drive the search for useful biomarkers (22). Biomarkers should thus not be viewed as a computational black box that exists outside of biology; rather, they can both drive, and profit from, specific hypothesis-driven research.

Whole genome expression profiling by microarray analysis of peripheral blood cells, which has been applied most widely (23–30), provides a large body of data tempting researchers to formulate novel hypotheses. Prominent in peripheral blood, active TB disease is reflected by an increased activity in interferon (IFN) signaling, mainly in circulating neutrophils (23). In a similar fashion, most studies on blood cells have identified up-regulated signaling through Fc gamma receptors and elevated activity of the complement system [Ref. (23–25, 29, 31), for a review see Ref. (2)]. Finally, several publications describe activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (23–25), and abnormal functionality of regulatory suppressors of cytokine signaling [SOCS3, Ref. (27)] in TB. Such findings point to dysregulation in several key biological pathways, marking chronic immune activation and immune pathology of TB disease. The biomarker studies performed over the past years have revealed several TB-related signatures as described herein. The current challenge is to identify markers that are specific and unique for TB (for

a short listing of markers in TB and other pulmonary diseases; see Table 1). For example, the first “TB-specific” signature identified in a comparison with other infectious diseases (23), turned out shortly afterward to be also present in sarcoidosis (26, 32), which has high clinical similarity to TB. Elevated IFN-signaling, Fc gamma receptor signaling and complement activation are also shared with other respiratory diseases (26, 32, 33). A recent study (34) identified blood transcriptome signatures that were identified in both HIV[−] and HIV⁺ donors. Such findings are encouraging that future TB-specific signatures could also be applied in HIV-endemic countries.

Although signatures, as described above, are related to elevated anti-microbial activities of the immune response, they can also have detrimental effects. One informative example is the preponderant role of IFNs in the inflammation sustained during TB disease. While the role of type II IFN (IFN II or IFN- γ) in protection against TB is well established (35, 36), the role of type I IFN (IFN I) is less clear. Rather IFN I responses have been generally associated with anti-viral defense (37). Yet, both IFN I and IFN II signaling pathways are markedly up-regulated in TB. This led several groups to embark on analysis of IFN I in experimental TB and investigate interference between IFN I and II in mycobacterial infections.

IFN I CROSS-REGULATES CYTOKINE NETWORKS IN TB

Non-redundant functions have been attributed to IFN I and II during pulmonary TB in TB-resistant mice (38). Both cytokines contribute to adequate differentiation, survival and/or recruitment of myeloid cells to the lungs early during infection. Anti-inflammatory roles affecting leukocyte recruitment were uncovered in animals with intact IFN I signaling. These observations, however, contrast data obtained with animals receiving IFN I inducers (39), which develop exacerbated lung inflammation. Others have proposed that similarly to IFN II, IFN I limits availability of IL-1, a key pro-inflammatory cytokine (40, 41). The anti-inflammatory roles of IFN I in murine TB, as described for TB-resistant mice, are partially in line with results recorded for acute bacterial infections (42). Yet, these effects are opposed to those reported for chronic insults and infections (43, 44), which are characterized by IFN I-triggered inflammation. These apparently contradictory findings suggest biphasic and perhaps context-specific activity of IFN I. In support of this notion, investigations focusing on lymphocytic choriomeningitis virus (LCMV) infection describe a paradoxical detrimental role of IFN I during the chronic phase of this viral disease (45, 46).

In addition, cross-regulation between IFN I and II was revealed during mycobacterial infection, thereby explaining, to some extent, divergent observations. Via IL-10, IFN I regulates expression of the receptor for IFN II and subsequently cellular responsiveness to protective IFN- γ (47). On the other hand, absence of the IFN I-inducible ubiquitin-like intracellular protein ISG15 limits release of IFN II (48). It appears that both positive and negative regulation loops between IFN I and II exist and additional regulatory check points will likely be identified in the future. Although considerable progress has been achieved to streamline the biomarker value of IFN I, there is still need to decipher in greater detail local and perhaps systemic effects of IFN I signaling in TB.

Table 1 | Genes identified as differentially expressed in TB compared to healthy controls and reported as a part of a biosignature.

Interferon signaling	Pattern recognition receptor and inflammation	Neutrophil response	Adaptive immunity	Chemokines and receptors	Complement system	Fc receptors	Other
IFIT2; 3 (s)	TLR5 (s ^a ; l)	MPO (p)	BATF2 (s)	CXCR3 (s)	C1QA	CD64 (s; p; l)	RAC1 (s)
IFI44L (s)	CD32 (s)	CTSG (s ^a)	CD4	CXCR4 (s)	C1QB (s ^a)	CD32 (s)	SEC14 (s)
GBP1; 2; 5; 6 (s)	IRAK1; 3; 4 (s)	LTF (s; p)	CD40 (s)	CXCR5 (s)	C2		KLF2 (s ^a)
OAS1 (s ^a)	ETS2 (s)	BPI (s ^a)	IGHM (s)	CXCL9 (s)	SERPING1 (s)		HIF1A (s)
SOCS1 (s)	NAMPT (s; p)	DEFA4 (p)	IGHD (s)	CXCL10 (s)			HLTF (s)
SOCS5 (s)	CD163 (s; l)	NCF1 (s)	IGJ	CXCL14 (s ^a)			PSMA 1–7 (s)
	TGFB1 (s)	LCN2		CCL23 (s)			UCN2 (s)
	TRAF5	MMP9 (s; l; p)					SMARCD3 (s)
		MMP8 (p)					FOXB1
							FOXC2
							TIMP (s)
							RAB13 (s ^a ; p)
							RAB33 (s)
							CASP8 (s)

Annotations in brackets denote whether a gene has also been reported as differentially expressed in another disease condition by Maertzdorf et al. (26) or Bloom et al. (32); s, sarcoidosis; p, pneumonia; l, lung cancer.

^aDenotes genes that, while identified as differentially expressed in sarcoidosis, differ significantly in their expression from TB.

miR-223 FINE-TUNES INFLAMMATION IN TB

In the beginning, gene expression profiling mainly targeted protein-coding genes. More recently, the potential value of microRNAs (miRs), as TB biomarkers, has gained increasing interest (49) (**Table 2**). miRs have a profound impact on the biological activity of proteins by regulating messenger RNA (mRNA) stability and translation (50). Parallel analysis of miRs and mRNAs revealed significant correlations between expression of protein-coding and regulatory small miRs, suggesting functional relevance in TB (26). Particular miRs have been tentatively identified as potential biomarkers based on their differential expression levels (51–55). Moreover, by means of HT analyses of TB mRNAs and miRs, we identified clusters of correlated miRs and mRNAs, which were differentially expressed between TB and controls (2, 26) and enriched for immune-related functions. Their direct biological function in TB, however, remains largely elusive. Whereas miRs are generally considered to fine-tune mRNA expression rather than performing unique functions, we recently demonstrated a unique biological role of one such miR in inflammatory processes in TB (56).

In our studies, we focused our attention on miR-223, which had been identified in one study as one of the most up-regulated miRs in peripheral blood of patients with active TB compared to individuals with LTBI (54). MiR-223 expression is induced during granulopoiesis (60), controlled by different myeloid transcription factors (61, 62), and reaches its highest level in mature neutrophils. Additionally, miR-223 modulates cell activation by targeting NLR family pyrin (NLRP) containing domain 3 (NLRP3) inflammasome and I-kappa-B-kinase (IKK) alpha (IKK- α) (63, 64). To address the biological role of miR-223 during TB, we employed the aerosol *Mtb* infection model of miR-223 mutant mice (60). MiR-223-deficient mice were highly susceptible to pulmonary TB. Lethality was preceded by profound alteration of the lung structure, high bacterial burden, and exacerbated inflammation, which

Table 2 | Candidate microRNA biomarkers for active TB.

Sample type	microRNA	Reference
Monocytes	hsa-mir-582-5p	(57)
CD4 ⁺ T cells	has-miR-21, has-miR-26a, has-miR-29a, and miR-142-3p	(52)
Serum	hsa-miR-361-5p, hsa-miR-889, and miR-576-3p	(53)
PBMCs	hsa-miR-146a and has-miR-424	(58)
Sputum	hsa-mir-3179, has-miR-147, and hsa-miR-19b-2-5p	(55)
Peripheral whole blood	hsa-miR-144	(26)
PBMCs	hsa-miR-155 and hsa-miR-155-3p	(59)
PBMCs	hsa-miR-144, hsa-miR-365, hsa-miR-424, and hsa-miR-451	(54)
Serum	hsa-miR-29a	(51)

was mostly due to uncontrolled neutrophil migration to the site of infection. Lung gene expression profiling highlighted genes involved in neutrophil recruitment and the immune response as potential targets for miR-223.

Corroboration of gene expression profiles with predicted targets of miR-223, combined with molecular investigations, allowed us to identify chemokine (C-X-C motif) ligand 2 (CXCL2), chemokine (C-C motif) ligand 3 (CCL3), and interleukin 6 (IL-6) as novel targets of miR-223. These proteins were abundant during *Mtb* infection and directed neutrophil activation, and/or recruitment. Indeed, CXCL2 and CCL3 were first identified as chemotactic molecules for neutrophils produced by activated macrophages

while IL-6 regulates granulopoiesis (65–67). Thus, in our model of experimental TB, the absence of miR-223 was responsible for uncontrolled expression of chemotactic mediators, CXCL2 and CCL3, and heightened neutrophil availability due to impaired granulopoiesis as a consequence of uncontrolled IL-6 expression. These conditions, concurrent with more abundant chemokine (C-X-C motif) receptor 2 (CXCR2) expression (60), were responsible for impaired neutrophil migration to the lung during TB and consequently tissue destruction.

These results add further knowledge to the role of miRs during TB and in particular suggest that miR-223 controls TB susceptibility by limiting neutrophil recruitment through regulation of pro-inflammatory chemokines.

CHEMOKINE-DEPENDENT NEUTROPHIL INFUX MODULATES TB PATHOLOGY AND SUSCEPTIBILITY

Our biomarker analysis focusing on miR-223 in experimental TB of mice as well as reports by others reveal a profound correlation between disease susceptibility, TB pathology, and magnitude of the neutrophil responses. DBA/2, CBA/J, and C3HeB/FeJ, all *Mtb*-susceptible mouse strains, suffer from increased pulmonary neutrophil influx (68–70). Treatment of these animals with anti-inflammatory agents alone or combined with anti-mycobacterial chemotherapy limits progression of active TB (70, 71), suggesting that targeting neutrophilic inflammation is a valid option for cure of TB. Likewise, susceptible gene-deletion mutant strains, such as *Card9*^{−/−} and *miR-223*^{−/−} mice can be rescued by antibody-mediated neutrophil depletion (56, 72). In patients with pulmonary TB, neutrophils are abundant in BAL fluid and sputum and show higher bacterial burden than macrophages (73). The potential beneficial role of neutrophils in TB through killing the pathogen remains a matter of debate. Reports on a role of neutrophils in human TB range from assigning them solely phagocytic capacities to *Mtb*-killing capacities (74). In the zebrafish model, neutrophils kill *M. marinum* by engulfing-infected macrophages (75). In mice, immune-advantageous functions of neutrophils in TB have been linked to dendritic cell migration and T cell priming (76, 77).

Neutrophils can rapidly enter sites of inflammation under the direction of humoral factors including chemokines, notably of the ELR⁺ CXC family, which bind to CXCR1 and CXCR2. ELR[−] CXC chemokines, for example, CXCL13, are primarily chemoattractants for lymphocytes and bind to CXCR3, CXCR4, CXCR5, and CXCR7. Further subfamilies, include the CC chemokine subfamily, which comprises various members and bind to CC receptors; their target cells include most types of leukocytes. The third and fourth chemokine subgroups contain few members: the (X)C chemokine family consists of XCL1 and XCL2, and both primarily target T cells expressing the receptor XCR1. The CX₃C chemokine subfamily comprises to date only one known member, CX3CL1, which serves in its soluble form as chemoattractant for T cells and monocytes and in its cell-bound form as adhesion molecule [reviewed in Ref. (78, 79)].

To better understand how excessive neutrophil influx participated in increased TB susceptibility, we embarked on studying the role of CXCR2/CXCL5 in neutrophil control. Different chemokine/chemokine receptor pairs and inflammatory

mediators are described to be involved in neutrophil influx into the lung (80). However, we and others had noted that the neutrophil-chemotactic receptor, CXCR2 and its ligand, CXCL5 [or LPS-induced CXC chemokine (LIX)] are strongly up-regulated early following *Mtb* infection (69, 78, 81). Other ligands of CXCR2 comprise ELR⁺ chemokines, such as CXCL1 [or keratinocyte-derived chemokine (KC)], CXCL2 [or macrophage inflammatory protein 2 (MIP-2)], and CXCL15 (or lungkine).

The potential of chemokines and their receptors in shaping immune responses against *Mtb* have thus far focused on lymphocyte recruitment and granuloma organization (78). The influence of the chemokine system on neutrophil responses and inflammation in TB is poorly characterized. CXCR2^{−/−} mice show normal pulmonary neutrophil influx following intratracheal infection with an atypical *Mycobacterium*, *M. avium*. After intraperitoneal challenge with *M. avium*, however, neutrophil recruitment was impaired in CXCR2^{−/−} mice albeit without affecting bacterial burden (82). We found that following aerosol *Mtb* infection, neutrophil influx into alveolar spaces depended on CXCR2 as well as CXCL5. Moreover, absence of either CXCR2 or CXCL5 rendered mice more tolerant to high-dose *Mtb* infection (83).

Since multiple chemokines can bind CXCR2, they have generally been regarded as redundant. However, kinetic analyses indicate their temporal regulation (78) and cell type-specific expression of different chemokines points toward their spatial regulation. In TB, lung epithelial cells served as the pulmonary source of CXCL5 (83). Neutrophil-attracting chemokines including CXCL2 and CXCL1 are abundantly secreted by macrophages and neutrophils. CXCL1, but not CXCL2, is also secreted by pneumocytes. CXCL15 is solely secreted by bronchial epithelial cells (80, 84). CXCL5 is produced by platelets and various tissue-resident cells, such as alveolar epithelial cells (85, 86), cardiac myocytes (87), enterocytes (88), and aortic endothelial cells (89).

Taking the pathologic potential of dysregulated neutrophil responses into account, it appears essential that the host employs mechanisms to tightly regulate their recruitment into sensitive tissues. We propose that one mechanism is the temporal and spatial regulation of neutrophil-attracting chemokines. Thus, rather than being redundant, we suggest that neutrophil-attracting chemokines targeting the same receptor express unique, often additive effects.

CONCLUDING REMARKS

Tuberculosis biomarker research continues to generate signatures with clinical applicability and additionally furnishes novel hypotheses related to disease pathophysiology. We followed several candidate pathways and molecules that emerged from transcriptomics studies, including miR-223. Murine experiments provided deeper insights into disease processes influenced by this molecule. Neutrophils represented key effector cells of pathogenesis. In a complementary approach, we investigated the pathways that control neutrophil recruitment to the lung in progressive TB and distinguished a hitherto unappreciated relevance of a unique chemokine, namely CXCL5.

These examples illustrate that reverse translation is a valid approach and perhaps most importantly that iterative cross-examining of basic research findings and patient “omics” data,

allows novel insights into TB pathogenesis. We envisage that additional aspects of disease pathophysiology will be uncovered by integrating information from multiple patient-driven HT studies, such as metabolomics, proteomics, lipidomics along with transcriptomics, and deep-sequencing. In a further step, host information could be complemented by pathogen screens. This strategy will facilitate insights into host-pathogen interplay and allow prediction of interaction algorithms to be experimentally validated. As TB vaccine research and development suffer from lack of rigorous correlates of protection, we propose that reverse translation can significantly contribute to better understanding of basic mechanisms underlying pathophysiology. Such an approach will not only form the basis for identification of biosignatures that predict risk of disease but also predict vaccine efficacy. In a similar vein, biosignatures derived from TB vaccine trials can provide novel insights into vaccine-induced protective mechanisms. The feasibility of such an approach has proven successful in trials with licensed vaccines (18). Reciprocally, experimental TB research can facilitate rational design of novel intervention measures.

AUTHOR CONTRIBUTIONS

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Immunoglobulin and T cell receptor genes: IMGT® and the birth and rise of immunoinformatics

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IMGT®, the international ImMunoGeneTics information system®¹, (CNRS and Université Montpellier 2) is the global reference in immunogenetics and immunoinformatics. By its creation in 1989, IMGT® marked the advent of immunoinformatics, which emerged at the interface between immunogenetics and bioinformatics. IMGT® is specialized in the immunoglobulins (IG) or antibodies, T cell receptors (TR), major histocompatibility (MH), and proteins of the IgSF and MhSF superfamilies. IMGT® has been built on the IMGT-ONTOLOGY axioms and concepts, which bridged the gap between genes, sequences, and three-dimensional (3D) structures. The concepts include the IMGT® standardized keywords (concepts of identification), IMGT® standardized labels (concepts of description), IMGT® standardized nomenclature (concepts of classification), IMGT unique numbering, and IMGT Colliers de Perles (concepts of numerotation). IMGT® comprises seven databases, 15,000 pages of web resources, and 17 tools, and provides a high-quality and integrated system for the analysis of the genomic and expressed IG and TR repertoire of the adaptive immune responses. Tools and databases are used in basic, veterinary, and medical research, in clinical applications (mutation analysis in leukemia and lymphoma) and in antibody engineering and humanization. They include, for example IMGT/V-QUEST and IMGT/JunctionAnalysis for nucleotide sequence analysis and their high-throughput version IMGT/HighV-QUEST for next-generation sequencing (500,000 sequences per batch), IMGT/DomainGapAlign for amino acid sequence analysis of IG and TR variable and constant domains and of MH groove domains, IMGT/3Dstructure-DB for 3D structures, contact analysis and paratope/epitope interactions of IG/antigen and TR/peptide-MH complexes and IMGT/mAb-DB interface for therapeutic antibodies and fusion proteins for immune applications (FPIA).

Keywords: IMGT, immunogenetics, immunoinformatics, IMGT-ONTOLOGY, IMGT Collier de Perles, immunoglobulin, T cell receptor, major histocompatibility

IMGT®: THE BIRTH OF IMMUNOINFORMATICS

IMGT®, the international ImMunoGeneTics information system®¹ (1), was created in 1989 by Marie-Paule Lefranc at Montpellier, France (CNRS and Université Montpellier 2). The founding of IMGT® marked the advent of immunoinformatics, a new science, which emerged at the interface between immunogenetics and bioinformatics. For the first time, immunoglobulin (IG) or antibody and T cell receptor (TR) variable (V), diversity (D), joining (J), and constant (C) genes were officially recognized as "genes" as well as the conventional genes (2–5). This major breakthrough allowed genes and data of the complex and highly diversified adaptive immune responses to be managed in genomic databases and tools.

The adaptive immune response was acquired by jawed vertebrates (or *gnathostomata*) more than 450 million years ago and is found in all extant jawed vertebrate species from fishes to humans. Understanding the basis for adaptive immunity, at the level of cell populations, individual cells, and molecules, has been a major

focus of immunology in the past century (6, 7). The adaptive immune response is characterized by a remarkable immune specificity and memory, which are the properties of the B and T cells owing to an extreme diversity of their antigen receptors. The specific antigen receptors comprise the immunoglobulins (IG) or antibodies of the B cells and plasmocytes (2) (Figure 1), and the T cell receptors (TR) (3) (Figure 2). The IG recognize antigens in their native (unprocessed) form, whereas the TR recognize processed antigens, which are presented as peptides by the highly polymorphic major histocompatibility (MH, in humans HLA for human leukocyte antigens) proteins (Figure 2).

The potential antigen receptor repertoire of each individual is estimated to comprise about 2×10^{12} different IG and TR, and the limiting factor is only the number of B and T cells that an organism is genetically programmed to produce (2, 3). This huge diversity results from the complex molecular synthesis of the IG and TR chains and more particularly of their variable domains (V-DOMAIN) which, at their N-terminal end, recognize and bind the antigens (2, 3). The IG and TR synthesis includes several unique mechanisms that occur at the DNA level: combinatorial rearrangements of the V, D, and J genes that code the

¹<http://www.imgt.org>

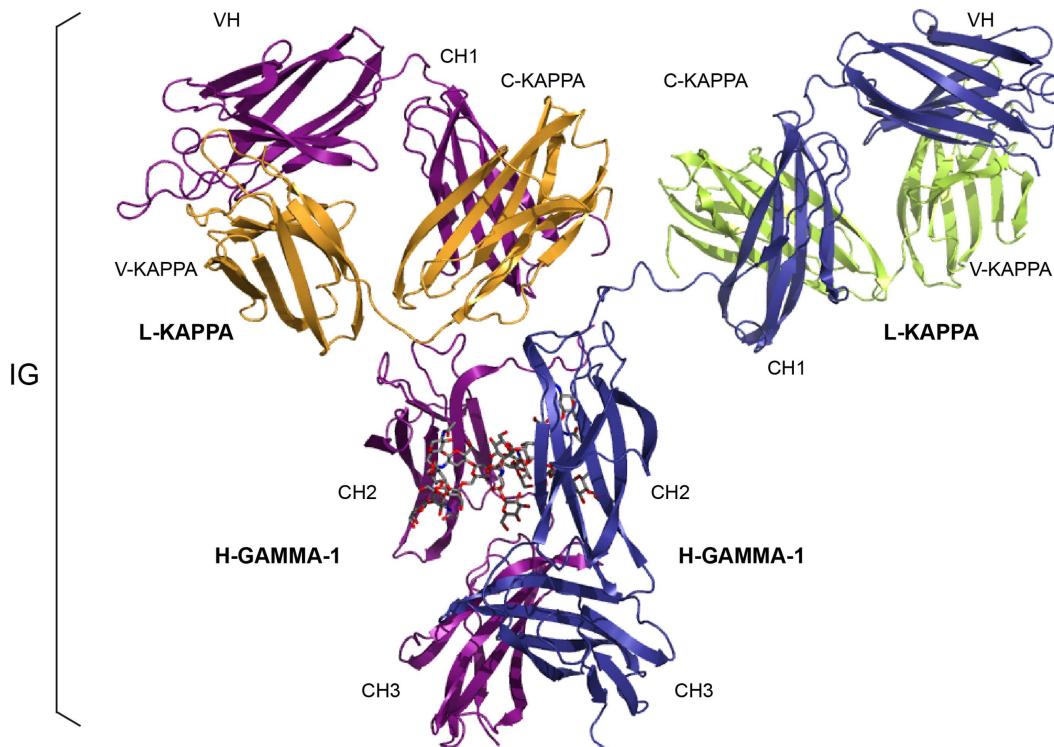


FIGURE 1 | An immunoglobulin (IG) or antibody. *In vivo*, an IG or antibody is anchored in the membrane of a B cell as part of a signaling B cell receptor (BcR = membrane IG+CD79) or, as shown here, is secreted (2). An IG is made of two identical heavy (H, for IG-HEAVY) chains and two identical light (L, for IG-LIGHT) chains (2). An IG comprises 12 domains (for example, IgG1, shown here) or 14 domains (IgM or IgE). The V-DOMAIN of each chain and the C-DOMAIN, one for each L chain and three for each H chain are highlighted.

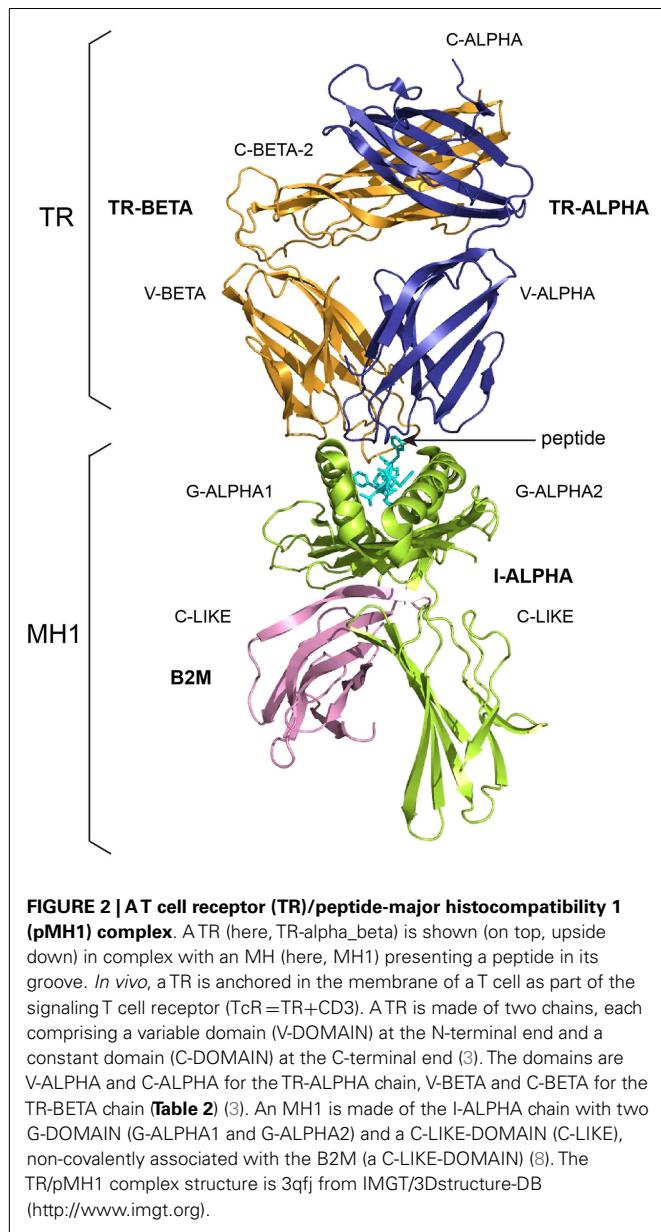
The light chain (here, L-KAPPA) is made of a variable domain (V-DOMAIN, here, V-KAPPA) at the N-terminal end and a constant domain (C-DOMAIN, here, C-KAPPA) at the C-terminal end. The heavy chain (here, H-GAMMA-1) is made of a VH (at the N-terminal end) and of three CH (four for H-MU or H-EPSILON) (Table 1) (2). The structure is that of the antibody b12, an IgG1-kappa, and so far the only complete human IG crystallized [1hz from IMGT/3Dstructure-DB (<http://www.imgt.org>)].

V-DOMAIN [the V-(D)-J being spliced to the C gene that encodes the C-REGION in the transcript], exonuclease trimming at the ends of the V, D, and J genes and random addition of nucleotides by the terminal deoxynucleotidyl transferase (TdT) that creates the junctional N-diversity regions, and later during B cell differentiation, for the IG, somatic hypermutations and class or subclass switch (2, 3).

IMGT® manages the diversity and complexity of the IG and TR and the polymorphism of the MH of humans and other vertebrates. IMGT® is also specialized in the other proteins of the immunoglobulin superfamily (IgSF) and MH superfamily (MhSF) and related proteins of the immune system (RPI) of vertebrates and invertebrates (1). IMGT® provides a common access to standardized data from genome, proteome, genetics, two-dimensional (2D), and three-dimensional (3D) structures. IMGT® is the acknowledged high-quality integrated knowledge resource in immunogenetics for exploring immune functional genomics. IMGT® comprises seven databases (for sequences, genes and 3D structures) (9–14), 17 online tools (15–30), and more than 15,000 pages of web resources [e.g., IMGT Scientific chart, IMGT Repertoire, IMGT Education > Aide-mémoire (31), the IMGT Medical page, the IMGT Veterinary page, the IMGT Biotechnology page, the IMGT Immunoinformatics page] (1).

IMGT® is the global reference in immunogenetics and immunoinformatics (32–47). Its standards have been endorsed by the World Health Organization–International Union of Immunological Societies (WHO–IUIS) Nomenclature Committee since 1995 (first IMGT® online access at the Ninth International Congress of Immunology, San Francisco, CA, USA) (48, 49) and the WHO–International Nonproprietary Names (INN) Programme (50, 51).

The accuracy and the consistency of the IMGT® data are based on IMGT-ONTOLOGY (52–54), the first, and so far, unique ontology for immunogenetics and immunoinformatics (8, 52–70). IMGT-ONTOLOGY manages the immunogenetics knowledge through diverse facets that rely on seven axioms: IDENTIFICATION, DESCRIPTION, CLASSIFICATION, NUMEROTATION, LOCALIZATION, ORIENTATION, and OBTENTION (53, 54, 58). The concepts generated from these axioms led to the elaboration of the IMGT® standards that constitute the IMGT Scientific chart: e.g., IMGT® standardized keywords (IDENTIFICATION) (59), IMGT® standardized labels (DESCRIPTION) (60), IMGT® standardized gene and allele nomenclature (CLASSIFICATION) (61), IMGT unique numbering (8, 62–66), and its standardized graphical 2D representation or IMGT Colliers de Perles (67–70) (NUMEROTATION).



The fundamental information generated from these IMGT-ONTOLOGY concepts, which led to the IMGT Scientific chart rules is reviewed. The major IMGT® tools and databases used for IG and TR repertoire analysis, antibody humanization, and Ig/Ag and TR/pMH structures are briefly presented: IMGT/V-QUEST (15–20) for the analysis of rearranged nucleotide sequence with the results of the integrated IMGT/JunctionAnalysis (21, 22), IMGT/Automat (23, 24) and IMGT/Collier-de-Perles tool (29), IMGT/HighV-QUEST, the high-throughput version for next-generation sequencing (NGS) (20, 25, 26), IMGT/DomainGapAlign (12, 27, 28) for amino acid (AA) sequence analysis, IMGT/3Dstructure-DB for 3D structures (11–13) and its extension, IMGT/2Dstructure-DB (for antibodies and other proteins for which the 3D structure is not available). IMGT® tools and databases run against IMGT reference

directories built from sequences annotated in IMGT/LIGM-DB (9), the IMGT® nucleotide database (175,406 sequences from 346 species in November 2013) and from IMGT/GENE-DB (10), the IMGT® gene database (3,117 genes and 4,732 alleles from 17 species, of which 695 genes and 1,420 alleles for *Homo sapiens* and 868 genes and 1,318 alleles for *Mus musculus* in November 2013).

An interface, IMGT/mAb-DB (14), has been developed to provide an easy access to therapeutic antibody AA sequences (links to IMGT/2Dstructure-DB) and structures (links to IMGT/3Dstructure-DB, if 3D structures are available). IMGT/mAb-DB data include monoclonal antibodies (mAb, INN suffix -mab; a -mab is defined by the presence of at least an Ig variable domain) and fusion proteins for immune applications (FPIA, INN suffix -cept) (a -cept is defined by a receptor fused to an Fc) from the WHO-INN Programme (50, 51). This database also includes a few composite proteins for clinical applications (CPCA) (e.g., protein or peptide fused to an Fc for only increasing their half-life, identified by the INN prefix ef-) and some related proteins of the immune system (RPI) used, unmodified, for clinical applications. The unified IMGT® approach is of major interest for bridging knowledge from Ig and TR repertoire in normal and pathological situations (71–74), Ig allotypes and immunogenicity (75–77), NGS repertoire (25, 26), antibody engineering, and humanization (35, 42–44, 46, 78–82).

IMGT-ONTOLOGY CONCEPTS

IDENTIFICATION: IMGT® STANDARDIZED KEYWORDS

More than 325 IMGT® standardized keywords (189 for sequences and 137 for 3D structures) were precisely defined (59). They represent the controlled vocabulary assigned during the annotation process and allow standardized search criteria for querying the IMGT® databases and for the extraction of sequences and 3D structures. They have been entered in BioPortal at the National Center for Biomedical Ontology (NCBO) in 2010².

Standardized keywords are assigned at each step of the molecular synthesis of an Ig. Those assigned to a nucleotide sequence are found in the “DE” (definition) and “KW” (keyword) lines of the IMGT/LIGM-DB files (9). They characterize for instance the gene type, the configuration type and the functionality type (59). There are six gene types: variable (V), diversity (D), joining (J), constant (C), conventional-with-leader, and conventional-without-leader. Four of them (V, D, J, and C) identify the Ig and TR genes and are specific to immunogenetics. There are four configuration types: germline (for the V, D, and J genes before DNA rearrangement), rearranged (for the V, D, and J genes after DNA rearrangement), partially-rearranged (for D gene after only one DNA rearrangement) and undefined (for the C gene and for the conventional genes that do not rearrange). The functionality type depends on the gene configuration. The functionality type of genes in germline or undefined configuration is functional (F), open reading frame (ORF), or pseudogene (P). The functionality type of genes in rearranged or partially-rearranged configuration is either productive [no stop codon in the V-(D)-J-region and in-frame junction]

²<http://bioportal.bioontology.org/ontologies/1491>

or unproductive [stop codon(s) in the V-(D)-J-region, and/or out-of-frame junction].

The 20 usual AA have been classified into 11 IMGT physicochemical classes (IMGT®, see footnote text 1, IMGT Education > Aide-mémoire > Amino acids). The AA changes are described according to the hydropathy (3 classes), volume (5 classes), and IMGT physicochemical classes (11 classes) (31). For example, Q1 > E (+ + -) means that in the AA change (Q > E), the two AA at codon 1 belong to the same hydropathy (+) and volume (+) classes but to different IMGT physicochemical properties (-) classes (31). Four types of AA changes are identified in IMGT®: very similar (+++), similar (++-, +-+), dissimilar (- +, - + -, +- -), and very dissimilar (---).

DESCRIPTION: IMGT® STANDARDIZED LABELS

More than 560 IMGT® standardized labels (277 for sequences and 285 for 3D structures) were precisely defined (60). They are written in capital letters (no plural) to be recognizable without creating new terms. Standardized labels assigned to the description of sequences are found in the “FT” (feature) lines of the IMGT/LIGM-DB files (9). Querying these labels represents a big plus compared to the generalist nucleotide databases [GenBank/European Nucleotide Archive (ENA)/DNA Data Bank of Japan (DDBJ)]. Thus it is possible to query for the “CDR3-IMGT” of the human rearranged productive sequences of IG-Heavy-Gamma (e.g., 1733 CDR3-IMGT obtained, with their sequences at the nucleotide or AA level). The core labels include V-REGION, D-REGION, J-REGION, and C-REGION, which correspond to the coding region of the V, D, J, and C genes. IMGT structure labels for chains and domains and their correspondence with sequence labels are shown for human IG (Table 1), for human TR (Table 2), and for MH (8) (Table 3). These labels are necessary for a standardized description of the IG, TR, and MH sequences and structures in databases and tools (60).

Highly conserved AA at a given position in a domain have IMGT labels (60). Thus three AA labels are common to the V and C-domains: 1st-CYS (cysteine C at position 23), CONSERVED-TRP (tryptophan W at position 41), and 2nd-CYS (C at position 104) (62–66). Two other labels are characteristics of the IG and TR V-DOMAIN and correspond to the first AA of the canonical F/W-G-X-G motif (where F is phenylalanine, W tryptophan, G glycine, and X any AA) encoded by the J-REGION: J-PHE or J-TRP (F or W at position 118) (62–64, 66).

CLASSIFICATION: IMGT® STANDARDIZED GENES AND ALLELES

The IMGT-ONTOLOGY CLASSIFICATION axiom was the trigger of immunoinformatics’ birth. Indeed the IMGT® concepts of classification allowed, for the first time, to classify the antigen receptor genes (IG and TR) for any locus [e.g., immunoglobulin heavy (IGH), T cell receptor alpha (TRA)], for any gene configuration (germline, undefined, or rearranged), and for any species (from fishes to humans). In higher vertebrates, there are seven IG and TR major loci (other loci correspond to chromosomal orphons sets, genes of which are orphans, not used in the IG or TR chain synthesis). The IG major loci include the IGH, and for the light chains, the immunoglobulin kappa (IGK), and the immunoglobulin lambda (IGL) in higher vertebrates, and the

immunoglobulin iota (IGI) in fishes (IMGT®, see footnote text 1, IMGT Repertoire).

Since the creation of IMGT® in 1989, at New Haven during the Tenth Human Genome Mapping Workshop (HGM10), the standardized classification and nomenclature of the IG and TR of humans and other vertebrate species have been under the responsibility of the IMGT Nomenclature Committee (IMGT-NC). IMGT® gene and allele names are based on the concepts of classification of “Group,” “Subgroup,” “Gene,” and “Allele” (61). “Group” allows to classify a set of genes that belong to the same multigene family, within the same species or between different species. For example, there are 10 groups for the IG of higher vertebrates: IGHV, IGHD, IGHJ, IGHC, IGKV, IGKJ, IGKC, IGLV, IGLJ, IGLC. “Subgroup” allows to identify a subset of genes, which belong to the same group, and which, in a given species, share at least 75% identity at the nucleotide level, e.g., *Homo sapiens* IGHV1 subgroup. Subgroups, genes, and alleles are always associated to a species name. An allele is a polymorphic variant of a gene, which is characterized by the mutations of its sequence at the nucleotide level, identified in its core sequence and compared to the gene allele reference sequence, designated as allele *01. For example, *Homo sapiens* IGHV1-2*01 is the allele *01 of the *Homo sapiens* IGHV1-2 gene that belongs to the *Homo sapiens* IGHV1 subgroup, which itself belongs to the IGHV group. For the IGH locus, the constant genes are designated by the letter (and eventually number) corresponding to the encoded isotypes (IGHM, IGHD, IGHG3...), instead of using the letter C. IG and TR genes and alleles are not italicized in publications. IMGT-ONTOLOGY concepts of classification have been entered in the NCBO BioPortal.

The IMGT® IG and TR gene names (2–5) were approved by the Human Genome Organisation (HUGO) Nomenclature Committee (HGNC) in 1999 (83, 84) and were endorsed by the WHO-IUIS Nomenclature Subcommittee for IG and TR (48, 49). The IMGT® IG and TR gene names are the official international reference and, as such, have been entered in IMGT/GENE-DB (10), in the Genome Database (GDB) (85), in LocusLink at the National Center for Biotechnology Information (NCBI) USA (86), in Entrez Gene (NCBI) when this database (now designated as “Gene”) superseded LocusLink (87), in NCBI MapViewer, in Ensembl at the European Bioinformatics Institute (EBI) (88), and in the Vertebrate Genome Annotation (Vega) Browser (89) at the Wellcome Trust Sanger Institute (UK). HGNC, Gene NCBI, Ensembl, and Vega have direct links to IMGT/GENE-DB (10). IMGT® human IG and TR genes were also integrated in IMGT-ONTOLOGY on the NCBO BioPortal and, on the same site, in the HUGO ontology and in the National Cancer Institute (NCI) Metathesaurus. AA sequences of human IG and TR constant genes (e.g., *Homo sapiens* IGHM, IGHG1, IGHG2) were provided to UniProt in 2008. Since 2007, IMGT® gene and allele names have been used for the description of the therapeutic mAb and FPIA of the WHO-INN Programme (50, 51).

The basis for the nomenclature of the MH of newly sequenced genomes has been set up on the same concepts. In IMGT®, MHC refers to the locus, which indeed is a complex of genes, particularly in the higher vertebrates. In contrast the letter “C” is dropped when referring to individual genes and proteins. Thus, the class I genes

Table 1 | Immunoglobulin (IG) receptor, chain, and domain structure labels and correspondence with sequence labels.

IG structure labels (IMGT/3Dstructure-DB)				Sequence labels (IMGT/LIGM-DB)
Receptor ^a	Chain ^b	Domain description type	Domain ^c	Region
IG-GAMMA-1_KAPPA	L-KAPPA	V-DOMAIN	V-KAPPA	V-J-REGION
		C-DOMAIN	C-KAPPA	C-REGION
		V-DOMAIN	VH	V-D-J-REGION
	H-GAMMA-1	C-DOMAIN	CH1	C-REGION ^d
		C-DOMAIN	CH2	
		C-DOMAIN	CH3	
IG-MU_LAMBDA	L-LAMBDA	V-DOMAIN	V-LAMBDA	V-J-REGION
		C-DOMAIN	C-LAMBDA-1	C-REGION
	H-MU	V-DOMAIN	VH	V-D-J-REGION
		C-DOMAIN	CH1	C-REGION ^d
		C-DOMAIN	CH2	
		C-DOMAIN	CH3	
		C-DOMAIN	CH4 ^e	

^aLabels are shown for two examples of IG (*Homo sapiens IgG1-kappa* and *IgM-lambda*). An IG ("Receptor") (Figure 1) is made of two identical heavy (H, for IG-HEAVY) chains and two identical light (L, for IG-LIGHT) chains ("Chain") and usually comprises 12 (e.g., IgG1) or 14 (e.g., IgM) domains. Each chain has an N-terminal V-DOMAIN (or V-(D)-J-REGION, encoded by the rearranged V-(D)-J genes), whereas the remaining of the chain is the C-REGION (encoded by a C gene). The IG C-REGION comprises one C-DOMAIN (C-KAPPA or C-LAMBDA) for the L chain, or several C-DOMAIN (CH) for the H chain (2).

^bThe kappa (L-KAPPA) or lambda (L-LAMBDA) light chains may associate to any heavy chain isotype (e.g., H-GAMMA-1, H-MU). In humans, there are nine isotypes, H-MU, H-DELTA, H-GAMMA-3, H-GAMMA-1, H-ALPHA-1, H-GAMMA-4, H-EPSILON, H-ALPHA-2 (listed in the order 5–3 in the IGH locus of the IGHC genes, which encode the constant region of the heavy chains (2)) (IMGT® <http://www.imgt.org>, IMGT Repertoire).

^cThe IG V-DOMAIN includes VH (for the IG heavy chain) and VL (for the IG light chain). In higher vertebrates, the VL is V-KAPPA or V-LAMBDA, whereas in fishes, the VL is V-IOTA. The C-DOMAIN includes CH [for the IG heavy chain, the number of CH per chain depending on the isotype (2)] and CL (for the IG light chain). In higher vertebrates, the CL is C-KAPPA or C-LAMBDA, whereas in fishes, the CL is C-IOTA.

^dThe heavy chain C-REGION also includes the HINGE-REGION for the H-ALPHA, H-DELTA, and H-GAMMA chains and, for membrane IG (mIG), the CONNECTING-REGION (CO), TRANSMEMBRANE-REGION (TM) and CYTOPLASMIC-REGION (CY); for secreted IG (sIG), the C-REGION includes CHS instead of CO, TM, and CY.

^eFor H-MU and H-EPSILON.

are designated as MH1 whereas the class II genes are designated as MH2. The IMGT nomenclature, with the MH1 and MH2 groups, has been used for the first time with the *Oncorhynchus mykiss* genes [see footnote text 1, IMGT Repertoire (MH) > Locus and genes > Gene tables]. It can also be applied to the human genes in databases, which deal with humans and other vertebrate species (for example, *Homo sapiens* MH1-A for HLA-A).

NUMEROTATION: IMGT UNIQUE NUMBERING AND IMGT COLLIER de PERLES

The IMGT-ONTOLOGY NUMEROTATION axiom is acknowledged as the "IMGT® Rosetta stone" that has bridged the biological and computational spheres in bioinformatics (40). The IMGT® concepts of numerotation comprise the IMGT unique numbering (8, 62–66) and its graphical 2D representation the IMGT Collier de Perles (67–70). Developed for and by the "domain," these concepts integrate sequences, structures, and interactions into a standardized domain-centric knowledge for functional genomics. The IMGT unique numbering has been defined for the variable V-domain (V-DOMAIN of the IG and TR, and V-LIKE-DOMAIN of IgSF other than IG and TR) (62–64), the constant C-domain (C-DOMAIN of the IG and TR, and C-LIKE-DOMAIN of IgSF other

than IG and TR) (65), and the groove G-domain (G-DOMAIN of the MH, and G-LIKE-DOMAIN of MhSF other than MH) (8, 90, 91). Thus the IMGT unique numbering and IMGT Collier de Perles provide a definitive and universal system across species including invertebrates, for the sequences and structures of the V, C, and G-domains of IG, TR, MH, IgSF, and MhSF (66, 70, 92, 93).

V-domain IMGT® definitive system

V-domain definition and main characteristics. In the IMGT® definitive system, the V-domain includes the V-DOMAIN of the IG and of the TR, which corresponds to the V-J-REGION or V-D-J-REGION encoded by V-(D)-J rearrangements (2, 3), and the V-LIKE-DOMAIN of the IgSF other than IG and TR. The V-domain description of any receptor, any chain, and any species is based on the IMGT unique numbering for V-domain (V-DOMAIN and V-LIKE-DOMAIN) (62–64, 66).

A V-domain (Figure 3) comprises about 100 AA and is made of nine antiparallel beta strands (A, B, C, C', C'', D, E, F, and G) linked by beta turns (AB, CC', C''D, DE, and EF), and three loops (BC, C'C'', and FG), forming a sandwich of two sheets [ABED] [GFCC'C''] (62–64, 66). The sheets are closely packed against each other through hydrophobic interactions giving a hydrophobic

Table 2 | T cell receptor (TR), chain, and domain structure labels and correspondence with sequence labels.

TR structure labels (IMGT/3Dstructure-DB)				Sequence labels (IMGT/LIGM-DB)
Receptor ^a	Chain	Domain description type	Domain ^b	Region
TR-ALPHA_BETA	TR-ALPHA	V-DOMAIN	V-ALPHA	V-J-REGION
		C-DOMAIN	C-ALPHA	Part of C-REGION ^c
	TR-BETA	V-DOMAIN	V-BETA	V-D-J-REGION
		C-DOMAIN	C-BETA	Part of C-REGION ^c
TR-GAMMA_DELTA	TR-GAMMA	V-DOMAIN	V-GAMMA	V-J-REGION
		C-DOMAIN	C-GAMMA	Part of C-REGION ^c
	TR-DELTA	V-DOMAIN	V-DELTA	V-D-J-REGION
		C-DOMAIN	C-DELTA	Part of C-REGION ^c

^aA TR (“Receptor”) (3) (**Figure 2**) is made of two chains (alpha and beta, or gamma and delta) (“Chain”) and comprises four domains. Each chain has an N-terminal V-DOMAIN [or V-(D)-J-REGION, encoded by the rearranged V-(D)-J genes (3)] whereas the remaining of the chain is the C-REGION (encoded by a C gene). The TR C-REGION comprises one C-DOMAIN (3). TR receptor, chain, and domain structure labels, and correspondence with sequence labels, are shown for two examples of TR (*Homo sapiens* TR-alpha_beta and TR-gamma_delta).

^bThe TR V-DOMAIN includes V-ALPHA, V-BETA, V-GAMMA, and V-DELTA. The TR C-DOMAIN includes C-ALPHA, C-BETA, C-GAMMA, and C-DELTA (there are two isotypes for the TR-BETA and TR-GAMMA chains in humans, TR-BETA-1 and TR-BETA-2, and TR-GAMMA-1 and TR-GAMMA-2, the C-REGION of these chains being encoded by the *TRBC1* and *TRBC2* genes, and *TRGC1* and *TRGC2* genes, respectively) (IMGT® <http://www.imgt.org>, IMGT Repertoire) (3).

^cThe TR chain C-REGION also includes the CONNECTING-REGION (CO), the TRANSMEMBRANE-REGION (TM), and the CYTOPLASMIC-REGION (CY), which are not present in 3D structures.

Table 3 | Major histocompatibility (MH) receptor, chain, and domain structure labels and correspondence with sequence labels.

MH group	MH structure labels (IMGT/3Dstructure-DB)				Sequence labels (IMGT/LIGM-DB)	
	Receptor ^a	Chain	Domain description type ^b	Domain	Domain number	Region
MH1	MH1-ALPHA_B2M	I-ALPHA	G-DOMAIN	G-ALPHA1	[D1]	Part of REGION ^c
			G-DOMAIN	G-ALPHA2	[D2]	
			C-LIKE-DOMAIN	C-LIKE	[D3]	
	B2M	II-ALPHA	C-LIKE-DOMAIN	C-LIKE	[D]	REGION
MH2	MH2-ALPHA_BETA	II-ALPHA	G-DOMAIN	G-ALPHA	[D1]	Part of REGION ^c
			C-LIKE-DOMAIN	C-LIKE	[D2]	
		II-BETA	G-DOMAIN	G-BETA	[D1]	Part of REGION ^c
			C-LIKE-DOMAIN	C-LIKE	[D2]	

^aAn MH (“Receptor”) (8) depending on the MH group is made of one chain (I-ALPHA) non-covalently associated to the beta2-microglobulin (B2M) (MH1 group, in the literature MHC class I) (**Figure 2**) or of two chains (II-ALPHA and II-BETA) (MH2 group, in the literature MHC class II). The I-ALPHA chain has two G-DOMAIN whereas each II-ALPHA and II-BETA has one G-DOMAIN. MH receptor, chain, and domain structure labels, and correspondence with sequence labels, are shown for examples of members of the MH1 and MH2 groups.

^bThe domain description type shows that the MH proteins belong to the MhSF by their G-DOMAIN and to the IgSF by their C-LIKE-DOMAIN. The B2M associated to the I-ALPHA chain in MH1 has only a single C-LIKE-DOMAIN and only belongs to the IgSF.

^cThe REGION of the I-ALPHA, II-ALPHA, and II-BETA chains also includes the CONNECTING-REGION (CO), the TRANSMEMBRANE-REGION (TM), and the CYTOPLASMIC-REGION (CY), which are not present in 3D structures.

core, and joined together by a disulfide bridge between a first highly conserved cysteine (1st-CYS) in the B strand (in the first sheet) and a second equally conserved cysteine (2nd-CYS) in the F strand (in the second sheet) (62–64, 66).

V-domain strands and loops (FR-IMGT and CDR-IMGT). The V-domain strands and loops and their delimitations and lengths, based on the IMGT unique numbering for V-domain (62–64, 66),

are shown in **Table 4**. In the IG and TR V-DOMAIN, the three hypervariable loops BC, C'C'', and FG involved in the ligand recognition (native antigen for IG and pMH for TR) are designated complementarity determining regions (CDR-IMGT), whereas the strands form the framework region (FR-IMGT), which includes FR1-IMGT, FR2-IMGT, FR3-IMGT, and FR4-IMGT (**Table 4**). In the IMGT® definitive system, the CDR-IMGT have accurate and unambiguous delimitations in contrast to the CDR described in

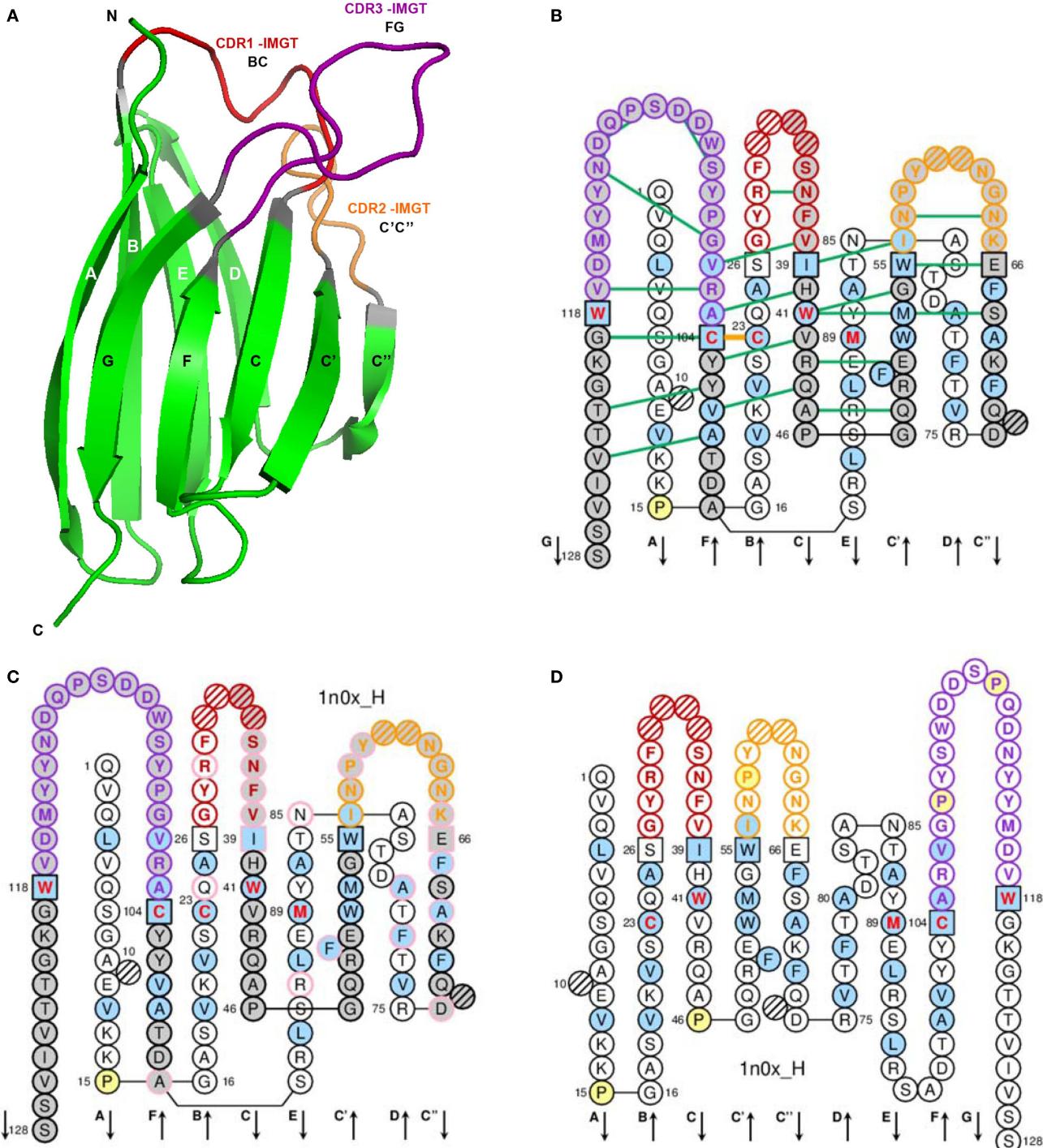


FIGURE 3 | Variable (V) domain. An Ig VH (V-DOMAIN) is shown as an example. **(A)** 3D structure ribbon representation with the IMGT strand and loop delimitations (64). **(B)** IMGT Collier de Perles on two layers with hydrogen bonds. The IMGT Collier de Perles on two layers show, in the forefront, the GFCC'C'' strands (forming the sheet located at the interface VH/VL of the Ig) and, in the back, the ABED strands. The IMGT Collier de Perles with hydrogen bonds (green lines online, only shown here for the GFCC'C'' sheet) is generated by the IMGT/Collier-de-Perles tool integrated in IMGT/3Dstructure-DB, from experimental 3D structure data (11–13). **(C)** IMGT Collier de Perles on two layers generated from IMGT/DomainGapAlign (12, 27, 28). Pink circles (online) indicate amino acid (AA) changes compared to the closest genes and alleles from the IMGT reference directory. **(D)** IMGT Collier de Perles on one layer. AA are shown in the one-letter abbreviation. All proline (P) are shown online in yellow. IMGT anchors are in square. Hatched circles are IMGT gaps according to the IMGT unique numbering for V-domain (64, 66). Positions with bold (online red) letters indicate the four conserved positions that are common to a V-domain and to a C-domain: 23 (1st-CYS), 41 (CONSERVED-TRP), 89 (hydrophobic), 104 (2nd-CYS) (62–66), and the fifth conserved position, 118 (J-TRP or J-PHE), which is specific to a V-DOMAIN (Continued)

27, 28). Pink circles (online) indicate amino acid (AA) changes compared to the closest genes and alleles from the IMGT reference directory. **(D)** IMGT Collier de Perles on one layer. AA are shown in the one-letter abbreviation. All proline (P) are shown online in yellow. IMGT anchors are in square. Hatched circles are IMGT gaps according to the IMGT unique numbering for V-domain (64, 66). Positions with bold (online red) letters indicate the four conserved positions that are common to a V-domain and to a C-domain: 23 (1st-CYS), 41 (CONSERVED-TRP), 89 (hydrophobic), 104 (2nd-CYS) (62–66), and the fifth conserved position, 118 (J-TRP or J-PHE), which is specific to a V-DOMAIN (Continued)

FIGURE 3 | Continued

and belongs to the motif F/W-G-X-G that characterizes the J-REGION (64, 66) (**Table 4**). The hydrophobic AA (hydropathy index with positive value: I, V, L, F, C, M, A) and tryptophan (W) (31) found at a given position in more than 50% of sequences are shown (online with a blue background color). Arrows indicate the direction of the beta strands and their designations in 3D structures. IMGT color menu for the CDR-IMGT of a V-DOMAIN indicates the type of rearrangement, V-D-J (for a VH here, red, orange,

and purple) or V-J (for V-KAPPA or V-LAMBDA (not shown), blue, green, and greenblue) (2). The identifier of the chain to which the VH domain belongs is 1n0x_H (from the *Homo sapiens* b12 Fab) in IMGT/3Dstructure-DB (<http://www.imgt.org>). The CDR-IMGT lengths of this VH are [8.8.20] and the FR-IMGT are [25.17.38.11]. The 3D ribbon representation was obtained using PyMOL (<http://www.pymol.org>) and “IMGT numbering comparison” of 1n0x_H (VH) from IMGT/3Dstructure-DB (<http://www.imgt.org>).

Table 4 | V-domain strands and loops, IMGT positions, and lengths, based on the IMGT unique numbering for V-domain (V-DOMAIN and V-LIKE-DOMAIN).

V-domain strands and loops ^a	IMGT positions ^b	Lengths ^c	Characteristic IMGT Residue@Position ^d	V-DOMAIN FR-IMGT and CDR-IMGT
A-STRAND	1–15	15 (14 if gap at 10)		FR1-IMGT
B-STRAND	16–26	11	1st-CYS 23	
BC-LOOP	27–38	12 (or less)		CDR1-IMGT
C-STRAND	39–46	8	CONSERVED-TRP 41	FR2-IMGT
C'-STRAND	47–55	9		
C/C"-LOOP	56–65	10 (or less)		CDR2-IMGT
C"-STRAND	66–74	9 (or 8 if gap at 73)		FR3-IMGT
D-STRAND	75–84	10 (or 8 if gaps at 81, 82)		
E-STRAND	85–96	12	Hydrophobic 89	
F-STRAND	97–104	8	2nd-CYS 104	
FG-LOOP	105–117	13 (or less, or more)		CDR3-IMGT
G-STRAND	118–128	11 (or 10)	V-DOMAIN J-PHE 118 or J-TRP 118 ^e	FR4-IMGT

^aIMGT® labels (concepts of description) are written in capital letters (no plural) (60). Beta turns (AB, CC, C'D, DE, or EF) are individualized only if they have additional AA compared to the standard description. If not, they are included in the strands.

^bBased on the IMGT unique numbering for V-domain (V-DOMAIN and V-LIKE-DOMAIN) (62–64, 66).

^cIn number of AA (or codons).

^dIMGT Residue@Position is a given residue (usually an AA) or a given conserved property AA class, at a given position in a domain, based on the IMGT unique numbering (66).

^eIn the IG and TR V-DOMAIN, the G-STRAND (or FR4-IMGT) is the C-terminal part of the J-REGION, with J-PHE or J-TRP 118, and the canonical motif F/W-G-X-G at positions 118–121 (2, 3). The JUNCTION refers to the CDR3-IMGT plus the two anchors 2nd-CYS 104 and J-PHE or J-TRP 118 (63, 64). The JUNCTION (positions 104–118) is therefore two AA longer than the corresponding CDR3-IMGT (positions 105–117) (63, 64).

the literature. Correspondences between the IMGT unique numbering with other numberings, e.g., Kabat (94) or Chothia (95), are available in the IMGT Scientific chart. The correspondences with these previous and heterogenous numberings are useful for the interpretation of previously published data but nowadays the usage of these numberings has become obsolete owing to the development of immunoinformatics based on the IMGT® standards (8, 62–70) (IMGT®, see footnote text 1, IMGT Scientific chart > Numbering > Correspondence between V numberings).

For a V-domain, the BC loop (or CDR1-IMGT in a V-DOMAIN) encompasses positions 27–38, the C'C" loop (or CDR2-IMGT in a V-DOMAIN) positions 56–65, and the FG loop (or CDR3-IMGT) positions 105–117. In a V-DOMAIN, the CDR3-IMGT encompasses the V-(D)-J junction that results from a V-J or V-D-J rearrangement (2, 3) and is more variable in sequence and length than the CDR1-IMGT and CDR2-IMGT that are encoded by the V gene region only. For CDR3-IMGT of length >13 AA, additional IMGT positions are added at the top of the loop between 111 and 112 (**Table 5**).

IMGT Colliers de Perles. The loop and strands are visualized in the IMGT Colliers de Perles (67–70), which can be displayed on one layer (closer to the AA sequence) or on two layers (closer to the 3D structure) (**Figure 3**). The three loops, BC, C'C", and FG (or CDR1-IMGT, CDR2-IMGT, and CDR3-IMGT for a V-DOMAIN) are delimited by the IMGT anchors, which are shown in square in the IMGT Colliers de Perles. IMGT anchors are positions, which belong to strands and represent anchors for the loops of the V-domains. IMGT anchors are the key and original concept of IMGT®, which definitively solved the ambiguous situation of different CDR lengths and delimitations found in the literature. The six anchors of a V-domain are positions 26 and 39 (anchors of the BC loop or CDR1-IMGT in V-DOMAIN), 55 and 66 (anchors of the C'C" loop or CDR2-IMGT in V-DOMAIN), 104 and 118 (anchors of the FG loop or CDR3-IMGT in V-DOMAIN). The CDR3-IMGT anchors are highly conserved, they are C104 (2nd-CYS, in F strand) and F118 or W118 (J-PHE or J-TRP in G strand). The JUNCTION of an IG or TR V-DOMAIN includes the anchors 104 and 118, and is therefore

Table 5 | IMGT additional positions for CDR3-IMGT.

CDR3-IMGT lengths	IMGT additional positions for CDR3-IMGT length > 13 AA ^a										
21	111	111.1	111.2	111.3	111.4	112.4	112.3	112.2	112.1	112	
20	111	111.1	111.2	111.3	–	112.4	112.3	112.2	112.1	112	
19	111	111.1	111.2	111.3	–	–	112.3	112.2	112.1	112	
18	111	111.1	111.2	–	–	–	112.3	112.2	112.1	112	
17	111	111.1	111.2	–	–	–	–	112.2	112.1	112	
16	111	111.1	–	–	–	–	–	112.2	112.1	112	
15	111	111.1	–	–	–	–	–	–	112.1	112	
14	111	–	–	–	–	–	–	–	112.1	112	

^aFor CDR3-IMGT length > 13 AA, IMGT additional positions are created between positions 111 and 112 at the top of the CDR3-IMGT loop in the following order 112.1, 111.1, 112.2, 111.2, 112.3, 111.3, etc., and as many positions can be added as necessary for very long CDR3-IMGT. For CDR3-IMGT length < 13 AA (not shown), IMGT gaps are created classically from the top of the loop, in the following order 111, 112, 110, 113, 109, 114, etc. (IMGT® <http://www.imgt.org>, IMGT Scientific chart > Numbering).

two AA longer than the corresponding CDR3-IMGT (positions 105–117).

In biological data, the lengths of the loops and strands are given by the number of occupied positions [unoccupied positions or “IMGT gaps” are represented with hatches in the IMGT Colliers de Perles (**Figure 3**) or by dots in alignments]. The CDR-IMGT lengths are given in number of AA (or codons), into brackets and separated by dots: for example [9.6.9] means that the BC, C'C'', and FG loops (or CDR1-IMGT, CDR2-IMGT, and CDR3-IMGT for a V-DOMAIN) have a length of 9, 6, and 9 AA (or codons), respectively. Similarly [25.17.38.11] means that the FR1-IMGT, FR2-IMGT, FR3-IMGT, and FR4-IMGT have a length of 25, 17, 38, and 11 AA (or codons), respectively. Together, the four FR of a VH domain usually comprise 91 AA and the individual FR-IMGT lengths are [25.17.38.11], whereas the four FR of a VL domain usually comprise 89 AA and the individual FR-IMGT lengths are [26.17.36.10].

Conserved AA. A V-domain has five characteristic AA at given positions (positions with bold (online red) letters in the IMGT Colliers de Perles). Four of them are highly conserved and hydrophobic (31) and are common to the C-domain: 23 (1st-CYS), 41 (CONSERVED-TRP), 89 (hydrophobic), and 104 (2nd-CYS). These AA contribute to the two major features shared by the V and C-domain: the disulfide bridge (between the two cysteines 23 and 104) and the internal hydrophobic core of the domain (with the side chains of tryptophan W41 and AA 89). The fifth position, 118, is an anchor of the FG loop. It is occupied, in the V-domains of IgSF other than IG or TR, by AA with diverse physicochemical properties (31). In contrast, in IG and TR V-DOMAIN, the position 118 is occupied by remarkably conserved AA, which consist in a phenylalanine or a tryptophan encoded by the J-REGION and therefore designated J-TRP or J-PHE 118. The bulky aromatic side chains of J-TRP and J-PHE are internally orientated and structurally contribute to the V-DOMAIN hydrophobic core (64).

Genomic delimitation. A last criterion used in the IMGT® definitive system for the characterization of a V-domain is its delimitation taking into account the exon delimitations, whenever appropriate. The exon rule is not used for the delimitation of the 5' end of the first N-terminal domain of proteins with a leader (this includes the V-DOMAIN of the IG and TR chains). In those cases, the 5'end of the first N-terminal domain of the chain corresponds to the proteolytic site between the leader (L-REGION) and the coding region of the mature protein. The IG and TR V-DOMAIN is therefore delimited in 5' by a proteolytic site and in 3' at the genomic level by the splicing site of the J-REGION (60). This IMGT® genomic approach integrates the strands A and G, in contrast to structural alignments that usually lack these strands due to their poor structural conservation, and thus bridges the gap between genomic data (exon) and 3D structure (domain).

C-domain IMGT® definitive system

C-domain definition and main characteristics. In the IMGT® definitive system, the C-domain includes the C-DOMAIN of the IG and of the TR (2, 3) and the C-LIKE-DOMAIN of the IgSF other than IG and TR. The C-domain description of any receptor, any chain, and any species is based on the IMGT unique numbering for C-domain (C-DOMAIN and C-LIKE-DOMAIN) (65, 66).

A C-domain (**Figure 4**) comprises about 90–100 AA and is made of seven antiparallel beta strands (A, B, C, D, E, F, and G), linked by beta turns (AB, DE, and EF), a transverse strand (CD) and two loops (BC and FG), and forming a sandwich of two sheets (ABED) (GFC) (65, 66). A C-domain has a topology and a three-dimensional structure similar to that of a V-domain but without the C' and C'' strands and the C'C'' loop, which is replaced by a transverse CD strand (65).

C-domain strands and loops. The C-domain strands, turns, and loops and their delimitations and lengths, based on the IMGT unique numbering for C-domain (65, 66), are shown in **Table 6**. Correspondences between the IMGT unique numbering with other numberings (Eu, Kabat) are available in the IMGT Scientific chart. The correspondences with these previous numberings are useful for the interpretation of previously published data but, as for the V-domain, the usage of these previous numberings has become obsolete owing to the development of immunoinformatics based on the IMGT® standards (8, 62–70) (IMGT®, see footnote text 1, IMGT Scientific chart > Numbering > Correspondence between C numberings).

IMGT Colliers de Perles. The lengths of the strands and loops are visualized in the IMGT Colliers de Perles (68–70), on one layer and two layers (**Figure 4**). There are six IMGT anchors in a C-domain (four of them identical to those of a V-domain): positions 26 and 39 (anchors of the BC loop), 45 and 77 [by extension, anchors of the CD strand as there is no C'-C'' loop in a C-domain (65)], and 104 and 118 (anchors of the FG loop).

Conserved AA. A C-domain has five characteristic AA at given positions [positions with bold (online red) letters in the IMGT Colliers de Perles]. Four of them are highly conserved and

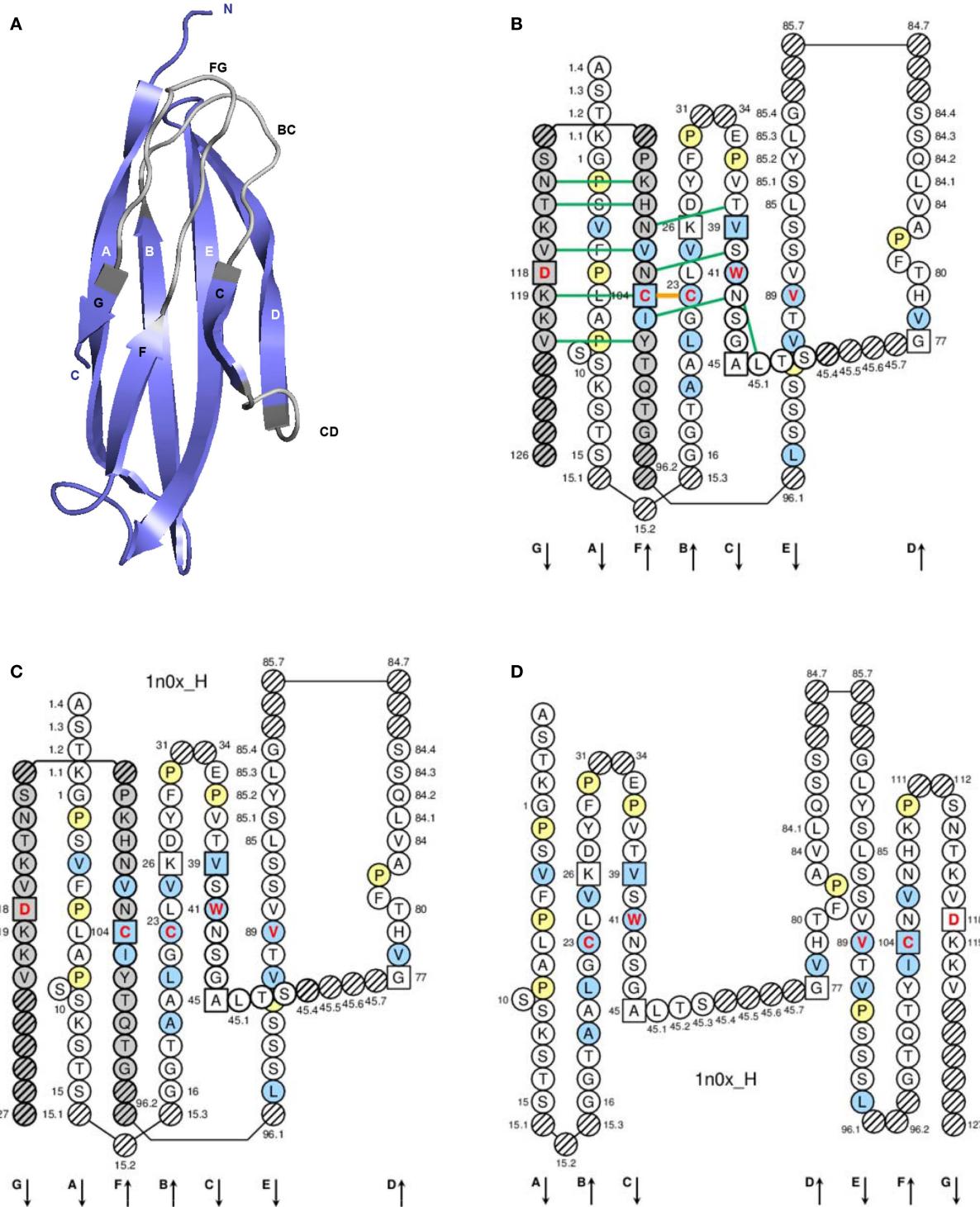


FIGURE 4 | Constant (C) domain. An Ig CH (C-DOMAIN) is shown as an example. **(A)** 3D structure ribbon representation with the IMGT strand and loop delimitations (65). **(B)** IMGT Collier de Perles on two layers with hydrogen bonds. The IMGT Colliers de Perles on two layers show, in the forefront, the GFC strands and, in the back, the ABED strands (located at the interface CH1/CL of the Ig), linked by the CD transverse strand. The IMGT Collier de Perles with hydrogen bonds (green lines online, only shown here for the GFC sheet) is generated by the IMGT/Collier-de-Perles tool integrated in

IMGT/3Dstructure-DB, from experimental 3D structure data (11–13). **(C)** IMGT Collier de Perles on two layers from IMGT/DomainGapAlign (12, 27, 28). **(D)** IMGT Colliers de Perles on one layer. Amino acids are shown in the one-letter abbreviation. All proline (P) are shown online in yellow. IMGT anchors are in square. Hatched circles are IMGT gaps according to the IMGT unique numbering for C-domain (65, 66). Positions with bold (online red) letters indicate the four conserved positions that are common to a V-domain
(Continued)

FIGURE 4 | Continued

and to a C-domain: 23 (1st-CYS), 41 (CONSERVED-TRP), 89 (hydrophobic), 104 (2nd-CYS) (62–66) (**Table 6**), and position 118, which is only conserved in V-DOMAIN. The identifier of the chain to which the CH domain belongs is

1n0x_H (from the *Homo sapiens* b12 Fab, in IMGT/3Dstructure-DB, <http://www.imgt.org>). The 3D ribbon representation was obtained using PyMOL and “IMGT numbering comparison” of 1n0x_H (CH1) from IMGT/3Dstructure-DB (<http://www.imgt.org>).

Table 6 | C-domain strands, turns, and loops, IMGT positions, and lengths, based on the IMGT unique numbering for C-domain (C-DOMAIN and C-LIKE-DOMAIN).

C-domain strands, turns, and loops ^a	IMGT positions ^b	Lengths ^c	Characteristic IMGT Residue@Position ^d
A-STRAND	1–15	15 (14 if gap at 10)	
AB-TURN	15.1–15.3	0–3	
B-STRAND	16–26	11	1st-CYS 23
BC-LOOP	27–31	10 (or less)	
	34–38		
C-STRAND	39–45	7	CONSERVED-TRP 41
CD-STRAND	45.1–45.9	0–9	
D-STRAND	77–84	8 (or 7 if gap at 82)	
DE-TURN	84.1–84.7	0–14	
	85.1–85.7		
E-STRAND	85–96	12	Hydrophobic 89
EF-TURN	96.1–96.2	0–2	
F-STRAND	97–104	8	2nd-CYS 104
FG-LOOP	105–117	13 (or less, or more)	
G-STRAND	118–128	11 (or less)	

^aIMGT® labels (concepts of description) are written in capital letters (no plural) (60).

^bBased on the IMGT unique numbering for C-domain (C-DOMAIN and C-LIKE-DOMAIN) (65, 66).

^cIn number of amino acids (AA) (or codons).

^dIMGT Residue@Position is a given residue (usually an AA) or a given conserved property AA class, at a given position in a domain, based on the IMGT unique numbering (66).

hydrophobic (31) and are common to the V-domain: 23 (1st-CYS), 41 (CONSERVED-TRP), 89 (hydrophobic), and 104 (2nd-CYS). As mentioned above, these AA contribute to the two major features shared by the V and C-domain: the disulfide bridge (between the two cysteines 23 and 104) and the internal hydrophobic core of the domain (with the side chains of tryptophan W41 and AA 89). The fifth position, 118, is diverse and is characterized as being an FG loop anchor.

Genomic delimitation. In the IMGT® definitive system, the C-domains (C-DOMAIN and C-LIKE-DOMAIN) are delimited taking into account the exon delimitation, whenever appropriate. As for the V-domain, this IMGT® genomic approach integrates the strands A and G, which are absent of structural alignments.

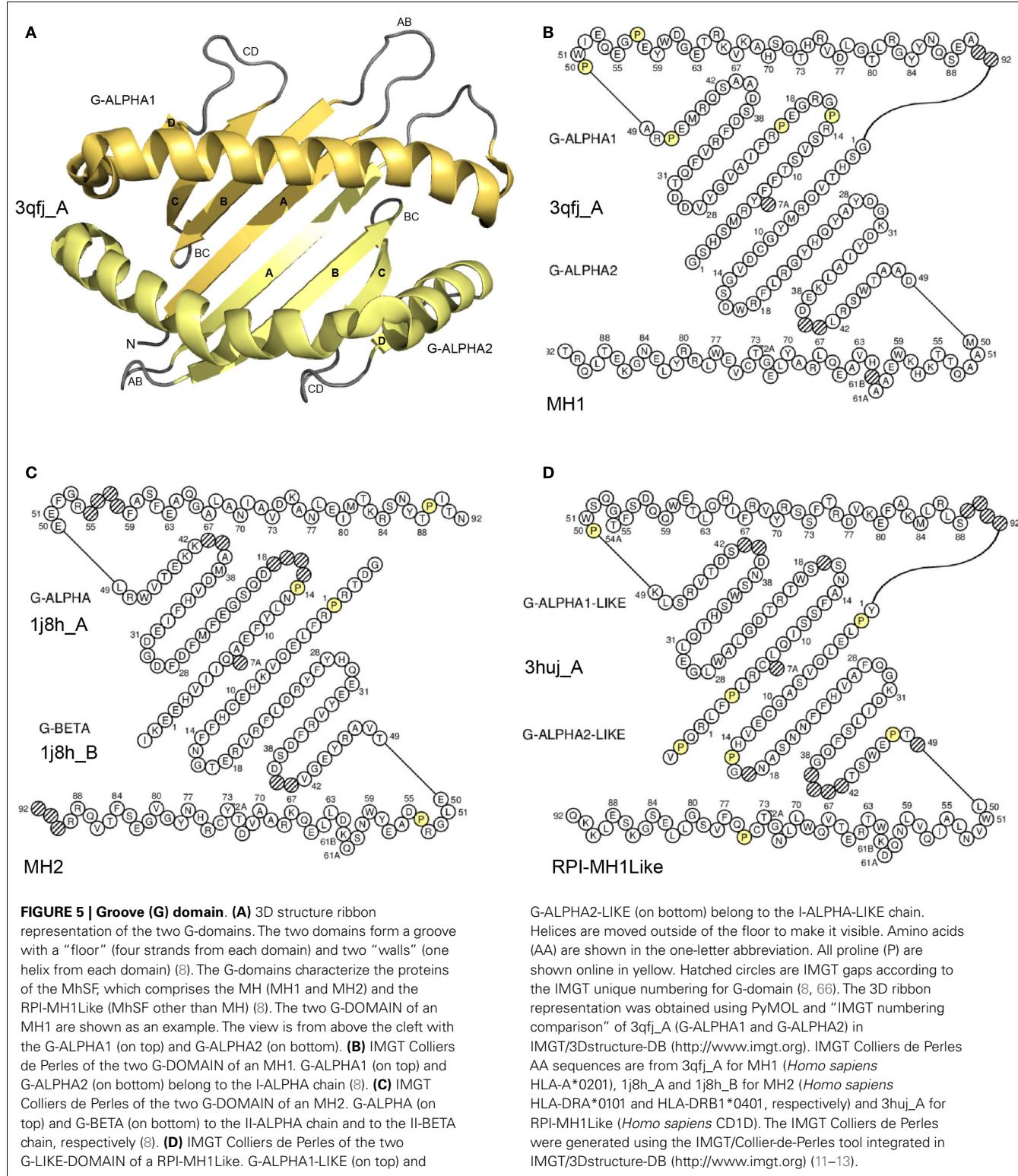
G-domain IMGT® definitive system

G-domain definition and main characteristics. In the IMGT® definitive system, the G-domain includes the G-DOMAIN of the

MH (Figure 5) (8, 66) and the G-LIKE-DOMAIN of the MhSF other than MH or RP1-MH1Like (there is no “RPI-MH2Like” identified so far) (96, 97). The RPI-MH1Like in humans comprise (97): AZGP1 (that regulates fat degradation in adipocytes), CD1A to CD1E proteins (that display phospholipid antigens to T cells and participate in immune defense against microbial pathogens), FCGRT (that transports maternal immunoglobulins through placenta and governs neonatal immunity), HFE (that interacts with transferring receptor and takes part in iron homeostasis by regulating iron transport through cellular membranes), MICA and MICB (that are induced by stress and involved in tumor cell detection), MR1 (that regulates mucosal immunity), PROCR, previously EPCR (that interacts with activated C protein and is involved in the blood coagulation pathway), RAET1E, RAET1G, and RAET1L (that are inducible by retinoic acid and stimulate cytokine/chemokine production and cytotoxic activity of NK cells), and ULBP1, ULBP2, and ULBP3 (that are ligands for NKG2D receptor). The G-domain description of any receptor, any chain, and any species is based on the IMGT unique numbering for G-domain (G-DOMAIN and G-LIKE-DOMAIN) (8, 66).

G-domain strands and helix. A G-domain (Figure 5) comprises about 90 AA and is made of four antiparallel beta strands (A, B, C, and D) linked by turns (AB, BC, and CD), and of a helix (98, 99); the helix sits on the beta strands, its axis forming an angle of about 40° with the strands (90, 91). Two G-domains are needed to form the MhSF groove made of a “floor” and two “walls” (8, 66). Each G-domain contributes by its four strands and turns to half of the groove floor and by its helix to one wall of the groove (8, 66, 90, 91). The MH groove in which the peptide binds is made of two G-DOMAIN belonging to a single chain or to two chains, depending on the MH group, MH1 or MH2, respectively. In the MH1, the groove is made of two G-DOMAIN (G-ALPHA1 and G-ALPHA2), which belong to the same chain I-ALPHA, whereas in the MH2, the groove is made of two G-DOMAIN (G-ALPHA and G-BETA), which belong to two different chains, II-ALPHA and II-BETA, respectively (8, 66). For the RPI-MH1Like, the two G-LIKE-DOMAIN also belong, as for the MH1, to the same chain (I-ALPHA-LIKE) (96, 97).

IMGT Colliers de Perles. The G-domain strands, turns, and helix and their delimitations and lengths, based on the IMGT unique numbering for G-domain (8, 66) are shown in Table 7. The strands and helix of each domain are visualized in the IMGT Collier de Perles (68–70, 90, 91) (Figure 5). The views are from above the cleft (with the helices displaced to show the floor) and with on top and on bottom, respectively, G-ALPHA1 and G-ALPHA2 (MH1), G-ALPHA and G-BETA (MH2), and G-ALPHA1-LIKE and G-ALPHA2-LIKE (RPI-MH1Like). There is no link between G-ALPHA and G-BETA because they belong to different chains (II-ALPHA and II-BETA).



Conserved AA. Two conserved cysteines, CYS-11 (in the A-strand) and CYS-74 (in the helix) (Table 7), are found in the G-ALPHA2, G-BETA, and G-ALPHA2-LIKE (Figure 5), where they form a disulfide bridge fixing the helix to the floor.

Genomic delimitation. In the IMGT® definitive system, the G-domains (G-DOMAIN and G-LIKE-DOMAIN) are delimited taking into account the exon delimitations, if appropriate. Alignment sequence comparison with previously identified genes

Table 7 | G-domain strands, turns, and helix, IMGT positions, and lengths, based on the IMGT unique numbering for G-domain (G-DOMAIN and G-LIKE-DOMAIN).

G-domain strands, turns, and helix ^a	IMGT positions ^b	Lengths ^c	Characteristic IMGT Residue@Position ^d and additional positions ^e
A-STRAND	1–14	14	7A, CYS-11
AB-TURN	15–17	3 (or 2 or 0)	
B-STRAND	18–28	11 (or 10 ^f)	
BC-TURN	29–30	2	
C-STRAND	31–38	8	
CD-TURN	39–41	3 (or 19 ^g)	
D-STRAND	42–49	8	49.1–49.5
HELIX	50–92	43 (or less or more)	54A, 61A, 61B, 72A, CYS-74, 92A

^aIMGT® labels (concepts of description) are written in capital letters (no plural) (60).

^bBased on the IMGT unique numbering for G-domain (G-DOMAIN and G-LIKE-DOMAIN) (8, 66).

^cIn number of AA (or codons).

^dIMGT Residue@Position is a given residue (usually an AA) or a given conserved property AA class, at a given position in a domain, based on the IMGT unique numbering (66).

^eFor details on additional positions, see Ref. (8).

^fOr 9 in some G-BETA (8).

^gOr 0 in some G-ALPHA2-LIKE (8).

is used when genomic data are not available, as recently done for the rainbow trout (*Oncorhynchus mykiss*) MH1 and MH2 [IMGT®, see footnote text 1, IMGT Repertoire (MH) > Proteins and alleles > Protein displays].

IMGT/Collier-de-Perles tool

The IMGT/Collier-de-Perles tool (29), on the IMGT® Web site at <http://www.imgt.org>, is a generic tool, which allows the users to draw IMGT Colliers de Perles (67–70) starting from their own domain AA sequences [sequences already gapped according to the IMGT unique numbering, using for example IMGT/DomainGapAlign (12, 27, 28)] (Table 8). IMGT Collier de Perles can be obtained for V and C-domains (on one or two layers) and for G-domains (with one or the two domains of the groove). IMGT/Collier-de-Perles tool online can be customized to display the IG and TR CDR-IMGT according to the IMGT color menu and the AA according to their hydrophathy or volume, or to the 11 IMGT physicochemical classes (31).

IMGT color menu for the CDR-IMGT of a V-DOMAIN indicates the type of rearrangement V-J or V-D-J (2, 3). Thus, the IMGT color menu for CDR1-IMGT, CDR2-IMGT, and CDR3-IMGT is red, orange, and purple for the IG VH and for the TR V-BETA or V-DELTA (encoded by a V-D-J-REGION resulting from a V-D-J rearrangement), and blue, green, and greenblue for the IG V-KAPPA or V-LAMBDA and for the TR V-ALPHA or V-GAMMA (encoded by a V-J-REGION resulting from a V-J rearrangement). Arbitrarily the red, orange, and purple is used for

the BC, C'C'' and FG loops of the V-domain of IgSF other than IG or TR.

The IMGT/Collier-de-Perles tool is integrated in IMGT/DomainGapAlign (12, 27, 28) (users start from V, C, or G AA sequences) and in IMGT/V-QUEST (15–20) (users start from IG and TR V-DOMAIN nucleotide sequences) (Table 8). IMGT Colliers de Perles for V, C, and G-domains are provided in IMGT/2Dstructure-DB (for AA sequences in the database), and in IMGT/3Dstructure-DB (on two layers with hydrogen bonds for the V or C-domains or with the pMH contact sites for the G-domains, for 3D structures in the database) (11–13) (Table 8).

IMGT® TOOLS FOR IG, TR, AND MH DOMAIN ANALYSIS

IMGT/V-QUEST

IMGT/V-QUEST for IG and TR V-domain analysis

IMGT/V-QUEST (15–20) is the IMGT® online tool for the analysis of nucleotide sequences of the IG and TR V-DOMAIN (Table 8). IMGT/V-QUEST identifies the variable (V), diversity (D), and junction (J) genes in rearranged IG and TR sequences and, for the IG, the nucleotide (nt) mutations and AA changes resulting from somatic hypermutations by comparison with the IMGT/V-QUEST reference directory. The tool integrates IMGT/JunctionAnalysis (21, 22) for the detailed characterization of the V-D-J or V-J junctions, IMGT/Automat (23, 24) for a complete sequence annotation, and IMGT/Collier-de-Perles (29).

The IMGT/V-QUEST most important functionalities include: introduction of IMGT gaps in the user nucleotide sequences (and in its translation), alignments, and identification of the genes and alleles with the closest germline V, D, and J genes, analysis of the junctions, analysis of somatic hypermutations, and AA changes and, if the option “Search for insertions and deletions” was selected, identification of insertions and deletions (indels) and their correction. Customized parameters and results provided by IMGT/V-QUEST and IMGT/JunctionAnalysis have been described elsewhere (15–20).

IMGT/V-QUEST reference directory

The IMGT/V-QUEST reference directory sets against which the IMGT/V-QUEST is running include IMGT reference sequences from all functional (F) genes and alleles, all ORF and all in-frame pseudogenes (P) alleles. By definition, the IMGT reference directory sets contain one sequence for each allele. By default, the user sequences are compared with all genes and alleles. However, the option “With allele *01 only” is useful for: (i) “Detailed view,” if the user sequences need to be compared with different genes, and (ii) “Synthesis view,” if the user sequences, which use the same gene need to be aligned together (independently of the allelic polymorphism) (17, 19).

The IMGT/V-QUEST reference directories have been set up for species, which have been extensively studied, such as human and mouse. This also holds for the other species or taxons with incomplete IMGT reference directory sets. In those cases, results should be interpreted considering the status of the IMGT reference directory (information on the updates on the IMGT® Web site). Links to the IMGT/V-QUEST reference directory sets are available from the IMGT/V-QUEST Welcome page (17, 19).

Table 8 | IMGT® tools and databases for the analysis of the IG, TR, and MH domains (<http://www.imgt.org>).

IMGT® tools	Results for V, C, or G-domains ^a	Entry types and protocol references
IMGT/Collier-de-Perles (29)	Graphical 2D representation of IMGT Colliers de Perles (67–70)	User “IMGT gapped” V, C, or G-domain amino acid (AA) sequences (one sequence per representation, two possible for G) (29)
IG AND TR REPERTOIRE ANALYSIS		
IMGT/V-QUEST (15–20)	<ol style="list-style-type: none"> 1. Introduction of IMGT gaps 2. Identification of the closest V, D, and J genes and alleles 3. IMGT/JunctionAnalysis results (21, 22) 4. Description of mutations and AA changes 5. Identification of indels and their correction (19) (option) 6. IMGT/Automat annotation (23, 24) 7. IMGT Colliers de Perles (29) 	User nucleotide sequences of V-DOMAIN (1–50 sequences per analysis, and 1–10 sequences with the option “Search for insertions and deletions”) (19) <i>Applications:</i> somatic mutations in chronic lymphocytic leukemia (CLL) prognostic
IMGT/HighV-QUEST (20, 25, 26)	<ol style="list-style-type: none"> 1. Introduction of IMGT gaps 2. Identification of indels and their correction (19) (by default) 3. Identification of the closest V, D, and J genes and alleles 4. IMGT/JunctionAnalysis results (21, 22) 5. Description of mutations and AA changes 6. IMGT/Automat annotation (23, 24) 7. Statistical analysis (25) 8. Characterization of the IMGT clonotypes (AA) (26) 	User NGS long (e.g., from 454) nucleotide sequences of V-DOMAIN (up to 500,000 sequences per run ^b) ^c (25, 26) <i>Applications:</i> IG and TR immune repertoires and clonotypes in NGS
IG, TR, MH DOMAIN AA SEQUENCE ANALYSIS		
IMGT/DomainGapAlign (12, 27, 28)	<ol style="list-style-type: none"> 1. Introduction of IMGT gaps 2. Identification of the closest genes and alleles 3. Delimitation of the domains 4. Description of AA changes 5. IMGT Colliers de Perles (67–70) with highlighted AA changes (pink circles online) 	User AA sequences of V, C, and G-domains (one to several sequences of same domain type) (27, 28) <i>Applications:</i> IMGT antibody engineering and humanization for V and C
IMGT® DATABASES		
IMGT/3Dstructure-DB (11–13)	<ol style="list-style-type: none"> 1. Identification of the closest genes and alleles 2. IMGT/DomainGapAlign results (12, 27, 28) 3. IMGT Collier de Perles (67–70) (on two layers with hydrogen bonds for V and C or with pMH contact sites for G) 4. Contact analysis between a pair of domains or between a domain and a ligand 5. Renumbered IMGT files 6. IMGT numbering comparison 	2,290 structure entries (1,987 IG, including 852 IG/Ag complexes, 151 TR, and 542 MH including 84 TR/pMH complexes) ^b <i>Applications:</i> identification of the paratope and epitope in IG/AG and TR/pMH complexes and pMH contacts
IMGT/2Dstructure-DB (13)*	<ol style="list-style-type: none"> 1. Identification of the closest genes and alleles 2. IMGT/DomainGapAlign results (12, 27, 28) 3. IMGT Collier de Perles (67–70) 4. Renumbered IMGT files 	512 AA sequence entries ^b (of which 506 IG)* <i>Applications:</i> from gene to structures in the absence of 3D

An asterisk (*) indicates that parts of the protocol dealing with 3D structures (hydrogen bonds in IMGT Colliers de Perles on two layers, Contact analysis) are not relevant, otherwise all other queries and results are similar to IMGT/3Dstructure-DB.

^aV: V-domain (includes V-DOMAIN of IG and TR and V-LIKE-DOMAIN of IgSF other than IG and TR) (64). C: C-domain (includes C-DOMAIN of IG and TR and C-LIKE-DOMAIN of IgSF other than IG and TR) (65). G: G-domain (G-DOMAIN of MH and G-LIKE-DOMAIN of MhSF other than MH) (8).

^bIn November 2013.

^cIn November 2013, more than 1.4 billions of sequences analyzed by IMGT/HighV-QUEST, by 702 users from 40 countries (43% users from USA, 38% from EU, 19% from the remaining world).

IMGT/HighV-QUEST

NGS IG and TR V-domain analysis

IMGT/HighV-QUEST (25), created in October 2010, is the high-throughput version of IMGT/V-QUEST. It is so far the only online tool available on the Web for the direct analysis of complete IG and TR domain sequences from NGS. It analyzes sequences obtained from the Roche 454 Life Sciences technology, without the need of computational read assembly (25, 26). IMGT/HighV-QUEST analyses up to 500,000 sequences per run in November 2013 (25, 26), with the same degree of resolution and high-quality results as IMGT/V-QUEST (15–20). IMGT/HighV-QUEST represents a major breakthrough for the analysis and the comparison of the antigen receptor V-DOMAIN repertoires and immunoprofilings of the adaptive immune response (25, 26).

The functionalities of IMGT/HighV-QUEST include: the introduction of IMGT gaps, the identification of indels and their correction (19) (by default), the identification of the closest V, D, and J genes and alleles, the IMGT/JunctionAnalysis results, the description of mutations and AA changes, the annotation by IMGT/Automat, the NGS statistical analysis, and the characterization of the IMGT clonotypes (AA) (25, 26) (**Table 8**). IMGT/HighV-QUEST provides results in different categories “1 copy” and “More than 1” to avoid redundancy of the analysis, “single allele” and “several alleles (or genes)” (with “single allele” sequences being usually longer than “several alleles”) (25). These categories have been fundamental in the characterization of clonotypes for NGS (26).

As for the other IMGT® databases and tools, IMGT/HighV-QUEST is freely available for academics. However, the IMGT/HighV-QUEST Welcome page requires user identification and provides, for new users, a link to register. User identification has been set to avoid non-relevant use and overload of the server, and to contact the user if needed. The user identification gives access to the IMGT/HighV-QUEST Search page.

NGS IMGT® clonotype identification

IMGT clonotype (AA) identification: clonal diversity. In the literature, clonotypes are defined differently, depending on the experiment design (functional specificity) or available data. Thus, a clonotype may denote either a complete antigen receptor (e.g., IgG1-kappa), or only one of the two chains of the receptor (e.g., H or L), or one domain (e.g., VH), or the CDR3 sequence of a domain. Moreover the sequence can be at the AA or nucleotide (nt) level, and this is rarely specified. Therefore, IMGT® goal was first of all to define clonotypes and their properties, which could be identified and characterized by IMGT/HighV-QUEST, unambiguously (26).

In IMGT®, the clonotype, designated as “IMGT clonotype (AA),” is defined by a unique V-(D)-J rearrangement (with IMGT gene and allele names determined by IMGT/HighV-QUEST at the nt level) and a unique CDR3-IMGT AA (in-frame) junction sequence (26). For identifying “IMGT clonotypes (AA)” in a given IMGT/HighV-QUEST dataset, the “1 copy” are filtered to select for sequences with in-frame junction, conserved anchors 104 and 118 (“C” is 2nd-CYS 104, and “F” or “W” is the J-PHE or J-TRP 118) and for V and J functional or ORF, and “single allele” (for V and J) (26).

By essence, an “IMGT clonotype (AA)” is “unique” for a given dataset. For that reason, each “IMGT clonotype (AA),” in a given dataset, has a unique set identifier (column “Exp. ID”) and, importantly, has a unique representative sequence (link in column “Sequence ID”) selected by IMGT/HighV-QUEST among the “1 copy” “single allele” (for V and J), based on the highest percent of identity of the V-REGION (“V%”) compared to that of the closest germline, and/or on the sequence length (thus the most complete V-REGION) (26).

Sequences assigned to IMGT clonotypes (AA): clonal expression.

Clonal expression is the number of sequences that can be assigned to each IMGT clonotype (AA). In our procedure, the high-quality and specific characterization of the “IMGT clonotype (AA)” (26) remains unaltered whereas the total number of sequences assigned to each given “IMGT clonotype (AA)” is calculated stepwise by adding:

1. The number of the “1 copy” “single allele” sequences not selected as representative. These sequences differ from the representative sequence by a different (usually shorter) length, and/or by sequencing errors in the V-REGION (lower “V%” of identity) or in the J-REGION, and/or by nt differences in the CDR3-IMGT. Sequences with nt differences in the CDR3-IMGT are identified as “IMGT clonotypes (nt),” the nt differences resulting from sequencing errors or, if this can be proven experimentally, from molecular convergence. For a given “IMGT clonotype (AA),” the number (nb) of different CDR3-IMGT (nt) or “IMGT clonotypes (nt),” the CDR3-IMGT sequence (nt) and the nb of different nt in the CDR3-IMGT are reported in the results (26).
2. The number of the “1 copy” “several alleles (or genes)” sequences that have the same V and J allele as the IMGT clonotype (AA), among their IMGT/HighV-QUEST results.
3. The number of “More than 1” (including those of the IMGT representative sequence) for each retained “1 copy” of steps 1 and 2 (25).

For the first time for NGS antigen receptor data analysis, the IMGT® standardized approach allows a clear distinction and accurate evaluation between the clonal diversity [nb of “IMGT clonotypes (AA)”,] and the clonal expression [total nb of sequences assigned, unambiguously, to a given “IMGT clonotype (AA)”,] (26). These assignments are clearly described and visualized in detail so the user always has the means of checking clonotypes individually. Indeed, the sequences of each “1 copy” assigned to a given “IMGT clonotype (AA)” are available in “Sequences file” (26). The user can easily perform an analysis of these sequences online with IMGT/V-QUEST (up to 10 sequences, selecting “Synthesis view display” and the option “Search for insertions and deletions”) and/or with IMGT/JunctionAnalysis (up to 5,000 junction sequences), which provide a visual representation familiar to the IMGT® users.

Clonal diversity is also visualized in the online results with histograms, which represent the number of IMGT clonotypes (AA) per V, D (for IGH, TRB or TRD), and J genes (in pink) (26). Clonal expression is visualized with histograms, which represent

the number of sequences assigned to IMGT clonotypes (AA) per V (in green), D (in red), and J (in yellow) genes (26). Values are normalized, respectively, for 10,000 IMGT clonotypes (AA) to represent IG diversity immunoprofiles per V, D (for IGH, TRB or TRD), and J genes, and for 10,000 sequences assigned to IMGT clonotypes (AA) to represent IG expression immunoprofiles per V, D (for IGH, TRB or TRD), and J genes (26). These normalized values allow comparative analysis studies performed with the same IMGT/HighV-QUEST standards (26).

IMGT/DomainGapAlign

V, C, and G-domain analysis of IG, TR, and MH

IMGT/DomainGapAlign (12, 27, 28) is the IMGT® online tool for the analysis of AA sequences and 2D structures of V and C-domains (for IG, TR, and other IgSF) and of G-domains (for MH and other MhSF) (Table 8). It analyzes domain AA sequences by comparison with the IMGT domain reference directory sets (translation of the germline V and J genes and of the C gene domains for IG and TR, AA domain sequences of MH and conventional genes). IMGT/DomainGapAlign functionalities include: introduction of IMGT gaps in the user AA sequences, alignments, and identification of the genes and alleles by comparison with the closest domain(s), delimitation of the domain(s) (e.g., V, C, or G) in the user sequence, description of the AA changes and IMGT Collier de Perles.

IMGT domain reference directory

The IMGT domain reference directory is the IMGT reference directory for V, C, and G-domains. Sequences are from the IMGT Repertoire (1) and from IMGT/GENE-DB (10). Owing to the particularities of the V-DOMAIN synthesis (2, 3), there is no V-DOMAIN in the IMGT reference directory. Instead, the directory comprises the translation of the IG and TR germline V and J genes (V-REGION and J-REGION, respectively). The IMGT domain reference directory provides the IMGT® “gene” and “allele” names. Data are comprehensive for human and mouse IG and TR whereas for other species and other IgSF and MhSF they are added progressively. The IMGT domain reference directory comprises domain sequences of functional (F), ORF, and in-frame pseudogene (P) genes. As IMGT® alleles are characterized at the nucleotide level, identical sequences at the AA level may therefore correspond to different alleles, in the IMGT domain reference directory. The sequences can be displayed by querying IMGT/DomainDisplay (see footnote text 1).

IMGT® DATABASES FOR IG, TR, AND MH DOMAIN ANALYSIS

IMGT/3Dstructure-DB

IMGT/3Dstructure-DB card

IMGT/3Dstructure-DB (11–13), the IMGT® structure database, provides IMGT® annotation and contact analysis of IG, TR, MH, IgSF, and MhSF 3D structures, and paratope/epitope description of IG/antigen and TR/pMH complexes (Table 8). There is one “IMGT/3Dstructure-DB card” per IMGT/3Dstructure-DB entry and this card provides access to all data related to that entry. The “PDB code” (four letters and/or numbers, e.g., 1n0x) is used as “IMGT entry ID” for the 3D structures obtained from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (100). The IMGT/3Dstructure-DB card

provides eight search/display options: “Chain details,” “Contact analysis,” “Paratope and epitope,” “3D visualization Jmol or Quick-PDB,” “Renumbered IMGT files,” “IMGT numbering comparison,” “References and links,” and “Printable card” (11–13).

IMGT chain and domain annotation

The “Chain details” section comprises information first on the chain itself, then per domain (11–13). Chain and domain annotation includes the IMGT gene and allele names (CLASSIFICATION), region and domain delimitations (DESCRIPTION) and domain AA positions according to the IMGT unique numbering (NUMEROTATION) (8, 62–66). The closest IMGT® genes and alleles (found expressed in each domain of a chain) are identified with the integrated IMGT/DomainGapAlign (12, 27, 28), which aligns the AA sequences of the 3D structures with the IMGT domain reference directory.

Contact analysis

“Contact analysis” gives access to a table with the different “Domain pair contacts” of the 3D structure [this table is also accessed from “Chain details” by clicking on “Domain contact (overview)”. “Domain pair contacts” refer to contacts between a pair of domains or between a domain and a ligand. Clicking on “DomPair” gives access to the contacts between AA for a given “Domain pair contacts.” Contacts between VH and the Ligand (antigen, Ag) and the V-KAPPA and the Ligand (Ag) of an IG/Ag complex are shown in Figure 6. These contact analysis representations are important as they demonstrate that most contacts with the ligand, if not all, involve the AA of the CDR-IMGT. They definitively confirmed the CDR-IMGT delimitations as the official reference standards (66, 70, 93).

In IMGT/3Dstructure-DB, all contacts are described as atom pair contacts. Atom pair contacts are obtained by a local program in which atoms are considered to be in contact when no water molecule can take place between them (11, 12). Atom pair contacts are provided by atom contact types (noncovalent, polar, hydrogen bond, nonpolar, covalent, disulfide) and/or atom contact categories [(BB) backbone/backbone, (SS) side chain/side chain, (BS) backbone/side chain, (SB) side chain/backbone] (11, 12, 90, 91).

Clicking on “R@P” gives access to the IMGT identity card of a given residue (usually an AA) at a given position or Residue@Position. The IMGT R@P card can also be accessed from the AA sequences of the IMGT/3Dstructure-DB card or from the IMGT Colliers de Perles, by clicking on one AA. In an IMGT R@P card, the Residue@Position is defined by the IMGT position numbering in a domain, or if not characterized, in the chain, the AA name (three-letter and between parentheses one-letter abbreviations), the IMGT domain description and the IMGT chain ID, e.g., “103 – TYR (Y) – VH – 1hz_H” (11–13). The IMGT R@P card includes (i) general information (PDB file numbering, IMGT file numbering, residue full name and formula), (ii) structural information “IMGT LocalStructure@Position” [secondary structure, Phi and Psi angles (in degrees) and accessible surface area (ASA) (in square angstrom)] and (iii) detailed contact analysis with AA of other domains (11–13).

A IMGT/3Dstructure-DB Domain pair contacts

Contacts of	Domain [D1] VH 1n0x_H	Chain	with	Domain [Ligand] 1n0x_P	Chain
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Summary:

Residue pair contacts	Number of residues	Atom pair contact types		
Total	From 1	From 2	Total	Polar Hydrogen Nonpolar
17	15	8	106	10 1 96

List of the Residue@Position pair contacts:
Click 'R@P' for IMGT Residue@Position cards

Order	Order				Atom pair contact types						
	IMGT Num	Residue	Domain	Chain	IMGT Num	Residue	Domain	Chain	Total	Polar	Hydrogen
R@P 55	TRP W	[D1] VH	1n0x_H	R@P 19	ORN	(Ligand)	1n0x_P	6	0	0	6
R@P 55	TRP W	[D1] VH	1n0x_H	R@P 20	LYS K	(Ligand)	1n0x_P	8	0	0	8
R@P 62	ASN N	[D1] VH	1n0x_H	R@P 19	ORN	(Ligand)	1n0x_P	1	0	0	1
R@P 64	ASN N	[D1] VH	1n0x_H	R@P 18	GLU E	(Ligand)	1n0x_P	1	1	0	0
R@P 64	ASN N	[D1] VH	1n0x_H	R@P 19	ORN	(Ligand)	1n0x_P	15	2	1	13
R@P 64	ASN N	[D1] VH	1n0x_H	R@P 20	LYS K	(Ligand)	1n0x_P	8	1	0	7
R@P 65	LYS K	[D1] VH	1n0x_H	R@P 19	ORN	(Ligand)	1n0x_P	1	1	0	0
R@P 65	LYS K	[D1] VH	1n0x_H	R@P 20	LYS K	(Ligand)	1n0x_P	8	1	0	7
R@P 66	GLU E	[D1] VH	1n0x_H	R@P 20	LYS K	(Ligand)	1n0x_P	9	2	0	7
R@P 66	GLU E	[D1] VH	1n0x_H	R@P 21	LYS K	(Ligand)	1n0x_P	2	0	0	2
R@P 112.3	PRO P	[D1] VH	1n0x_H	R@P 4	SER S	(Ligand)	1n0x_P	5	0	0	5
R@P 112.3	PRO P	[D1] VH	1n0x_H	R@P 5	TYR Y	(Ligand)	1n0x_P	9	1	0	8
R@P 112.3	PRO P	[D1] VH	1n0x_H	R@P 6	MET M	(Ligand)	1n0x_P	19	0	0	19
R@P 112.3	PRO P	[D1] VH	1n0x_H	R@P 17	ALA A	(Ligand)	1n0x_P	2	0	0	2
R@P 112.2	GLN Q	[D1] VH	1n0x_H	R@P 6	MET M	(Ligand)	1n0x_P	10	0	0	10
R@P 112.2	GLN Q	[D1] VH	1n0x_H	R@P 17	ALA A	(Ligand)	1n0x_P	1	0	0	1
R@P 112.2	GLN Q	[D1] VH	1n0x_H	R@P 21	LYS K	(Ligand)	1n0x_P	1	1	0	0

B IMGT/3Dstructure-DB Domain pair contacts

Contacts of	Domain [D1] V-KAPPA 1n0x_L	Chain	with	Domain [Ligand] 1n0x_P	Chain
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Summary:

Residue pair contacts	Number of residues	Atom pair contact types		
Total	From 1	From 2	Total	Polar Hydrogen Nonpolar
24	19	12	7	195 32 163

List of the Residue@Position pair contacts:
Click 'R@P' for IMGT Residue@Position cards

Order	Order				Atom pair contact types						
	IMGT Num	Residue	Domain	Chain	IMGT Num	Residue	Domain	Chain	Total	Polar	Hydrogen
R@P 1	GLU E	[D1] V-KAPPA	1n0x_L	R@P 10	LEU L	(Ligand)	1n0x_P	4	0	0	4
R@P 2	ILE I	[D1] V-KAPPA	1n0x_L	R@P 9	ASP D	(Ligand)	1n0x_P	6	0	0	6
R@P 2	ILE I	[D1] V-KAPPA	1n0x_L	R@P 10	LEU L	(Ligand)	1n0x_P	11	0	0	11
R@P 27	HIS H	[D1] V-KAPPA	1n0x_L	R@P 9	ASP D	(Ligand)	1n0x_P	7	1	0	6
R@P 27	HIS H	[D1] V-KAPPA	1n0x_L	R@P 10	LEU L	(Ligand)	1n0x_P	11	1	0	10
R@P 28	SER S	[D1] V-KAPPA	1n0x_L	R@P 9	ASP D	(Ligand)	1n0x_P	10	3	0	7
R@P 29	ILE I	[D1] V-KAPPA	1n0x_L	R@P 9	ASP D	(Ligand)	1n0x_P	26	3	0	23
R@P 30	ARG R	[D1] V-KAPPA	1n0x_L	R@P 7	PHE F	(Ligand)	1n0x_P	21	0	0	21
R@P 30	ARG R	[D1] V-KAPPA	1n0x_L	R@P 9	ASP D	(Ligand)	1n0x_P	10	2	0	8
R@P 30	ARG R	[D1] V-KAPPA	1n0x_L	R@P 12	ASP N	(Ligand)	1n0x_P	11	4	0	7
R@P 36	SER S	[D1] V-KAPPA	1n0x_L	R@P 7	PHE F	(Ligand)	1n0x_P	8	1	0	7
R@P 36	SER S	[D1] V-KAPPA	1n0x_L	R@P 9	ASP D	(Ligand)	1n0x_P	14	4	2	10
R@P 37	ARG R	[D1] V-KAPPA	1n0x_L	R@P 9	ASP D	(Ligand)	1n0x_P	1	1	0	0
R@P 38	ARG R	[D1] V-KAPPA	1n0x_L	R@P 6	MET M	(Ligand)	1n0x_P	2	0	0	2
R@P 38	ARG R	[D1] V-KAPPA	1n0x_L	R@P 7	PHE F	(Ligand)	1n0x_P	10	4	1	6
R@P 38	ARG R	[D1] V-KAPPA	1n0x_L	R@P 8	SER S	(Ligand)	1n0x_P	3	1	0	2
R@P 38	ARG R	[D1] V-KAPPA	1n0x_L	R@P 9	ASP D	(Ligand)	1n0x_P	9	5	0	4
R@P 108	ALA A	[D1] V-KAPPA	1n0x_L	R@P 6	MET M	(Ligand)	1n0x_P	8	0	0	8
R@P 109	ALA A	[D1] V-KAPPA	1n0x_L	R@P 7	PHE F	(Ligand)	1n0x_P	2	0	0	2
R@P 109	ALA A	[D1] V-KAPPA	1n0x_L	R@P 8	SER S	(Ligand)	1n0x_P	6	1	0	5
R@P 109	ALA A	[D1] V-KAPPA	1n0x_L	R@P 10	LEU L	(Ligand)	1n0x_P	2	0	0	2
R@P 109	ALA A	[D1] V-KAPPA	1n0x_L	R@P 15	ILE I	(Ligand)	1n0x_P	3	0	0	3
R@P 114	SER S	[D1] V-KAPPA	1n0x_L	R@P 10	LEU L	(Ligand)	1n0x_P	2	0	0	2
R@P 115	SER S	[D1] V-KAPPA	1n0x_L	R@P 10	LEU L	(Ligand)	1n0x_P	8	1	0	7

FIGURE 6 | IMGT/3Dstructure-DB Domain pair contacts between VH and V-KAPPA and the ligand from an IG/Ag complex. The IG/Ag complex structure is 1n0x from IMGT/3Dstructure-DB (<http://www.imgt.org>) (11–13). The ligand is a synthetic peptide. **(A)** Domain pair contacts between VH and the ligand. The Summary shows that there are a total of 106 atom pair contacts (10 polar including 1 hydrogen bond and 96 non-polar) for 17 pair contacts between the VH (1n0x_H) and the ligand (1n0x_P). Seven amino acids (AA) of the VH interact with the ligand. The list of the pair contacts show that three of them belong to the CDR2-IMGT (orange color online) and two of them to the CDR3-IMGT (purple color online), and together contribute to 81 atom pair contacts (including the hydrogen bond). The VH binds the ligand primarily by the N64 of the CDR2-IMGT and the P112.3 and Q112.2 of the CDR3-IMGT that are localized next to the top of the loops

(Figure 3). The only two positions of the FR-IMGT that have contacts with the ligand are the anchors 55 and 66 of the CDR2-IMGT. In that structure, there is no contact of the CDR1-IMGT. **(B)** Domain pair contacts between V-KAPPA and the ligand. The Summary shows that there are a total of 195 atom pair contacts (32 polar including 3 hydrogen bonds and 163 non-polar) for 24 pair contacts between the V-KAPPA (1n0x_L) and the ligand (1n0x_P). Twelve AA of the VH interact with the ligand. The list of the pair contacts show that seven of them belong to the CDR1-IMGT (blue color online) and three of them to the CDR3-IMGT (green/blue color online) and together contribute to 174 atom pair contacts (including the three hydrogen bonds). The only two positions of the FR-IMGT that have contacts with the ligand are the positions 1 and 2 of the strand A of the FR1-IMGT. In that structure, there is no contact with the CDR2-IMGT.

Paratope and epitope

In an IG/Ag complex, the AA in contact at the interface between the IG and the Ag constitute the paratope on the IG V-DOMAIN surface, and the epitope on the Ag surface. Similarly, in an TR/pMH complex, the AA in contact at the interface between the TR and the pMH constitute the paratope on the TR V-DOMAIN surface, and the epitope on the pMH surface. For IG/Ag and TR/pMH, the paratope and epitope are displayed in Contact analysis, but for each V-domain, separately. Clicking on the “Paratope and epitope” tag (displayed in the IMGT/3Dstructure-DB card, only if relevant), gives access to “IMGT paratope and epitope details”, which are described in a standardized way. Each AA that belongs to the paratope is defined by its position in a V-DOMAIN. Each AA that belongs to the epitope in an IG/Ag complex is defined by its position in the chain in the 3D structure or, if the antigen belongs to an IgSF or MhSF protein and if the epitope is part of a characterized V, C, or G-domain, by its position in the domain according to the IMGT unique numbering. The epitope in a TR/pMH complex includes AA of the peptide and of the two G-DOMAIN helices.

Renumbered flat file and IMGT numbering comparison

“Renumbered IMGT file” allows to view (or download) an IMGT coordinate file renumbered according to the IMGT unique numbering, and with added IMGT specific information on chains and domains (added in the “REMARK 410” lines (blue online), and identical to the “Chain details” annotation).

“IMGT numbering comparison” provides, per domain, the IMGT DOMAIN numbering by comparison with the PDB numbering, and the residue (three-letter and one-letter names), which allows standardized IMGT representations using generic tools (Figures 3A and 4A).

IMGT/3Dstructure-DB associated tools

Tools associated to IMGT/3Dstructure-DB include IMGT/StructuralQuery (11) and IMGT/DomainSuperimpose, available online. IMGT/StructuralQuery allows to retrieve the IMGT/3Dstructure-DB entries, based on specific structural characteristics of the intramolecular interactions: phi and psi angles, ASA, type of atom contacts, distance in angstrom between

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AA, IMGT Residue@Position contacts and, for V-DOMAIN, CDR-IMGT length or pattern (11). IMGT/DomainSuperimpose allows to superimpose the 3D structures of two domains from IMGT/3Dstructure-DB.

IMGT/2Dstructure-DB

IMGT/2Dstructure-DB was created as an extension of IMGT/3Dstructure-DB (11–13) to describe and analyze AA sequences of chains and domains for which no 3D structures were available (**Table 8**). IMGT/2Dstructure-DB uses the IMGT/3Dstructure-DB informatics frame and interface, which allow one to analyze, manage, and query IG, TR, and MH, as well as other IgSF and MhSF and engineered proteins (FPIA, CPCCA), as polymeric receptors made of several chains, in contrast to the IMGT/LIGM-DB sequence database that analyzes and manages sequences individually (9). The AA sequences are analyzed with the IMGT® criteria of standardized identification (59), description (60), nomenclature (61), and numerotation (8, 62–66).

The current IMGT/2Dstructure-DB entries include AA sequences of antibodies from Kabat (94) (those for which there were no available nucleotide sequences), and AA sequences of mAb and FPIA from the WHO-INN Programme (14, 50, 51). Queries can be made on an individual entry, using the Entry ID or the Molecule name. The same query interface is used for IMGT/2Dstructure-DB and IMGT/3Dstructure-DB. Thus a “trastuzumab” query in “Molecule name” allows to retrieve three results: two INN (“trastuzumab” and “trastuzumab emtansine”) from IMGT/2Dstructure-DB, and one 3D structure (“1nz8”) from IMGT/3Dstructure-DB.

The IMGT/2Dstructure-DB cards provide standardized IMGT information on chains and domains and IMGT Colliers de Perles on one or two layers, identical to that provided for the sequence analysis in IMGT/3Dstructure-DB, however the information on experimental structural data (hydrogen bonds in IMGT Collier de Perles on two layers, Contact analysis) is only available in the corresponding IMGT/3Dstructure-DB cards, if the antibodies have been crystallized.

IMGT® V AND C-DOMAIN FOR ANTIBODY HUMANIZATION AND ENGINEERING

CDR-IMGT DELIMITATION FOR GRAFTING

The objective of antibody humanization is to graft at the DNA level the CDR of an antibody V-domain, from mouse (or other species) and of a given specificity, onto a human V-domain framework, thus preserving the specificity of the original (murine or other species) antibody while decreasing its immunogenicity (101). IMGT/DomainGapAlign (12, 27, 28) is the reference tool for antibody humanization design based on CDR grafting. Indeed, it precisely defines the CDR-IMGT to be grafted and helps selecting the most appropriate human FR-IMGT by providing the alignment of the AA sequences between the mouse (or other species) and the closest human V-DOMAIN.

Analyses performed on humanized therapeutic antibodies underline the importance of a correct delimitation of the CDR and FR. As an example, two AA changes were required in the first version of the humanized VH of alemtuzumab, in order to restore the specificity and affinity of the original rat antibody. The positions

of these AA changes (S28 > F and S35 > F) are now known to be located in the CDR1-IMGT and should have been directly grafted, but at the time of this mAb humanization they were considered as belonging to the FR according to the Kabat numbering (94). In contrast, positions 66–74 were, at the same time, considered as belonging to the CDR according to the Kabat numbering, whereas they clearly belong to the FR2-IMGT and the corresponding sequence should have been “human” instead of being grafted from the “rat” sequence (IMGT®, see footnote text 1, The IMGT Biotechnology page > Antibody humanization > Alemtuzumab).

IGHG1 ALLELES AND G1m ALLOTYPES

Allotypes are polymorphic markers of an IgG subclass that correspond to AA changes and are detected serologically by antibody reagents (76). In therapeutic antibodies (human, humanized, or chimeric) (14), allotypes may represent potential immunogenic residues (75), as demonstrated by the presence of antibodies in individuals immunized against these allotypes (76). The allotypes of the human heavy gamma chains of the IgG are designated as Gm (for gamma marker).

The allotypes G1m, G2m, and G3m are carried by the constant region of the gamma1, gamma2, and gamma3 chains, encoded by the IGHG1, IGHG2, and IGHG3 genes, respectively (76). The gamma1 chains may express four G1m alleles (combinations of G1m allotypes): G1m3, G1m3,1, G1m17,1, and G1m17,1,2 (and in Negroid populations two additional G1m alleles, Gm17,1,28 and Gm17,1,27,28) (76) (**Table 9**). The C-region of the G1m3,1, G1m17,1, and G1m17,1,2 chains differ from that of the G1m3 chains by two, three, and four AA, respectively (76). The correspondence between the G1m alleles and IGHG1 alleles is shown in **Table 9**. Thus, IGHG1*01 and IGHG1*02 are G1m17,1, IGHG1*03 is G1m3, IGHG1*04 is G1m17,1,2 and IGHG1*05 is G1m3,1.

In the IGHG1 CH1, the lysine at position 120 (K120) in strand G corresponds to the G1m17 allotype (76) (**Figure 4D**). The isoleucine I103 (strand F) is specific of the gamma1 chain isotype. If an arginine is expressed at position 120 (R120), the simultaneous presence of R120 and I103 corresponds to the expression of the G1m3 allotype (76). For the gamma3 and gamma4 isotypes (which also have R120 but T in 103), R120 only corresponds to the expression of the nG1m17 isoallotype (an isoallotype or nGm is detected by antibody reagents that identify this marker as an allotype in one IgG subclass and as an isotype for other subclasses) (76).

In the IGHG1 CH3, the aspartate D12 and leucine L14 (strand A) correspond to G1m1, whereas glutamate E12 and methionine M14 correspond to the nG1m1 isoallotype (76) (**Table 9**). A glycine at position 110 corresponds to G1m2, whereas an alanine does not correspond to any allotype (G1m2-negative chain) (**Table 9**). Therapeutic antibodies are most frequently of the IgG1 isotype, and to avoid a potential immunogenicity, the constant region of the gamma1 chains are often engineered to replace the G1m3 allotype by the less immunogenic G1m17 (CH1 R120 > K) (G1m17 is more extensively found in different populations) (76).

CONCLUSION

IMGT-ONTOLOGY and the IMGT® information system, which are at the origin of immunoinformatics, have provided the

Table 9 | Correspondence between the IGHG1 alleles and G1m alleles.

IGHG1 alleles	G1m alleles ^a		IMGT amino acid (AA) positions ^b					Populations (76)	
	Allotypes	Isoallotypes ^c	CH1		CH3				
			103	120	12	14	110		
			G1m3 ^d	G1m17/nG1m1	G1m1/nG1m1	G1m2/-			
IGHG1*01 ^e	G1m17,1		I	K	D	L	A	Caucasoid Negroid	
IGHG1*02 ^e								Mongoloid	
IGHG1*03	G1m3	<i>nG1m1, nG1m17</i>	I	R	E	M	A	Caucasoid	
IGHG1*04 ^f	G1m17,1,2		I	K	D	L	G	Caucasoid	
								Mongoloid	
IGHG1*05 ^f	G1m3,1	<i>nG1m17</i>	I	R	D	L	A	Mongoloid	

^aIn Negroid populations, the G1m17,1 allele frequently includes G1m27 and G1m28, leading to two additional G1m alleles, G1m17,1,27 and G1m17,1,27,28 (76).

^bAA corresponding to G1m allotypes are shown in bold.

^cThe *nG1m1* and *nG1m17* isoallotypes present on the Gm1-negative and Gm17-negative gamma1 chains (and on other gamma chains) are shown in italics.

^dThe presence of R120 is detected by anti-*nG1m17* antibodies whereas the simultaneous presence of I103 and R120 in the gamma1 chains is detected by anti-Gm3 antibodies (76).

^eThe IGHG1*01 and IGHG1*02 alleles only differ at the nucleotide level (codon 85.1 in CH2).

^fIGHG1*04 and IGHG1*05 AA are expected (76) but not yet sequenced at the nucleotide level and therefore the IGHG1*04 and IGHG1*05 alleles are not shown in IMGT Repertoire, Alignments of alleles: *Homo sapiens IGHG1* (<http://www.imgt.org>).

concepts, the knowledge environment, and the informatics frame for a standardized and integrated analysis of IG, TR, and MH, extended to other IgSF (102–106) and MhSF (96, 97), from gene to structure and function (32–47). IG and TR repertoire analysis, antibody humanization, IG and TR engineering for immunotherapy, paratope/epitope characterization represent major current fields of immunoinformatics at the forefront of basic, clinical, and pharmaceutical research owing to major methodological advances and medical implications.

The IMGT® standards are used in clinical applications. Thus, IMGT/V-QUEST is frequently used by clinicians for the analysis of IG somatic hypermutations in leukemia, lymphoma, and myeloma, and more particularly in chronic lymphocytic leukemia (CLL) (18, 72–74) in which the percentage of mutations of the rearranged IGHV gene in the VH of the leukemic clone has a prognostic value for the patients. For this evaluation, IMGT/V-QUEST is the standard recommended by the European Research Initiative on CLL (ERIC) for comparative analysis between laboratories (72). The sequences of the V–(D)–J junctions determined by IMGT/JunctionAnalysis (21, 22) are also used in the characterization of stereotypic patterns in CLL (73, 74) and for the synthesis of probes specific of the junction for the detection and follow-up of minimal residual diseases (MRD) in leukemias and lymphomas. A new era is opening in hemato-oncology with the use of NGS for analysis of the clonality and MRD identification, making IMGT® standards use more needed as ever. More generally, the IMGT/HighV-QUEST web portal is a paradigm for identification of IMGT clonotype diversity and expression in NGS immune repertoire analysis of the adaptive immune response in infectious diseases, in vaccination, and for next-generation repertoire immunoprofiling (26).

The therapeutic monoclonal antibody engineering field represents the most promising potential in medicine. A standardized

analysis of IG genomic and expressed sequences, structures, and interactions is crucial for a better molecular understanding and comparison of the mAb specificity, affinity, half-life, Fc effector properties, and potential immunogenicity. IMGT-ONTOLOGY concepts have become a necessity for IG loci description of newly sequenced genomes, antibody structure/function characterization, antibody engineering [single chain Fragment variable (scFv), phage displays, combinatorial libraries] and antibody humanization (chimeric, humanized, and human antibodies) (35, 42, 44, 46, 75–77, 82). IMGT® standardization allows repertoire analysis and antibody humanization studies to move to novel high-throughput methodologies with the same high-quality criteria. The CDR-IMGT lengths are now required for mAb INN applications and are included in the WHO-INN definitions (51), bringing a new level of standardized information in the comparative analysis of therapeutic antibodies.

AVAILABILITY AND CITATION

Authors who use IMGT® databases and tools are encouraged to cite this article and to quote the IMGT® Home page, <http://www.imgt.org>. Online access to IMGT® databases and tools are freely available for academics and under licenses and contracts for companies.

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Serum microRNAs as biomarkers of human lymphocyte activation in health and disease

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Induction of the adaptive immune system is evaluated mostly by assessment of serum antibody titers and T lymphocyte responses in peripheral blood, although T and B cell activation occurs in lymphoid tissues. In recent years, the release of microRNAs (miRNAs) in the extra-cellular environment has been exploited to assess cell functions at distance via measurement of serum miRNAs. Activated lymphocytes release a large amount of nano-sized vesicles (exosomes), containing miRNA, however there are insufficient data to determine whether this phenomenon is reflected in modulation of serum miRNAs. Interestingly, miRNA signatures of CD4⁺ T cell-derived exosomes are substantially different from intracellular miRNA signatures of the same cells. We have recently identified serum circulating miR-150 as a sensor of general lymphocyte activation and we strongly believe that miRNAs differentially released by specific CD4⁺ effector T cell subsets (Th1, Th2, Th17, and Treg) may serve as serum biomarkers of their elicitation in lymphoid tissues but also in damaged tissues, potentially providing clinically relevant information about the nature of immune responses in health and disease.

Keywords: T lymphocytes, circulating microRNAs, exosomes, vaccination

BLOOD-CIRCULATING miRNAs AS BIOMARKERS IN HEALTH AND DISEASE

MicroRNAs (miRNAs) are small single-stranded RNA molecules (18–25 nt), that once loaded into the Argonaute protein of the silencing complex, pair with messenger RNAs, directly effecting post-transcriptional regulation (1). In recent years, it has been found that most cells release miRNAs in the extra-cellular environment, predominantly in association with either vesicles or protein complexes that protect them from RNases (2–4). This release can be a passive phenomenon that results from tissue damage, or an active process as in the case of miRNAs actively secreted into the surrounding environment by healthy cells, where once outside, miRNAs can reach the bloodstream and constitute what it is now referred to as the “blood-circulating extra-cellular miRNome.” Extra-cellular miRNAs can be extracted from serum, plasma, and other body fluids and profiled through microarray, real time quantitative PCR or sequencing. This possibility has been exploited to assess cell functions at distance via measurement of serum miRNAs and nowadays blood-circulating miRNAs are regarded among the most promising clinical biomarkers for the diagnosis, prognosis, and therapeutic options of a variety of pathological conditions such as cancer (5–7), cardiovascular diseases (8, 9), diabetes (10), liver pathologies (11, 12), and sepsis (13, 14), among others [reviewed in Ref. (15)].

Circulating miRNAs as clinical biomarkers are not without some technical challenges. First, dilution effects in blood limit the amount of RNA per volume of starting material. Second, cellular detritus, hemolysis, and the presence of contaminating components constitute pre-analytical challenges, potentially impacting reproducibility and sensitivity. Finally, as miRNAs are released by virtually all cells in the body and most of the blood miRNAs are

released by large organs as well as highly dividing cells, specificity is impacted by high background. However, the fact that serum miRNAs circulate in different compartments might provide an advantage. miRNAs circulate in association with vesicles of nanometric size (20–100 nm) called exosomes, that are formed by the inward budding and subsequent fusion to the plasma membrane of multivesicular endosomes (16); vesicles of larger size (0.2–1 μm) that bud directly from the plasma membrane, are called microvesicles and comprise also apoptotic and senescent bodies (17); in association with Argonaute protein in a vesicle-free form (3); or linked to high-density lipoproteins (18). This compartmentalization facilitates the purification of specific isolates, enriching for biomarkers of interest. Moreover, the possibility of isolating cell lineage-specific exosomes based upon their parental protein expression patterns, could in principle represent a further advantage in the identification and validation of biomarkers in lymphocyte activation.

RELEASE OF EXOSOME-ASSOCIATED miRNAs UPON ACTIVATION OF LYMPHOCYTES: BIOLOGICAL ASPECTS

The presence of RNA, such as messengers and regulatory RNAs (among which miRNAs) within exosomes was initially described in 2007 (19) but the role of exosomes in conveying intercellular communication had already been extensively investigated in the immune system [reviewed in Ref. (20, 21)]. In 1996, it was first demonstrated that B lymphocytes release exosomes with a significantly different overall surface protein profile from that of the plasma membrane and that they do contain MHC class II and are able to induce antigen-specific MHC class II-restricted T cell responses, demonstrating a specific role for exosomes in antigen presentation *in vivo* (22). Later it was also shown that upon

TCR triggering, T lymphocytes produce a large amount of exosomes that bear TCR from the pool of activated complexes. It was suggested that exosomes may work as powerful vehicles to specifically deliver signals to cells with a specific combination of peptide/MHC complexes (23). Furthermore, T lymphocytes have been observed killing target cells by CD95 engagement through membrane CD95L containing exosomes (24). Exosomes can be viewed as communication modules between cells of the immune system, and are regarded by some authors as important to the microenvironment as the release of cytokines and chemokines (25). While the role of intracellular miRNAs has long been recognized as a key level of gene-expression regulation in cells of the immune system (26, 27), its role in exosomes has only begun to be explored. Initial data indicate an exosome-mediated transfer of RNA between T cells and antigen presenting cells during antigen recognition, as well as the capacity of miRNAs transferred during immune synapse to modulate gene-expression in recipient cells (28, 29). To fully elucidate the role of exosome-associated miRNAs, it will be necessary to better characterize the fundamental processes of extra-cellular RNA biogenesis, distribution, uptake, and how they contribute to overall function. In principle, since exosomes contain myriad miRNAs in varying numbers they possess the potential to regulate the expression of multiple genes leading to very effective paracrine control over neighboring cells (30).

LYMPHOCYTE SIGNATURES OF EXOSOME-ASSOCIATED miRNAs: A NEW LANDSCAPE TO BE UNVEILED

The release of RNA through exosomes is not a passive phenomenon, but actually a regulated, active process demonstrated by the fact that exosomal RNA content is not at all a mere reflection of that found within the intracellular milieu. By next-generation sequencing of small RNA species present in vesicles released in co-culture of T lymphocytes and dendritic cells, it has been described that distinct RNA categories, such as small ribosomal RNA and specific tRNA fragments, long interspersed elements (LINEs), and long terminal repeats (LTRs) are conveyed in, and released by vesicles in significantly greater numbers than other RNA types, such as lincRNAs (31). A quantitative analysis shows the selective enrichment of some miRNAs in purified exosomes compared to cells. The differential rate of release for various RNA molecules was further demonstrated by our finding that the intracellular miRNome of CD4⁺ T lymphocytes is more similar to the intracellular miRNome of B lymphocytes than to their own exosomal miRNome (Figure 1A) (32). Indeed, we have identified a discrete set of miRNAs whose intracellular concentrations, compared to that found within their cognate lymphocyte-derived exosomes, was significantly different (32).

As previously reported, we have identified 20 intracellular miRNAs that are able to distinguish between different subpopulations of CD4⁺ T cells, defining the development and differentiation of this lineage (Figure 1B) (33). Only two of these miRNAs were independently demonstrated to be differentially represented in Th cell-derived compared to B cell-derived exosomes (Figure 1C); further 17 miRNAs with a signature specifically associated to exosomes released by CD4⁺ T cells have been subsequently identified (32). These observations tell us that even a thorough analysis of the biological relevance of miRNAs at the intracellular level is not helpful

in deciphering the spectrum of differences at the extra-cellular level and that only a high-throughput quantitative investigation of miRNA in exosomes will define lymphocyte-specific exosome-associated miRNA signatures. Consequently, this endeavor commenced by showing that CD4⁺ T and B lymphocytes display significantly different selective enrichment of specific extra-cellular miRNAs, and we are now in the process of fully elucidating the differential exosomal miRNomes of each CD4⁺ T subset.

These studies will potentially have two different and equally relevant impacts on the field. On the one side, the analysis of extra-cellular lymphocyte signatures may shed light on the role of miRNA disposal during activation. For example, as previously described, when primary CD4⁺ T cells are activated *in vitro*, they dramatically down-regulate intracellular miR-150 while accumulating it in extra-cellular vesicles, suggesting that this type of release may represent an additional layer of post-transcriptional regulation for miRNAs with very rapid effects on target genes of the discarded miRNAs (32). We thus believe that correlating data of intracellular modulation upon activation with data of extra-cellular disposal will tell us if the case of miR-150 is isolated or not, and if this type of regulation is specific for different lymphocyte subsets. Furthermore, should exosome-associated miRNAs display a paracrine control over neighboring cells, the full description of extra-cellular miRNAs differentially released by different subsets of effector cells will profoundly change the knowledge we have on how these cells impact on the extra-cellular environment.

On the other side, these same studies will pave the way for the identification and validation of potentially powerful biomarkers of lymphocyte activation. Indeed, upon the identification of miRNAs that are differentially released by various lymphocyte effector cells (e.g., Th1, Th2, Th17, and Treg), the assessment of their modulation in serum may render possible to mark the elicitation of these cells, which occurs in lymphoid tissues or damaged organs (34).

THE GROWING NEED FOR NEW BIOMARKERS OF VACCINATION

Vaccinations are based on the activation of the adaptive immune system. Their efficacy is evaluated mostly by assessing serum antibody titers and lymphocyte responses in peripheral blood, despite T and B cell activation occurring within the lymphoid tissues. While the protective role of vaccination is primarily conferred by the generation of B cells-derived antigen-specific antibodies, T cells are fundamental for the induction of high-affinity antibodies and immune memory, and in certain types of vaccines, these cells must be regarded as prime effectors. Then, the identification of novel immune correlates of vaccine efficacy that take into account parameters different than antibody titers will become increasingly more important in the development of new adjuvants and the optimization of current vaccines (35). Moreover, as some subsets of effector T helper cells can trigger adverse events from allergy to autoimmunity, the ability to easily monitor the induction of these subsets by new vaccines is becoming critical concern in clinical development.

Toward these ends, a pilot study to address the feasibility of following the generation of an immune response through the profiling of serum-associated miRNAs has been undertaken (32).

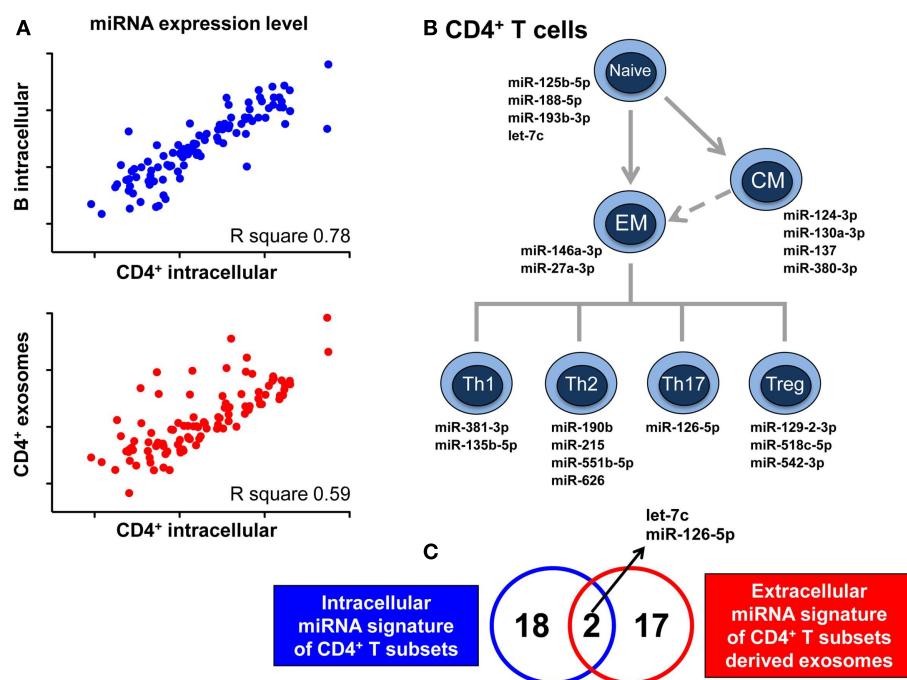


FIGURE 1 | MicroRNAs signature of CD4⁺ T cell-derived exosomes cannot be predicted by relevant intracellular miRNA signatures of CD4⁺ T cells development and differentiation. (A) Pearson correlation between miRNA expression level in CD4⁺ lymphocytes and either miRNA expression level in B lymphocytes (upper panel) or miRNA representation in CD4⁺ lymphocyte-derived exosomes (lower panel). *R* square values are reported. (B) Schematic view of CD4⁺ T lymphocytes with the relative intracellular miRNA signatures identified as relevant for

the development and differentiation of these subsets (EM, effector memory; CM, central memory). (C) Venn diagram showing the partial overlap between the 20 miRNAs composing the intracellular signatures of CD4⁺ T subsets of panel B and the 19 miRNAs at later stage identified for being either exclusive or differentially up-represented in exosomes derived from CD4⁺ T subsets compared to exosomes derived from B lymphocytes. Data shown are adapted from previous published studies (32, 33).

EXOSOME-ASSOCIATED miR-150 AS A GENERAL SENSOR OF VACCINATION

Over the past few years, one specific miRNA, namely miR-150, has been confirmed to play a critical role in the development of lymphoid and myeloid lineages in both mice and humans (36). In particular, miR-150 is expressed at a low level in B- and T-progenitor cells, but gets highly up-regulated in mature lymphocytes. Moreover, as naïve T cells differentiate into effector Th cells, the level of miR-150 is down-modulated again (37).

miR-150 has been also frequently observed to be dramatically dysregulated in several types of leukemias and lymphomas (36). When the level of intracellular miR-150 decreases, some critical targets get de-repressed, e.g., c-Myb, a transcription factor that promotes lymphocyte survival by inducing Bcl2 (38, 39). Another target of miR-150 action is Notch3, a member of the Notch family of receptors, which plays a pivotal role in T cell differentiation and leukemogenesis (40).

We recently described that when primary human CD4⁺ Th cells dramatically down-regulate intracellular miR-150 upon activation, it's released by exosomes, suggesting that this process of extra-cellular miRNA "disposal" may represent an additional layer of post-transcriptional down-regulation for miRNAs with very rapid effects on target genes that critically control lymphocyte responses (32).

In parallel with the biological implications of these observations, we decided to investigate miRNA-150 as a potential candidate for the optimal biomarker of lymphocyte activation because: (i) it is highly expressed in both human lymphocyte cells and lymphocyte-derived exosomes; (ii) it is expressed specifically in spleen compared to other human tissues, supporting the idea that the major source of serum miR-150 are lymphoid cells and (iii) it is easily detectable and significantly enriched in exosomes circulating in human blood (32). Hence, our working hypothesis is based on the assumption that when the immune system is activated by vaccination, the lymphocytes that participate in the response will release an easily detectable number of exosomes into the bloodstream and consequently a readily measureable level of a lymphocyte-derived exosomal miRNA (as it is the case for miR-150) (Figure 2A). Before proceeding to analyze precious human samples of vaccinated individuals, we made use of the mouse model, and discovered that, as correctly hypothesized, serum miR-150 levels increase significantly in mice upon vaccination with adjuvant-OVA. Instead, miR-150 serum concentrations remain unchanged in immunized mice that are depleted of mature CD4⁺ T lymphocytes [major histocompatibility complex class II-deficient mice (41)] showing that serum miR-150 modulation is a specific phenomenon strictly dependent on adaptive immune responses elicited by effective vaccination (Figure 2B) (32).

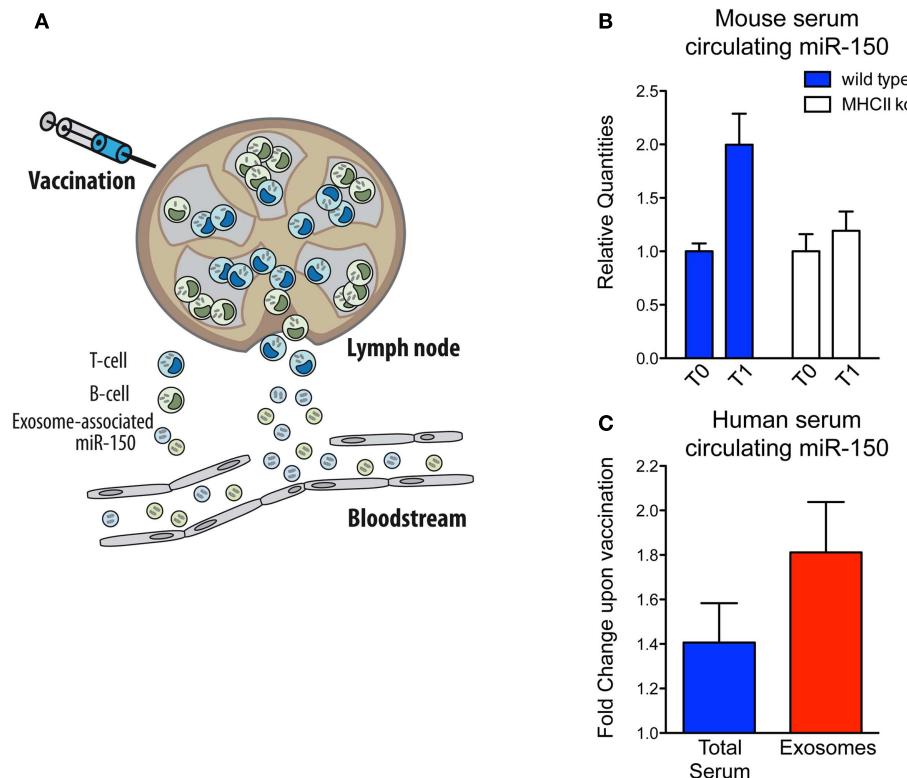


FIGURE 2 | Serum circulating miR-150 increases in both human and mice upon vaccination. **(A)** Schematic view of exosome-associated miR-150 release upon induction of adaptive immune response in secondary lymphoid organs upon vaccination. **(B)** Mouse serum circulating miR-150 increase expressed as relative quantities at time T0 (pre-vaccination) and T1 (post-vaccination) in wild type compared to CD4⁺ cell-depleted MHCII knock

out mice (four animals/group; the result shown is representative of two independent experiments). **(C)** Human circulating miR-150 increase expressed as fold change upon vaccination in total serum compared to serum purified exosomes (results come from 17 paired samples of sera collected at the time of vaccination and 4 weeks after that). Data shown are adapted from previous published studies (32).

Sera from adults or children vaccinated with the 2009 pandemic flu (H1N1) vaccine adjuvanted with MF-59 (42) was then assessed and, similarly to what was found in mice, the level of extra-cellular miR-150 increased significantly in human serum upon vaccination and this increase was significantly more evident upon purification of exosomes (Figure 2C) (32). This observation suggests that serum miR-150 modulation is specifically compartmentalized to lymphocyte-derived vesicles and that exosome purification strategies from blood may serve to increase lymphocyte biomarker sensitivity by enriching immune-related circulating extra-cellular RNAs, as suggested for other conditions (7, 43). Furthermore, in flu vaccinated individuals, miR-150 serum levels post-vaccination have been found to be significantly higher in people mounting higher antibody response, showing a quantitative correlation between the modulation of a circulating miRNA and the adaptive immune response (32).

In a recent study, reduced miR-150 serum concentrations have also been found to be associated with an unfavorable outcome in critically ill patients with sepsis. It has been hypothesized that lower circulating miR-150 levels might lead to de-repression of genes such as CXCR4 and c-Myb, both linked to immune response activation and poor prognosis (14).

Consistently, we do speculate that the significant uptick in circulating miR-150 levels that coincides with vaccination may play a role in down-modulating adaptive immune responses by carrying extra-cellular messages to other immune cells and consequently regulating miR-150 target genes.

Obviously, as miR-150 is ubiquitous across all lymphocyte populations, it may only serve as a generic lymphocyte activation sensor, devoid of more insights into the lymphocyte subsets involved. Nonetheless, this study has provided the first proof-of-concept that serum miRNAs can be readily detected, from a minimally invasive serum sample, toward their validation as sensitive and specific biomarkers of vaccination, and more generally of the adaptive immune response.

SUMMARY AND PERSPECTIVES

Blood-circulating extra-cellular miRNAs have the potential to become highly valuable biomarkers in the near future. In particular, the identification of serum miRNA signatures able to directly report the differential activation state of clinically relevant lymphocytic subsets may become an innovative tool to provide pivotal information about the nature of the immune responses occurring in health (e.g., vaccination) and disease (e.g., auto-immune

and immune-mediated disorders). We have recently described a significant increase of circulating miR-150 serum concentrations 1 month post-vaccination, and shown to be the release of exosome-associated miRNAs by lymphocytes activated *in vivo* by vaccines. This study gives support to the idea that profiling of serum miRNA levels may lead to the identification of new biomarkers of immune responses and that exosome purification represents a facile yet powerful step toward increased sensitivity, with possible increased specificity.

In conclusion, the compilation of a catalog of exosome-associated miRNAs derived from specific lymphocyte subsets should help confirm whether serum miRNAs are indeed able to report the activation of specific T cell subsets (e.g., Th1, Th2, Th17, Treg, etc.) occurring at distant sites. These lines of enquiry should benefit the assessment of pathogenic immune response during the course of auto-immune diseases and their therapies, as well as significantly contributing to the close, rapid monitoring of clinical trials with new immune-regulatory drugs, new vaccines, and/or adjuvants, particularly in the earlier stages of clinical development.

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Human NK cells: from surface receptors to the therapy of leukemias and solid tumors

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Natural Killer (NK) cells are major effector cells of the innate immunity. The discovery, over two decades ago, of major histocompatibility complex-class I-specific inhibitory NK receptors and subsequently of activating receptors, recognizing ligands expressed by tumor or virus-infected cells, paved the way to our understanding of the mechanisms of selective recognition and killing of tumor cells. Although NK cells can efficiently kill tumor cells of different histotypes *in vitro*, their activity may be limited *in vivo* by their inefficient trafficking to tumor lesions and by the inhibition of their function induced by tumor cells themselves and by the tumor microenvironment. On the other hand, the important role of NK cells has been clearly demonstrated in the therapy of high risk leukemias in the haploidentical hematopoietic stem cell (HSC) transplantation setting. NK cells derived from donor HSC kill leukemic cells residual after the conditioning regimen, thus preventing leukemia relapses. In addition, they also kill residual dendritic cells and T lymphocytes, thus preventing both GvH disease and graft rejection.

Keywords: NK cells, killer Ig-like receptors, alloreactive NK cells, activating NK receptors, hematopoietic stem cell transplantation, acute leukemias, tumor microenvironment

INTRODUCTION

Natural Killer (NK) cells play a central role in innate immunity as they mediate early defenses against viral infections and, more in general, against pathogens. However, NK cells are also involved in immune surveillance against tumors and prevent dissemination of metastatic tumors (1, 2). The NK effector function against tumors and virus-infected cells is mostly related to their cytolytic activity. In addition, by the secretion of various cytokines and chemokines, NK cells promote inflammatory responses and exert a regulatory control on downstream adaptive immune responses by influencing not only the strength, but also the quality of T cell responses. T helper-1 responses, favored by NK cells, further contribute to anti-tumor and anti-virus defenses. In turn, NK cell function is regulated by cytokines, including IL-15, IL-2, and IL-18 (3) as well as by cell-to-cell interactions involving different cell types primarily dendritic cells (DC) (3–5), macrophages (6), and mesenchymal stromal cells (7, 8). NK cells migrate to inflamed tissue and to secondary lymphoid organs where they can encounter tumor cells and participate to the first line of defense against pathogens. NK cells originate from hematopoietic stem cells (HSC) and undergo maturation primarily in the bone marrow (BM). However, evidence has been accumulated during the past several years that NK precursors at different stages of differentiation are present in tonsils (9), lymph nodes (10), decidua (11), and gut-associated lymphoid tissues (12). In addition, precursors capable of undergoing *in vitro* differentiation toward NK cells were isolated from human thymus over two decades ago (13).

INHIBITORY AND ACTIVATING NK RECEPTORS: PAST AND PRESENT

In spite of their functional relevance in defenses against viruses and tumors, NK cells remained mysterious and poorly considered for many years after their discovery (14–16) so that core questions regarding the molecular mechanisms involved in their ability to discriminate between normal and tumor or virus-infected cells remained unanswered. However, starting in early 90s, we began to gain a fair idea on the mechanisms regulating NK cell activation and function. In late 80s, Ljunggren and Kärre had proposed the “missing self hypothesis” (17), based on the observation that NK cells could efficiently kill a murine lymphoma cell line that had lost major histocompatibility complex (MHC)-class I, while the parental MHC-class I⁺ lymphoma cells were resistant to lysis. Thus, it appeared that NK cells could sense MHC-class I molecules, sparing MHC-class I⁺ cells while killing MHC-class I⁻ cells. In addition, a clue that NK cells could sense even allelic differences on hematopoietic target cells was provided by the hybrid resistance phenomenon in which NK cells could reject parental BM graft in F1 hybrid mice (18). Another experiment suggesting that MHC-class I molecules could influence NK cell function was the detection of human NK cell proliferation in mixed lymphocyte culture against stimulating cells from unrelated donors (in the presence of IL-2). In addition, such cultured NK cells could lyse phytohemagglutinin (PHA) blasts isolated from the same stimulating donor (19). Taken together, these data were compatible with the expression, at the NK cell surface, of

inhibitory receptors sensing MHC-class I molecules. The discovery of surface molecules expressed by human NK cell subsets that could inhibit the NK cell cytotoxicity upon monoclonal antibody (mAb)-mediated crosslinking (20, 21), was the first step toward the identification of human leukocytes antigen (HLA)-class I-specific inhibitory receptors recognizing allelic forms of HLA-C (22). Remarkably, in parallel, Yokoyama et al. had identified Ly49 molecules as the murine receptors for MHC-class I (23). A number of novel receptors belonging to the same Ig-superfamily of the two HLA-C-specific prototypes (named p58.1 and p58.2) were identified and collectively called killer Ig-like receptors (KIRs). They also recognized allelic forms of HLA-B or -A allotypes (24–27). In addition, activating KIRs were discovered (28) that were similar to the corresponding inhibitory KIRs in the extracellular Ig-domains, but substantially differed in the transmembrane and in the intracytoplasmic portions (29). Both inhibitory and activating KIRs have been shown to play an important role in the cure of high risk leukemias in the haploidentical HSC transplantation setting (see below). Genetic analysis revealed that KIR-encoding genes evolved and diversified rapidly in primates and humans (30). Likewise the HLA loci, KIR sequences were found to be highly polymorphic. KIR genes are organized as a family in the leukocyte receptor complex in chromosome 19 and are inherited as haplotypes. KIR haplotypes exhibit variability in the number and type of genes and in allelic polymorphism of the individual KIR genes, resulting in extensive genetic diversity. On the basis of their gene content, KIR haplotypes have been divided into group A (with a fixed gene pattern mainly including inhibitory KIR) and group B (more variable and including several activating KIR) (31). Other receptors with different HLA-I specificities, including CD94/NKG2A and LIR-1, were discovered and characterized (32, 33). Since inactivation of NK cell function represents a central fail-safe mechanism to prevent killing of normal self HLA-class I⁺ cells, the existence of activating receptors that are triggered upon interaction with normal cells had to be postulated. Experiments aimed at identifying these receptors were successful and three important activating NK receptors named NKp46 (34, 35), NKp44 (36, 37), and NKp30 (38) were discovered and molecularly characterized (39). These molecules, collectively termed natural cytotoxicity receptors (NCRs), were found to play a central role in tumor cell recognition and killing. Additional surface molecules functioning as activating receptors or co-receptors were subsequently identified. Some of these molecules, primarily NKG2D and DNAM-1, were also shown to play an important role in target cell recognition and lysis (40, 41). Remarkably, the known ligands of such receptors are over-expressed or expressed *de novo* upon cell stress, particularly when consequent to tumor transformation or viral infection (40, 42, 43). The fact that NK cell activation may occur only upon interaction with abnormal target cells represents an important checkpoint to control unnecessary NK cell activation (44). In case of NK cell interaction with ligand-positive stressed cells, the latter are protected from lysis because of the engagement of HLA-I-specific inhibitory NK receptors by HLA-I molecules expressed normally, or even upregulated in these cells. On the contrary, virus-infected or tumor cells lack the expression of HLA-I molecules and upregulate the expression of NK activating receptor ligands becoming susceptible to NK cell lysis. The ligands of

the main activating NK receptors include the human leukocyte antigen-B-associated transcript 3 (BAT-3) and B7H6 for NKp30 (45, 46), a novel isoform of the mixed-lineage leukemia-5 protein (MLL5) for NKp44 (47), PVR (CD155) and Nectin-2 (CD112) for DNAM-1 (42), and MICA/B and ULBPs for NKG2D (43). Direct identification of such ligands in tumor cells may allow predicting whether a given tumor may be susceptible to NK-mediated killing (see below for details).

NK CELLS AND SOLID TUMORS

Besides specific T lymphocytes, also NK cells are thought to play an important role in cancer immunosurveillance. NK cells are capable of recognizing and killing a wide variety of tumor cells. NK cells are potentially capable of eliminating tumors with reduced or absent MHC-class I expression that evade CD8⁺ T cell-mediated control. Therefore, they are playing a complementary role in anti-tumor activity. Recent studies also suggest that NK cells recognize and kill cancer stem cells (CSCs) (48, 49). Within the tumor mass, CSCs represent a small subpopulation of quiescent, self-renewing, chemo- and radio-resistant cells and hence they are responsible for tumor relapses after cytoreductive therapies.

In clinical studies, the degree of NK-mediated cytotoxic activity has been inversely correlated with cancer incidence in long survey subjects (50). In addition, several studies have provided evidence that, in a variety of different solid tumors, such as lung, gastric, colorectal, and head and neck cancers, the presence of high numbers of tumor-infiltrating NK cells correlates with improved prognosis of cancer patients (51–53). Despite the fact that NK cells represent a potential tool to eliminate tumor cells, NK cell-based immunotherapy has resulted in limited clinical benefit (54). In particular, this holds true in the case of solid tumors, suggesting that mechanisms of resistance at the level of the tumor microenvironment may be prevailing in many cases. This may reflect the limited capacity of adoptively transferred NK cells to traffic to tumor sites (55, 56).

Of note, factors regulating NK cell recruitment into neoplastic tissues are highly influenced by the tumor type, and by the chemokine profile of the tumor microenvironment. Several studies suggested that certain solid malignancies are infiltrated by variable numbers of NK cells. Those include, non-small cell lung cancers (NSCLC), gastrointestinal sarcoma (GIST), colorectal and renal cell carcinoma, and lung metastases (57–59). A recent study suggested that CD56⁺ NK cells could scarcely infiltrate melanomas, hepatocellular carcinomas, breast cancers, and renal cell carcinomas (60). Other studies reported that NK cells in solid tumors are often not located in direct contact with tumor cells but within the stroma (55, 61) and usually functionally anergic.

Thus, tumor cells may have developed various escape mechanisms to avoid NK-mediated killing. Hence, the tumor cells themselves or even tumor stromal cells may be actively involved in inhibition of NK cell function. Indeed, the tumor microenvironment may greatly influence NK-mediated defenses by a number of immunosuppressive strategies. Similar to T cells, tumor-infiltrating NK cells may be inhibited in their functional capability (57, 62–64). It has been shown that impaired NK cell function is often associated with down-modulation of activating

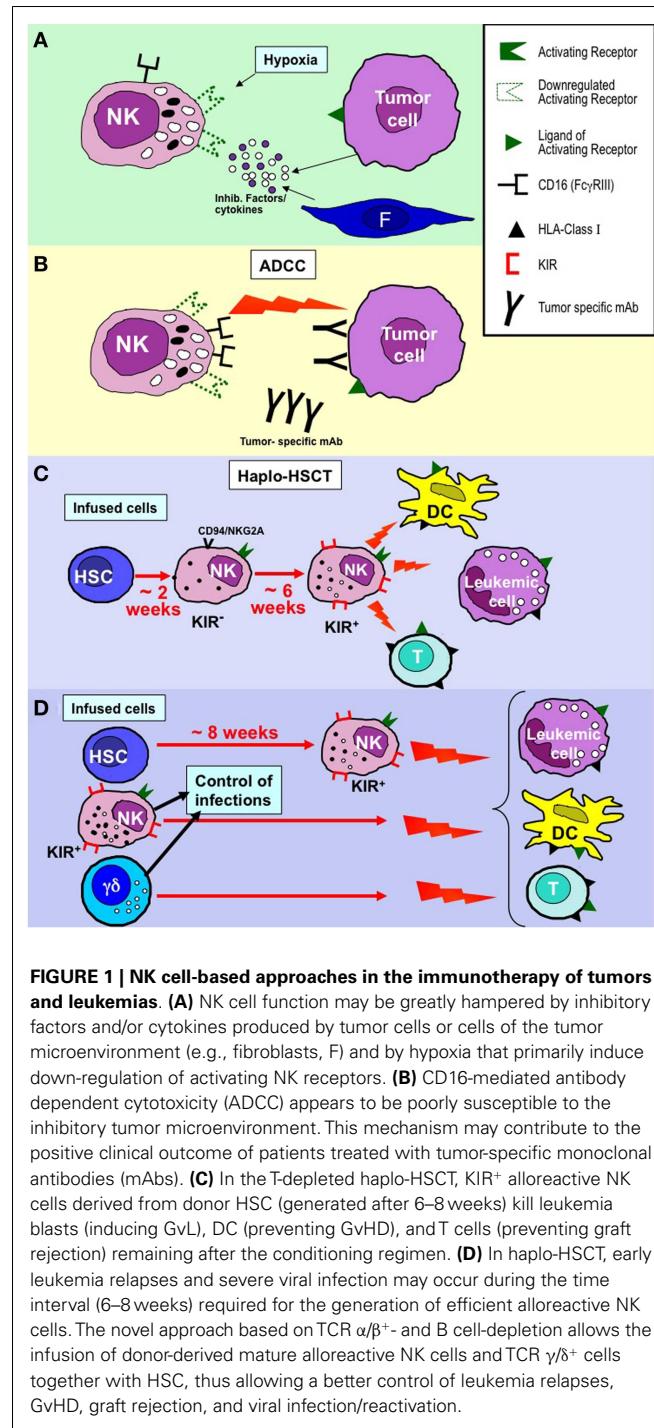
NK receptors. The molecular mechanisms underlying this down-regulation are only partially understood. In this context, ligand-induced receptor down-regulation may play a relevant role. This may be consequent to receptor blocking by ligand shed from tumor cells or to intercellular transfer (a phenomenon known as trogocytosis) (65, 66). In addition, chronic ligand-induced stimulation of NK cells may account for the down-regulation of activating receptors such as NKG2D (67). Surface molecules expressed by tumor cells could also inhibit NK cell function. For example, MUC16, a glycoprotein expressed on the surface of ovarian cancer cells inhibits synapse formation between tumor cells and NK cells (68). In addition, cytokines or soluble mediators such as TGF- β and PGE2, synthesized either by tumor or by stromal cells down-regulate the surface expression of NKp30, NKp44, and NKG2D and, consequently, NK cell cytotoxicity and cytokine production (69, 70). Furthermore, the enzyme indoleamine 2,3-dioxygenase (IDO) (over-expressed by some tumor cells including melanomas) may also contribute to the establishment of immune tolerance in the tumor microenvironment. In this context, a recent study by our group in melanomas reported that NK cell function may be suppressed by IDO-generated L-kynurenone (a tryptophan-derived toxic metabolite) (71). Finally, also the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) has been shown to inhibit the NKG2D expression in peripheral blood (PB) NK cells derived from ovarian cancer patients (72) (Figure 1A).

The hypoxic condition in cancer tissues may also contribute to tumor escape from NK cells. In a recent study, we observed that hypoxia can significantly impair both the surface expression and the function of major activating NK receptors involved in tumor recognition, including NKp46, NKp30, NKp44, and NKG2D. Accordingly, the NK-mediated cytotoxicity against tumor cells was sharply decreased under hypoxia conditions (Figure 1A). Interestingly, hypoxia did not affect CD16 (Fc γ RIII) expression and function. Therefore, NK cells maintained the ability to efficiently kill mAb-coated target cells. These data imply that even at low oxygen tension, targeting of tumors with mAbs may be effective by NK cell-mediated antibody dependent cellular cytotoxicity (ADCC) (73) (Figure 1B).

The described mechanisms of inhibition help to better understand how tumors and their microenvironment can alter the ability of NK cells to elicit an effective anti-tumor response. In view of the immunosuppressive effect exerted by tumor cells at the tumor site, new strategies are required to prevent inhibition of potentially efficient effector mechanisms, for example by blocking the soluble mediators with immunosuppressive activity. Notably, these strategies may be applied to design novel protocols of NK cell-based adoptive immunotherapy to treat solid tumors.

NK CELLS IN THE THERAPY OF HIGH RISK LEUKEMIAS

Over the past 40 years, allogeneic hematopoietic BM or HSC transplantation from HLA-matched donors has been increasingly used to treat thousands of patients with malignant (primarily leukemias) or non-malignant disorders (e.g., severe combined immunodeficiencies) (74, 75). However, approximately one-third of patients in need of an allograft do not find a compatible donor, including matched-unrelated donors (MUD) and umbilical cord blood (UCB). However, the majority of patients, particularly



children or young adults, have a family member identical for one HLA haplotype and mismatched for the other (the so-called haploididentical donor), who could serve as donor of HSC. This, haplo-HSC transplantation offered a promptly available treatment to any patient lacking a matched donor or suitable UCB units (76–78). However, because of the incompatibility at three major HLA loci, it became clear that an extensive T cell depletion was strictly necessary to prevent fatal graft versus host (GvH) reactions (79).

T cell-depletion associated to high intensity immunosuppressive/myeloablative conditioning regimens and the use of very large numbers (“megadoses”) of highly purified PB-derived CD34⁺ cells resulted in: (a) the successful engraftment of HSC across the HLA barrier; (b) a very low incidence of grade II–IV acute GvH disease (GvHD), even in the absence of post-transplant prophylactic immune suppression (80–82). However, removal from the graft of mature T cells that, in HLA-matched transplants, are mainly responsible for protection from severe infections resulted in a state of immune deficiency for several months after transplantation. In order to overcome, at least in part, this major disadvantage, the adoptive infusion of T cell lines or clones specific for common life-threatening pathogens, including cytomegalovirus, Epstein–Barr virus, adenovirus, and *Aspergillus*, has been applied successfully in pilot trials (83–85). Another possible consequence of the extensive T cell depletion was a higher rate of leukemia relapses. However, milestone studies in acute myeloid leukemia (AML) adult patients receiving a haplo-HSCT revealed that the graft versus leukemia (GvL) effect was mediated by NK cells generated from donor HSC. This effect was detectable almost exclusively in patients transplanted with donors who had NK cells alloreactive toward recipient cells. These studies clearly indicated that also cells of the innate immunity, such as NK cells, may guarantee a successful clinical outcome in this transplantation setting (81, 82).

The noticeable beneficial effect of alloreactive NK cells, first assessed in adult AML, was subsequently reported in children with high risk acute lymphoid leukemia (ALL) (82, 86, 87). Indeed, the probability of leukemia relapse was very low and the survival rate was at least as good as that of patients receiving a HLA-matched sibling or unrelated donor. Notably, the NK-mediated GvL effect is separated by the occurrence of GvHD, thus clearly indicating that alloreactive NK cells kill leukemia blasts while sparing normal tissues, despite the KIR–HLA-I mismatch. In view of the favorable clinical outcome and the immediate availability of a family haploidentical donor, haplo-HSCT has been included as a valuable option for treating pediatric patients with life-threatening leukemias (88).

In haplo-HSCT, the first wave (occurring after 2–3 weeks) of NK cells derived from donor CD34⁺ HSC cells is composed of CD56^{bright} cells expressing CD94/NKG2A as the only HLA-I-specific receptor. These cells are relatively immature and display low levels of cytolytic activity. The appearance of KIR⁺ NK cells (containing the alloreactive subset) requires four to six additional weeks. Therefore, it is conceivable that an efficient NK-mediated anti-leukemic effect occurs only after this time interval from transplantation (87, 89–91) (**Figure 1C**).

Given the central role of alloreactive NK cells in preventing leukemia relapses, information on the size of the alloreactive subset in potential donors appeared particularly relevant for optimal donor selection (92). In addition, this information was crucial to assess the generation of this subset in the recipient and its persistence over time. The basic criteria applied for donor selection have been the phenotypic identification of the alloreactive NK cell subset and the assessment of the NK cytotoxicity against leukemia cells (87, 93). Cytofluorimetric analysis, using appropriate combinations of monoclonal antibodies conjugated with

different fluorochromes, allowed to identify the alloreactive subset. While only inhibitory KIRs were originally assessed, the more recent availability of mAbs, capable of discriminating between activating and inhibitory KIRs, allowed to extend the analysis to activating KIRs and to better define the size of this subset. This revealed to be particularly important for prevention of leukemia relapses, primarily in donors expressing the activating KIR2DS1, provided that patient's cells express the ligand of such activating receptor (i.e., HLA-C2 alleles) (87, 93, 94). Other selection criteria have been added that are fundamental particularly in donor–patient pairs in whom no alloreactive NK cells can be found. One is based on KIR genotype analysis, since selection of donors with KIR B haplotypes was associated with significant improvement in disease free survival in adult AML patients. This suggests that activating KIRs, particularly those located in the centromeric portion, play a positive role in GvL (95, 96). In addition, mothers were found to be better donors than fathers (97). By applying all these criteria to donor selection, the survival rate of patients receiving a haplo-HSCT is now over 70% in children with high risk, otherwise fatal, ALL.

As specified above, in haplo-HSCT, the appearance of KIR⁺ NK cells may require 6–8 weeks after donor CD34⁺ cell transplantation. Therefore, their anti-leukemia effect is relatively delayed. In case of rapidly proliferating leukemia blasts and/or of high tumor burden residual after the conditioning regimen, this delay may result in leukemic relapses as well as in impaired control of infections (74). In order to minimize this risk, donor-derived mature alloreactive NK cells, either resting or expanded *in vitro*, can be infused at transplantation or shortly after. A particularly promising approach based on the negative selection of T lymphocytes expressing the $\alpha\beta$ TCR associated with B cell depletion has recently been applied (98) (**Figure 1D**). This approach allows the accurate removal of $\alpha\beta$ T cells, responsible for the occurrence of GvHD. In addition, in this novel transplantation setting, it is possible not only to transfer to the recipient high numbers of CD34⁺ cells, but also mature NK cells and $\gamma\delta$ T cells. Thus, mature, alloreactive NK cells can promptly exert their anti-leukemia activity and prevent GvHD. A similar effect can be mediated by $\gamma\delta$ T cells in virtue of their ability to kill leukemia blasts (which express ligands recognized by NK cells and/or $\gamma\delta$ T cells). In addition, both cell types can control viral infections or reactivation that may represent life-threatening complications in these patients (99). Additional donor selection criteria can be based also on the higher proportion of NK and $\gamma\delta$ T cells in their PB. Preliminary data are particularly encouraging even against pediatric AML that were not cured efficiently by the conventional haplo-HSCT approach upon infusion of CD34⁺ cells (Locatelli et al. study in progress). An additional particularly promising approach resides in NK cell manipulation using anti-KIR mAbs (100). These mAbs, now studied in phase II clinical trials in patients with multiple myeloma or AML, can stably block KIRs and allow NK-mediated killing of autologous or HLA-matched tumor or leukemia cells, thus conferring alloreactivity to any KIR⁺ NK cell.

In conclusion, the discovery of NK cell receptors and of the NK alloreactivity represented a true revolution in allo-HSCT and in the cure of otherwise fatal leukemias.

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IFITs: emerging roles as key anti-viral proteins

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Interferon-induced proteins with tetratricopeptide repeats (IFITs) are a family of proteins, which are strongly induced downstream of type I interferon signaling. The molecular mechanism of IFIT anti-viral activity has been studied in some detail, including the recently discovered direct binding of viral nucleic acid, the binding to viral and host proteins, and the possible involvement in anti-viral immune signal propagation. The unique structures of some members of the IFIT family have been solved to reveal an internal pocket for non-sequence-specific, but conformation- and modification-specific, nucleic acid binding. This review will focus on recent discoveries, which link IFITs to the anti-viral response, intrinsic to the innate immune system.

Keywords: IFIT, innate immune system, anti-viral immune response, TPR, PAMPs

INTRODUCTION

The germline-encoded innate immune system initiates a fast and targeted response upon recognition of an invading virus. Most research on the innate immune system is focused on how the host senses and detects pathogen-associated molecular patterns (PAMPs), including viral nucleic acids. Foreign nucleic acid within the cytosol is an extremely potent PAMP, and the detection elicits a strong innate immune response (1). The host proteins that sense viral PAMPs, termed pattern recognition receptors (PRRs), range in their specific targets enormously and are located in endosomes and the cell cytosol (2–4). Some important PRRs that are specific to virus nucleic acid recognition include Toll-Like Receptors (TLRs) TLR3, TLR7, TLR8, and TLR9 (4, 5). Absent in Melanoma 2 (AIM2)-like receptors such as the AIM2 inflammasome and IFI16 (6–9), and RIG-I-like receptors including MDA5 (melanoma differentiation associated gene 5) and RIG-I (retinoic acid inducible gene I) (10, 11). The detection of various viral nucleic acid species by these receptors elicits signaling cascades that include the production of anti-viral genes and pro-inflammatory cytokines, including type I interferons (IFNs) (1). These responses slow virus replication by setting in motion a systematic anti-viral response.

Type I IFNs are comprised of IFN α and IFN β ; these are responsible for an array of biological and immunological functions [reviewed in Ref. (12)]. Type I IFN signaling is mediated via the IFN α/β receptor (IFNAR), and downstream signaling results in the upregulation of IFN-stimulated genes (ISGs) (12). Beginning with the innate immune sensing of virus infection, ISGs encode many important protective and anti-viral pathways. ISGs are directly responsible for blocking virus infection and priming pro-inflammatory and adaptive immune response systems (12).

While the ISG class is quite large and diverse, this review will focus on a family of proteins that recently emerged as having a wide range of anti-viral functions: interferon-induced proteins

with tetratricopeptide repeats (IFITs). The detection of virus infection by receptors and downstream pathways is of fundamental importance for our understanding of innate immunity processes regulating cellular homeostasis. Moreover, innate mechanism leading to inhibition of virus replication is particularly worth being investigated as they can possibly be harnessed for anti-viral therapy design.

THE IFIT PROTEIN FAMILY

The IFIT family includes four canonical human members (IFIT1, IFIT2, IFIT3, and IFIT5) and three mouse members (IFIT1, IFIT2, and IFIT3), which are induced upon simulation with IFN, virus infection, or other PAMP recognition (13, 14). Another human IFIT, IFIT1B, is thought to be expressed in a non-IFN dependent manner due to lack of an interferon-stimulated response element (ISRE), which typically are present in two to three copies within the promoters of the other IFIT genes (15, 16). IFIT5 is a paralog of IFIT1, which is absent in the murine genome. Instead, another closely related gene seems to be present in the mouse, Ifit1c (17). Moreover, two additional genes, Ifit1b and Ifit3b, are part of the mouse repertoire. IFIT homologs have been discovered in many vertebrate species: birds, fishes, and amphibians (15, 18). Their conserved role throughout evolution hints to their general importance. Since fish commonly contain multiple copies of IFIT genes that most resemble IFIT1/5, it is tempting to speculate that these IFITs are part of an ancient immune defense mechanism in vertebrates (19).

While IFITs generally are not expressed in cells at high basal levels, the transcription of IFIT genes rapidly increases during virus infection or IFNAR signaling (16). Low levels of IFIT5 expression, however, have been detected in HEK cells, which further increase several-fold upon IFN stimulation (13). The presence of the ISREs within the IFIT promoter region explains their low baseline-transcriptional levels and fast IFN-dependent induction (16).

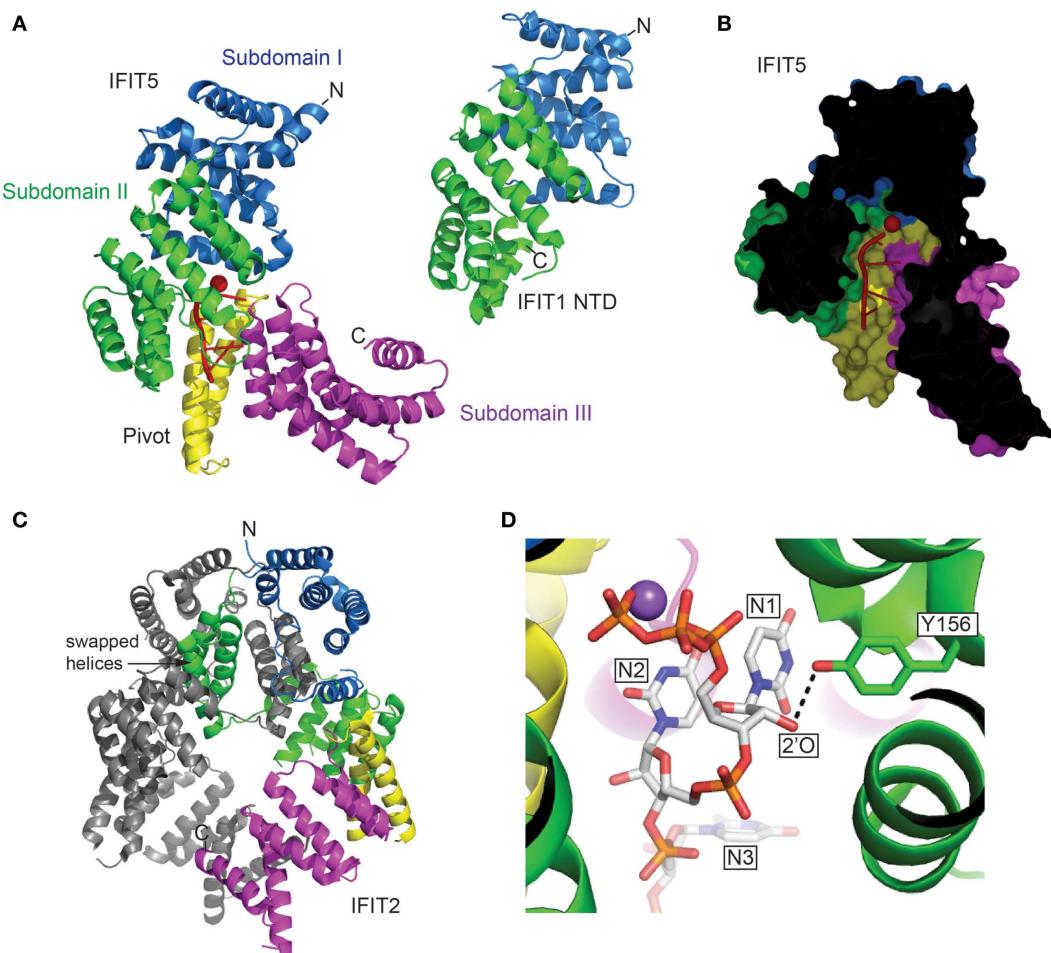


FIGURE 1 | (A) The structures of human IFIT5 in complex with oligoA and human IFIT1 NTD in cartoon representation (PDB entries 4HOT and 4HOU). The subdomains identified in IFIT5 are color coded, and RNA is in red. **(B)** Cross-section of the complex of IFIT5 with oligoA, showing a narrow pocket that binds four nucleotides. **(C)** The structure of human IFIT2 (PDB entry 4G1T) colored according to the corresponding IFIT5 subdomains. Indicated are helices 7–9 that are swapped with the other protomer. **(D)** The Y156 of IFIT5 forms a hydrogen bond (dotted line) with the 2'-O of the first ribose (IFIT5 in complex with oligoU, PDB entry 4HOS). Metal ions are depicted as spheres (Red, Mg²⁺; Purple, Na²⁺).

However, the kinetics of the transcriptional levels of specific IFITs can be cell line and tissue dependent (20, 21). Moreover, the transcriptional profile in different cell types could imply that specific IFITs have various functions during virus infections in the host.

IFIT STRUCTURE

All IFIT proteins consist of repeats of the eponymous tetra-tripeptide (TPR) motif, which typically contains 34 amino acids with the consensus sequence [WLF]-X(2)-[LIM]-[GAS]-X(2)-[YLF]-X(8)-[ASE]-X(3)-[FYL]-X(2)-[ASL]-X(4)-[PKE] that adopts a basic helix-turn-helix fold. Adjacent TPR motifs usually form a sheet of antiparallel helices that curves into a super-helix, and this unique fold presents concave and convex curved surfaces that allow for binding of diverse ligands. TPR domains are conserved in all kingdoms of life and are generally believed to serve as protein and peptide recognition domains; with the discovery of RNA-binding IFITs, the known ligand spectrum of TPR motifs is broadened to also include nucleic acids.

The recent crystallographic structures of IFIT5 and the N-terminal half of IFIT1 (NTD) (22–24) reveal that the usual TPR super-helix is interrupted by an upside-down flip of the N-terminal subdomain, but nevertheless the remainder of the protein forms a concave surface that binds the 5' end of RNA (Figure 1A). The narrow pocket can accommodate up to four nucleotides of exclusively single-stranded RNA (Figure 1B), and the C-terminal subdomain tightens slightly around the aperture upon ligand binding. Most notably is the pocket that engages the 5' triphosphate extension of the single-stranded RNA. Whereas, IFIT5 is monomeric, the structure of IFIT2 reveals a dimer (25), and IFIT1, IFIT2, and IFIT3 form homodimers in solution (13). The dimerization of IFIT2 occurs through swapping of three helices belonging to TPR motifs 3 and 4 (Figure 1C). IFIT2 might have a preference for double-stranded RNA (25), but it is not clear where the RNA-binding interface is located. With IFIT1 and IFIT5 targeting the 5' end, and IFIT2 binding the body of the RNA, the IFIT proteins have diversified in the features of the non-self

RNA that they recognize, described below, but it remains to be shown whether they complement each other and act in concert in a synergistic manner.

IFIT ANTI-VIRAL FUNCTION

Since IFITs are swiftly induced following virus infection, it is hypothesized that IFITs play a role in the anti-viral milieu of cells. Over the past years, many investigations have alluded to the important anti-viral mechanisms of each IFIT family member. Below, we will discuss the most important findings.

5'-TRIPHOSPHYLATED AND 2'-O-UNMETHYLATED CAPPED RNA BINDING

In general, cellular cytoplasmic RNAs are single stranded and contain a 5'-monophosphate or *N*-7-methylated guanosine cap linked by a 5'-to-5' triphosphate bridge to the first base: rRNAs/tRNAs and mRNAs, respectively. In higher eukaryotes, mRNA is further modified with a methylated 2'-*O* position of the first ribose (26, 27). These modifications and secondary additions assist in not only translational control, but the lack thereof plays a role in the detection of foreign nucleic acid. In contrast, viruses may form long double-stranded RNA, and/or generate triphosphorylated RNA (PPP-RNA) during their life cycle, which elicits a strong anti-viral response (28, 29). Using a proteomics approach, with PPP-RNA as bait, a mass spectrometry analysis revealed IFIT1 as a major binding partner in HEK cells (13); thereby revealing a role for IFIT1 in recognizing and potentially sequestering viral PPP-RNA, preventing it from being translated by the host machinery (Figure 2A). From the proteomic and subsequent biochemical analysis, it appeared as if only IFIT1 would bind the PPP-RNA directly while IFIT2 and IFIT3 bind IFIT1 in a multi-protein complex required for anti-viral activity (13). Knocking down IFIT1, IFIT2, and IFIT3 in HeLa cells with siRNA resulted in an increase rate of infection by viruses known to display a PPP-RNA nucleic acid species during their life cycle such as Rift Valley fever virus (RVFV), vesicular stomatitis virus (VSV), and influenza A. In contrast, growth of Encephalomyocarditis virus (EMCV), which does not produce a PPP-RNA species, was unaffected by the presence or absence of IFIT1 (13). Moreover, studies with IFIT1^{-/-} mouse fibroblasts and myeloid cells also resulted in increased replication of VSV, with no changes detected in pro-inflammatory cytokines (13). Also, other studies determined that IFIT2 protects mice from VSV neuropathogenesis (30), and IFIT3 expression in human A549 cells is required for IFN α -dependent anti-viral activity against VSV (31).

As described above, higher eukaryotes and many viral RNAs are not only methylated at the *N*-7 position, but also the 2'-*O* of the 5' guanosine cap. The lack of the latter cap, common for foreign nucleic acids, elicits a strong anti-viral response (32). Viruses lacking this 2'-*O*-methylation, such as a West Nile virus (WNV) that lacks 2'-*O*-methyltransferase activity, were unable to infect wild type cells, but could replicate in cells lacking IFIT1 expression (33, 34). Again, using proteomics, IFIT1 was discovered to have a much stronger affinity for 2'-*O* uncapped vs. capped RNA, which explains the IFIT1 mediated control of 2'-*O*-methyltransferase deficient WNV (17). The study found that IFIT1 bound 2'-*O*-unmethylated RNA, which resulted in an inhibition of translation,

and therefore decreased virus infection (Figure 2A). Human IFIT5 has also been described to bind PPP-RNA (outside of the IFIT1–IFIT2–IFIT3 complex), and also to uncapped 2'-*O*-unmethylated RNA (13, 17) (Figure 2A). IFIT1 binding of 2'-*O*-unmethylated RNA was also supported by another study that described a role for IFIT1 in controlling Japanese Encephalitis Virus (JEV) 2'-MTase mutant by binding preferentially to capped 2'-*O*-unmethylated viral mRNAs (35).

IFIT1 can possibly accommodate capped RNA due to the larger size of the binding cavity. Mutagenesis of IFIT1 in the residue, which in IFIT5 makes contact with the 2'-OH of the first nucleotide (Figure 1D), followed by pulldowns on RNA, suggests that IFIT1 should be highly sensitive to the methylation status of this moiety (22), with methylated RNA being a poor ligand. Additionally, IFIT5 seems less sensitive to the disruption of 2'-*O* binding, which enforces the notion of its high specificity for 5'PPP-RNA, since 2'-*O*-methylation usually occurs in conjunction with capping; there is also little evidence of sequence specificity in binding, as demonstrated by the structures of IFIT5 in complex with oligoU, oligoC, and oligoA (22). Furthermore, IFIT family members have been shown to effect translation by binding to mRNA with various 5'-modifications (36).

These studies nicely define a role for IFITs in the preferential binding to mis- or un-modified RNA in the cytoplasm; using a key evolutionarily conserved feature of transcriptional regulation to decipher self- vs. non-self nucleic acid.

INHIBITION OF VIRAL PROTEIN TRANSLATION

Eukaryotic cap-dependent protein translation relies on an *N*-7-methylguanoside cap at the 5' end of mRNA, compared to the 5'-PPP-modified viral RNA species described above (37). Evidence has shown that IFIT family members can lessen host cap-dependent protein translation by binding to subunits of the eukaryotic initiation factor 3 (eIF3) translation complex (38) (Figure 2B). The eIF3 protein complex is required for translation initiation in several ways, including: mRNA recruitment, scanning mRNA for the start codon, and tRNA delivery to the translation machinery (37). Human IFIT1 and IFIT2 may perhaps block function of eIF3 tRNA delivery while human IFIT2 and mouse IFIT1 and IFIT2 may block mRNA recruitment (38–40). The decrease in mRNA translation can have detrimental effects on the host, though this also yields host-dependent virus replication. Viruses can also use internal ribosome entry sites (IRESs) during their replication for cap-independent translation, which also requires eIF3 (37). It was discovered that human IFIT1 can suppress this IRES-dependent viral RNA translation during Hepatitis C virus (HCV) infection (41). These studies, for which the molecular mechanism remains to be conformed, would suggest that IFIT family members may, on top of the indirect effects through RNA engagement, also affect virus translation directly, by altering translational processes itself.

DIRECT VIRAL PROTEIN BINDING

While it has become clear that IFIT mechanism of anti-viral activity is directed through binding foreign nucleic acid, yeast-two-hybrid studies have suggested that IFITs can bind other viral proteins. IFIT1 binds to E1, a viral helicase from Human

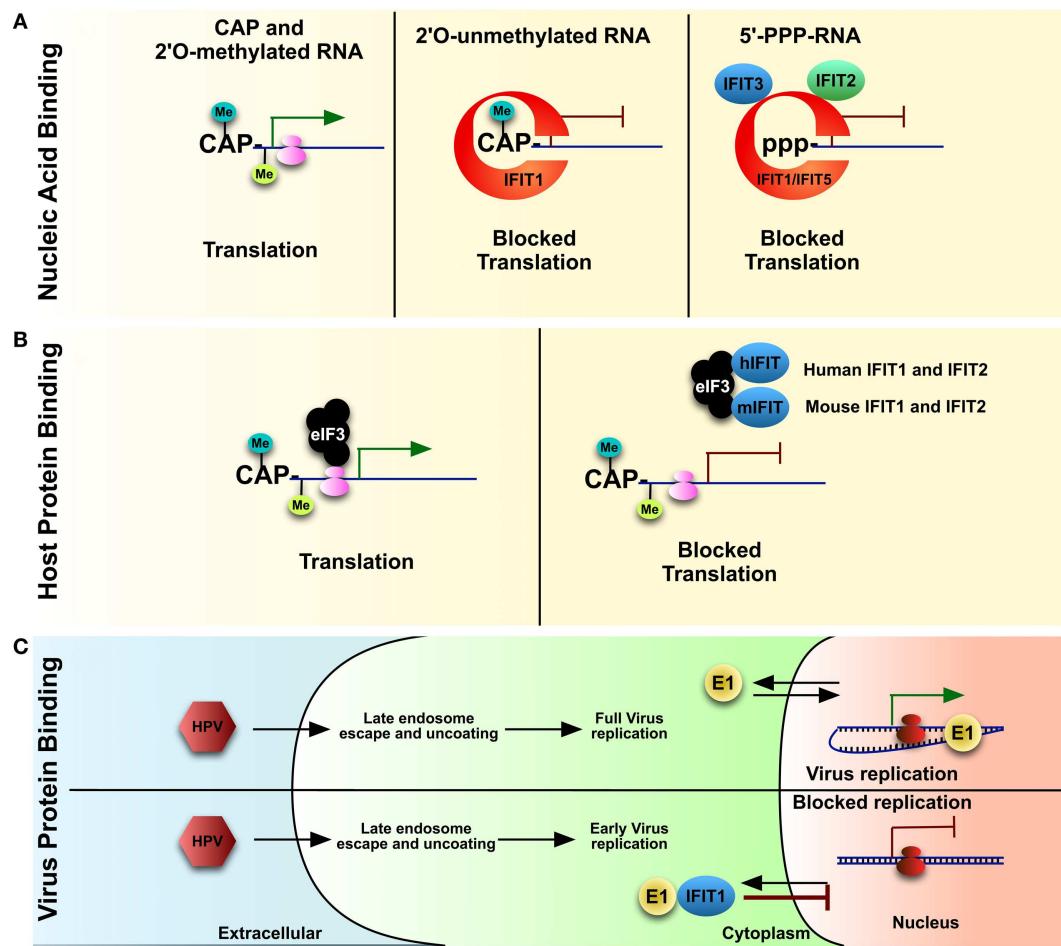


FIGURE 2 | IFITs, once upregulated due to IFN signaling, play various roles in blocking virus and host protein translation. IFIT1 can bind to the (A) 5' of mis-modified RNA either which is 2'-O-unmethylated or has 5'-PPP-RNA (features of foreign nucleic acid) vs. properly capped host mRNA. IFIT1, along with a complex of IFIT2 and IFIT3, can block translation of these nucleic acids. Multiple IFITs have been described to

bind to various subunits of host (B) eIF3, a key component of mRNA translation. Though the target of eIF3 is properly processed mRNA, including host mRNA, IFIT1 may block translation cell-wide during virus infection. As well, IFIT1 can bind (C) a key virulence factor of HPV, helicase E1, and sequester it into the cytoplasm, thereby preventing virus replication.

papillomavirus (HPV), which is required for replication (42, 43). IFIT1 binds E1 and sequesters it within the cytoplasm, preventing it from aiding in viral replication within nucleus (Figure 2C). This was supported using a HPV virus expressing an E1 helicase with a deleted F399amino acid residue, which was required for IFIT1 binding; the resulting virus had no loss of replication (43).

IFIT ROLE IN ANTI-VIRAL SIGNAL PATHWAY TRANSDUCTION

As well as being described as effector proteins in anti-viral replication, IFITs may also control downstream signaling, though some controversies exist. Pichlmair et al., who originally described the role of IFIT1 in binding PPP-RNA, noticed no decrease in type I IFNs produced in mouse fibroblasts, macrophages, or dendritic cells lacking IFIT1 (13). Later, IFIT1 was proposed as one of many innate immune “bottlenecks” and that the knocking down of IFIT1 resulted in decreased pro-inflammatory responses after LPS treatment of cells (44). In determining IFIT-mediated immune

pathways, a role for IFIT3 to interact with TBK1 (TNFR-associated factor family member-associated NF- κ B activator-binding kinase 1), an important innate immune modulating kinase (45), was outlined. This interaction of IFIT3 with TBK1 bridges the kinase with mitochondrial anti-viral signaling (MAVS) on mitochondria; over-expression or knock down of IFIT3 resulted in the increase or decrease of anti-viral gene expression, respectively.

In contrast, groups have also reported immune suppressive function of IFITs. By over-expressing IFIT2 in mouse macrophages, Berchtold et al. observed reduced LPS-induced expression of multiple pro-inflammatory cytokines including TNF and IL-6 (46). This was associated with reduced mRNA stability of the cytokine transcripts in the presence of increased IFIT2, suggesting post-transcriptional regulation of inflammatory responses (46). This phenomenon, however, could be due to the natural function of IFIT family members to bind RNA and therefore an over-expression could cause intrinsic cellular issues, as well

as cell growth defects (47). Furthermore, IFIT1 and IFIT2 were proposed to interact with stimulator of IFN genes (STING) (48), which recruits TBK1 (described above to bind IFIT3 by Liu et al.) and propagates phosphorylation of the transcription factor IRF3 (IFN regulatory transcription factor 3), activation of NF- κ B, and IFN production together with MAVS (49, 50). However, in this case, over-expression of IFIT1 in HEK cells resulted in a decrease of IRF3 activation and IFN β promoter activation in response to Sendai virus. Here, IFIT1 was described to disrupt the interaction of STING with MAVS or TBK1 (48). Given the conflicting results in pathway propagation and downstream immunological effects, more investigative work must be done on the individual IFITs, both *in vitro* and *in vivo*, in order to draw conclusions. Moreover, the extensive characterization of protein complexes formed by IFIT family members in various cell lines using affinity purification and mass spectrometry has failed to confirm any of these interactions.

CONCLUSION

The IFIT family of proteins has recently been described as major players in anti-viral innate immunity, and their huge cellular abundance within the cell after ISG induction underscores their importance. Currently, the molecular mechanism for which high-resolution structure evidence exists, clearly defines a mechanistic role of IFITs by binding to foreign nucleic acid: interfering with viral processes, which expose a foreign 5' configurations of RNA, such as protein translation. However, more work must be focused on determining the fate of the bound RNA: Are IFITs trafficking foreign nucleic acid for destruction, or is the natural turnover of IFIT naturally ridding the cell of foreign material? Moreover, greater detailed investigation of IFIT effect on host-translational machinery could lead to understanding of IFIT function in absence of virus infection. Following-up on these unanswered questions will better allow us to harness the potential activity of IFITs for anti-viral treatments by exploiting the direct effect of halting transcription of foreign nucleic acid, independently of sequence.

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Importance of both innate immunity and acquired immunity for rapid expulsion of *S. venezuelensis*

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In the first part of this review, we described the relevant roles of endogenous IL-33 for accumulation of ILC2 and eosinophils even in the lungs of Rag2^{-/-} mice. Type II alveolar epithelial (ATII) cells express IL-33 in their nucleus and infection with *Strongyloides venezuelensis* induces IL-33 production by increasing the number of ATII cells possibly by the action of chitin. IL-33 from ATII cells induces ILC2 proliferation and at the same time activates them to produce IL-5 and IL-13, which in combination induce lung eosinophilic inflammation, aiding to expel infected worms in the lungs. In the second part, we showed that, although AID^{-/-} mice normally develop Th2 cells and intestinal mastocytosis after infection with *S. venezuelensis*, they need adoptive transfers of immune sera from *S. venezuelensis* infected mice to obtain the capacity to promptly expel *S. venezuelensis*. Thus, intestinal nematode infection induces various Th2 immune responses (e.g., Th2 cell, ILC2, goblet cell hyperplasia, intestinal mastocytosis, smooth muscle cell contraction, local and systemic eosinophilia, and high serum level of IgE and IgG1). However, all of them are not necessary for rapid expulsion of intestinal nematodes. Instead, some combinations of Th2 immune responses are essentially required.

Keywords: intestinal nematode, Th2 cell, ILC2, IgE, mast cell, eosinophils, IL-33, chitin

When animals are infected with intestinal nematodes, resistant hosts develop Th2 immune responses, which induce IgG1 and IgE production, intestinal mastocytosis, pulmonary eosinophilia (e.g., Loeffler syndrome), and systemic eosinophilia. *Nippostrongylus brasiliensis* is a gut-dwelling nematode. Goblet cell hyperplasia and intestinal smooth muscle contraction, both of which are induced by the action of Th2 cytokines (IL-4 and IL-13), are indispensable for rapid expulsion of *N. brasiliensis* (1, 2). However, B cells and antibody (Ab) production are not needed for this expulsion (3). Thus, host animals expel *N. brasiliensis* in a T cell but not B cell-dependent manner.

Strongyloides venezuelensis, a counterpart of human pathogen *Strongyloides stercoralis*, naturally infects rodents and has been used as an experimental intestinal parasite model (4). In contrast to *N. brasiliensis* expulsion, intestinal mastocytosis is indispensable for rapid expulsion of *S. venezuelensis* (5–7). Furthermore, FcRy-induced mucosal mast cell (MMC) activation is important for rapid expulsion of *S. venezuelensis* (8), suggesting that Ab-dependent MMC activation is essential for rapid expulsion of *S. venezuelensis* from intestine.

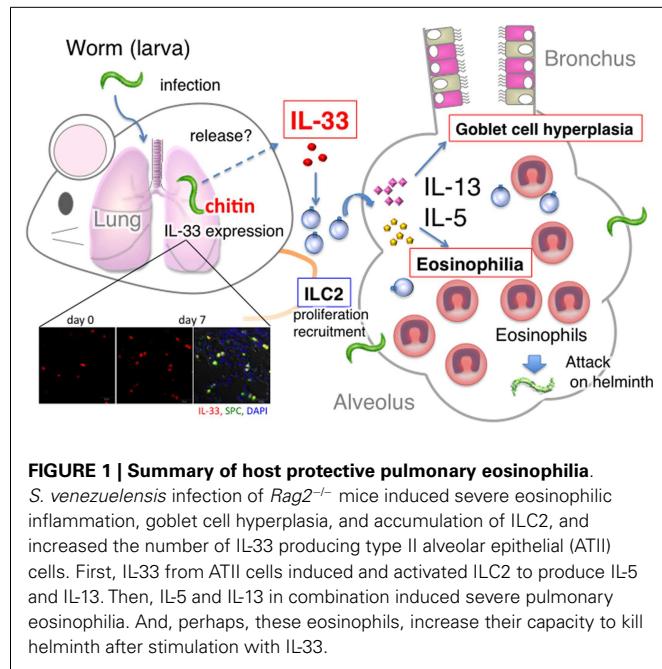
In the life cycle of *S. venezuelensis*, third stage larvae (L3) migrate to the lung, where they induce severe inflammatory change (mouse Loeffler syndrome), characterized by severe eosinophilic infiltration and goblet cell hyperplasia. Then, they leave lungs, migrate to oral cavity, and go down to small intestine, where they become adult worms and induce severe intestinal mastocytosis. Host animals try to expel them by the action of mast cells in intestine. Therefore, there are two inflammatory sites; one in the lung and the other in the intestine. We

speculated that these two sites are important for protection against intestinal nematode. Here, we show the importance of innate immunity and acquired immunity for rapid expulsion of *S. venezuelensis*.

IS LOEFFLER SYNDROME A PROTECTIVE IMMUNE RESPONSE?

We first examined the mechanism how *S. venezuelensis* infection induces pulmonary eosinophilia. Loeffler syndrome is severe pulmonary eosinophilia, and parasite-infected patients often develop this syndrome (9). However, we still do not know why only lungs develop such severe eosinophilic inflammation after infection with intestinal nematodes, such as round worms, hook worms, and *Strongyloides* spp. (9). To understand this mechanism, we used *S. venezuelensis* infected-animal model. As intranasal administration of IL-33 induces severe pulmonary eosinophilia and goblet cell hyperplasia in the lungs of animals (10), we speculated that *S. venezuelensis* infection induces Loeffler syndrome in an IL-33-dependent manner (11).

IL-33 is a member of IL-1 family cytokine (12), stored in the nucleus of cells (13), released when cells are damaged (14), and binds to ST2 (IL-1RL1) on Th2 cells and various types of innate immune cells including mast cells, basophils, eosinophils, and group 2 innate lymphoid cells (ILC2s) (10, 15–19). In the first part of this review, we demonstrate that worms increase IL-33 expression in the lung, which in turn not only induces the accumulation of ILC2s in the lung but also stimulates them to produce IL-5 and IL-13, which in combination induce pulmonary eosinophilia.



S. VENEZUELENESIS INFECTION FAILED TO INDUCE LUNG EOSINOPHILIA IN IL33^{-/-} MICE

Wild type (WT) C57BL/6 mice, infected with *S. venezuelensis*, developed pulmonary eosinophilia and lung goblet cell hyperplasia at days 5 and 7 after infection. In contrast, *S. venezuelensis* infected IL-33^{-/-} mice failed to develop these changes. These results strongly indicated that *S. venezuelensis* infection induced lung eosinophilic infiltration and goblet cell hyperplasia by induction of IL-33. Therefore, we next tried to determine what type of cells express IL-33. We detected IL-33-expressing cells even before infection. Their number increased and peaked at day 7. We could determine these IL-33-expressing cells as type II alveolar epithelial (ATII) cells, because they are positive for ATII cell marker Pro-Surfactant protein C (Figure 1). Other investigators also reported that influenza virus infection induces IL-33 expression in alveolar epithelial and endothelial cells (20). Influenza virus infection also induces IL-33 expression in the alveolar macrophages (21). However, we could not detect IL-33 expression in F4.80⁺ macrophages in the lungs, suggesting selective activation of ATII cells by *S. venezuelensis*.

INTRANASAL ADMINISTRATION OF CHITIN INDUCES IL-33 IN THE LUNG

Chitin is a component of the outer membrane of parasites (22), and intranasal administration of chitin beads induces eosinophilic accumulation in the lungs by the action of macrophage and leukotriene B₄ (23). Thus, we speculated *S. venezuelensis* infection induced pulmonary eosinophilia by the action of chitin. We administered chitin into WT mice and IL-33^{-/-} mice, and found that this treatment increased the number of IL-33-expressing ATII cells and IL-33 protein level in the BALF of WT mice. Expectedly, only WT mice developed pulmonary eosinophilia after chitin treatment, suggesting that *S. venezuelensis* infection induces pulmonary

eosinophilia at least by the action of chitin to induce an increase in the number of IL-33-expressing ATII cells.

INDUCTION OF ILC2 IN THE LUNGS BY *S. VENEZUELENESIS* INFECTION

We next examined whether *S. venezuelensis* infection induces pulmonary eosinophilia without help from Th2 cells. Thus, we infected WT and *Rag2^{-/-}* mice with *S. venezuelensis*. Both types of mice after infection almost equally increased the number of IL-33-expressing ATII cells and developed lung eosinophilia, indicating that acquired immune cells are dispensable for IL-33-induced eosinophil accumulation in the lungs. IL-33 is a potent inducer of IL-5 and IL-13, which are strongly related with the accumulation of eosinophils (24). Th2 cell is a candidate for the source of these cytokines, however, as we showed previously (10), intranasal administration of IL-33 induces pulmonary eosinophilia even in *Rag2^{-/-}* mice, excluding contribution of Th2 cells to lung eosinophilic inflammation. ILC2 is another candidate for Th2 cytokine-producing cell in response to IL-33 (25). Therefore, we examined whether *S. venezuelensis* infection induced ILC2s in the lung. We found that *S. venezuelensis* infection induced ILC2s in the lungs of *Rag2^{-/-}* mice. ILC2s in the BALF started to increase at least at day 7 and increased even beyond day 10. Compared to WT mice, ST2 deficient mice showed little induction of ILC2s. IL-33^{-/-} mice also showed very modest increase of ILC2s. And, administration of IL-33 strikingly increased this proportion (11, 26).

PULMONARY EOSINOPHILA IS INVOLVED IN HOST DEFENSE AGAINST *S. VENEZUELENESIS* INFECTION

Consistent with the above results, IL-33^{-/-} mice developed modest eosinophilia in their lungs, but they became to develop severe pulmonary eosinophilia after IL-33 treatment. Thus, we examined the contribution of IL-33-induced eosinophilia to worm expulsion. We measured their egg deposition. Compared to IL-33^{-/-} mice, WT mice significantly reduced their egg deposition at day 8. Importantly, IL-33^{-/-} mice could reduce their egg deposition in response to IL-33 treatment. Interestingly, they more rapidly reduced egg deposition than PBS-treated WT mice. These results are consistent with previous reports that eosinophils are required for the rapid expulsion of larvae, which is demonstrated by anti-IL-5 Ab-treated mice and IL-5 transgenic mice (27, 28). The importance of eosinophils in nematode protection was also suggested by a functional study of leukotriene B₄ in *S. venezuelensis* infected mice. Numbers of adult worms and eggs/g/feces were greater in 5-lipoxygenase^{-/-} mice or in WT mice treated with leukotriene synthesis inhibitor (MK886) than that in their WT control mice or in PBS-treated WT mice, respectively (29).

Taken together, worms induced IL-33 in the lung by the action of chitin. IL-33 from ATII cells induces and activates ILC2s to produce IL-5 and IL-13, which in combination induce pulmonary eosinophilia and possibly kill helminth (Figure 1). Thus, ILC2s in the lung play an important role in host defense against helminth.

DOES IgE ACT IN CONCERT WITH IgG TO EXPEL *S. VENEZUELENESIS*?

Th2 cells induced goblet cell hyperplasia and smooth muscle contraction are essential for rapid expulsion of *N. brasiliensis* (1, 2).

However, B cells and antibodies are not required for this expulsion (3). In contrast, Ab-dependent MMC activation is essential for rapid expulsion of *S. venezuelensis* from intestine (8). Thus, host deploys a subset of immune response to expel intestinal nematode, which differs depending on the nature of helminth. As Th2 cells induce various immune responses (e.g., goblet cell hyperplasia, intestinal mastocytosis, local and systemic eosinophilia, and high production of IgG1 and IgE) in mice infected with intestinal nematodes, we tried to determine which components of Th2 immune responses are essentially required for rapid expulsion of *S. venezuelensis*. Thus, in the last part of this review, we show the importance of acquired immunity for rapid expulsion of *S. venezuelensis* from intestine.

RELEVANCE OF INTESTINAL MASTOCYTOSIS AND Abs AGAINST MURINE STRONGYLOIDIASIS

The relevance of intestinal mastocytosis has been well documented in the host defense against murine Strongyloidiasis (5–7, 30). We previously reported that administration of IL-18 induces intestinal mastocytosis and such IL-18-pretreated mice gain the capacity to strongly expel implanted adult worms (7). We further demonstrated that identically pretreated mast cell-deficient WBB6F1-W/W^v failed to acquire this capacity. These results strongly indicate that intestinal mastocytosis is required for rapid expulsion of *S. venezuelensis* (7). However, other investigators suggested that induction of intestinal mastocytosis is not sufficient and that FcR γ -induced MMC activation is essentially required for rapid expulsion of *S. venezuelensis* (8). Abraham's group demonstrated the contribution of Abs (IgM and IgG) and complement to the protection of mice against larval infection with *S. stercoralis* (31, 32). But, because human pathogen *S. stercoralis* can not grow into adult worms in the mice, the role of Abs against adult worms have not been examined in detail.

AID^{−/−} MICE DEVELOP Th2 CELLS BUT LACK THE CAPACITY TO EXPEL *S. VENEZUELENSIS*

To solve these issues, we employed activation-induced cytidine deaminase (AID)-deficient mice devoid of Ig class switching (33). *AID*^{−/−} mice produce more IgM than do WT mice, but lack IgA, IgG, and IgE (34). Although WT mice showed strong production of IgG1 and IgE following *S. venezuelensis* infection, *AID*^{−/−} mice were unable to produce IgG1 and IgE. WT mice completed expulsion of *S. venezuelensis* by day 12, whereas *AID*^{−/−} mice required additional 9 days to do so. IL-4 production *in vivo*, which was detected by the *in vivo* cytokine capture assay (35), did not differ between WT mice and *AID*^{−/−} mice at day 10 after infection. Adult worms of *S. venezuelensis* found in the small intestines of *AID*^{−/−} mice outnumbered those of WT mice at day 10 after infection. In contrast, like WT mice, *AID*^{−/−} mice completed expulsion of *N. brasiliensis* by day 10 after infection, excluding contribution of IgG1 and IgE for expulsion of *N. brasiliensis*. The accumulations of mast cells in *AID*^{−/−} intestines outnumbered those in WT controls at day 14 after infection. *AID*^{−/−} mice also produced comparable levels of mouse mast cell protease-1 (mMCP-1) to those by WT mice at day 10 after infection, and produced twice as much as did WT mice at day 14 after infection. These data indicated that although Th2-type immune responses and MMC proliferation occurred normally in *AID*^{−/−} mice, worm expulsion was retarded compared to WT mice due to their no production of class-switched antibodies.

FcR γ -INDUCED MMC ACTIVATION IS ESSENTIALLY REQUIRED FOR RAPID EXPULSION OF *S. VENEZUELENSIS*

Thus, we investigated the capacity of immune sera from mice infected with *S. venezuelensis*. *AID*^{−/−} mice transferred with *S. venezuelensis*-immune sera diminished egg deposition in the feces at day 11, which was 11 days earlier than the normal

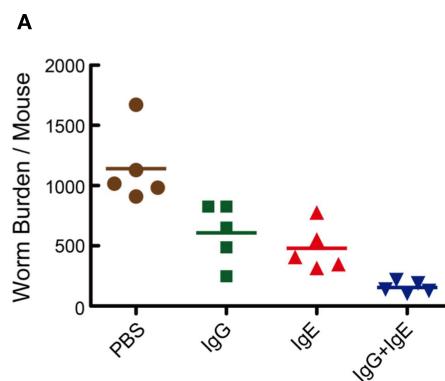
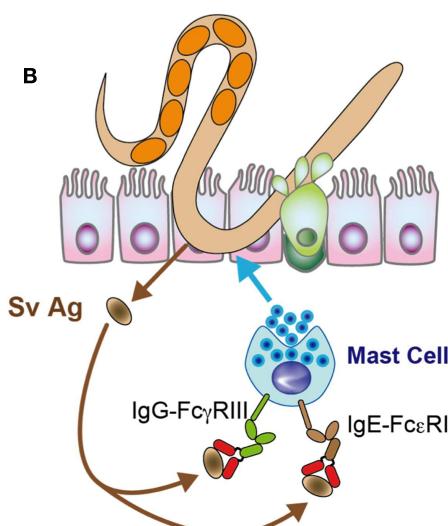


FIGURE 2 | IgG and IgE collaboratively accelerate expulsion of *S. venezuelensis* infection. (A) To examine which classes of Ig are able to induce worm expulsion. We injected IgG Fr (1.8 mg), IgE Fr (5 μ g), or a mixture of IgG Fr (1.8 mg) and IgE Fr (5 μ g) into *AID*^{−/−} mice



on day 7 after infection with 4,000 L3, and adult worms were recovered at day 8. As shown here, IgG and IgE reduced worm burdens collaboratively. **(B)** Hypothetical mechanism of IgG- and IgE-mediated worm expulsion.

sera-treated AID^{-/-} mice. Consistently, AID^{-/-} mice transferred with *S. venezuelensis*-immune sera almost completely expelled worms. These effects were due to expulsion of adult worms from the small intestines, but not due to the suppression of fecundity. Because *N. brasiliensis*-immune sera exerted no activities of eliciting worm expulsion, specific Abs against *S. venezuelensis* could play essential roles in expelling worms from the small intestine. Immune sera-derived IgG and IgE induced worm expulsion via Fcγ receptor III (FcγRIII) and Fcε receptor I (FcεRI), respectively. Although FcγRIII^{-/-} mice or FcεRIα^{-/-} mice could normally expel *S. venezuelensis*, FcγRIII^{-/-} mice, when their IgE was neutralized by anti-IgE, or FcεRIα^{-/-} mice, when their IgG-binding to FcγRIII was blocked by anti-FcγRIII, showed markedly reduced ability to expel *S. venezuelensis*. Additionally, combined administration of IgG and IgE showed a collaborative effect on *S. venezuelensis* expulsion (Figure 2A). These data revealed that IgG and IgE played redundant roles but acted in concert to accelerate *S. venezuelensis* expulsion. IgG or IgE was not able to promote worm expulsion in mast cell-deficient WBB6F1-W/W^v mice, indicating that mast cells are cellular targets of IgG and IgE.

In summary, *S. venezuelensis* infection induced IgG and IgE activate FcγRIII and FcεRI, respectively, expressed on mast cells. Then, mast cells get fully activated and expel *S. venezuelensis* promptly from the small intestine (Figure 2B). Recently, El-Malky et al. also reported the importance of B cells in immunity against surgically transferred adult worms of *S. venezuelensis* using B cell-deficient JHD mice (36).

In this review, we showed that acquired lymphoid cells, exemplified by Th2 cells and B cells and innate lymphoid cells, exemplified by ILC2s, are important for rapid expulsion of intestinal nematode. First, IL-33-induced ILC2s induce pulmonary eosinophilia (Loeffler syndrome), which is the lung-based first defense line for *S. venezuelensis*. Second, Th2 cells induce intestinal mastocytosis and Ab production by B cells (e.g., IgG and IgE), which in combination build up the second defense line based on IgG1 plus IgE-mediated MMC activation in the small intestine.

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The dopaminergic system in autoimmune diseases

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Bidirectional interactions between the immune and the nervous systems are of considerable interest both for deciphering their functioning and for designing novel therapeutic strategies. The past decade has brought a burst of insights into the molecular mechanisms involved in neuroimmune communications mediated by dopamine. Studies of dendritic cells (DCs) revealed that they express the whole machinery to synthesize and store dopamine, which may act in an autocrine manner to stimulate dopamine receptors (DARs). Depending on specific DARs stimulated on DCs and T cells, dopamine may differentially favor CD4⁺ T cell differentiation into Th1 or Th17 inflammatory cells. Regulatory T cells can also release high amounts of dopamine that acts in an autocrine DAR-mediated manner to inhibit their suppressive activity. These dopaminergic regulations could represent a driving force during autoimmunity. Indeed, dopamine levels are altered in the brain of mouse models of multiple sclerosis (MS) and lupus, and in inflamed tissues of patients with inflammatory bowel diseases or rheumatoid arthritis (RA). The distorted expression of DARs in peripheral lymphocytes of lupus and MS patients also supports the importance of dopaminergic regulations in autoimmunity. Moreover, dopamine analogs had beneficial therapeutic effects in animal models, and in patients with lupus or RA. We propose models that may underlie key roles of dopamine and its receptors in autoimmune diseases.

Keywords: dendritic cell, regulatory T cell, Th17, Crohn's disease, multiple sclerosis, ulcerative colitis, systemic lupus erythematosus, rheumatoid arthritis

Regulation of the immune system's activities by other organs occurs primarily through interactions with receptors expressed on immune cells. Over the last years, studies of the neuroendocrine and immune systems have indicated that neuropeptides, neurotransmitters, hormones, and cytokines, as well as their respective receptors, can be used as common mediators in a neuroendocrine-immune network, allowing the body to mount proper responses to changes of the internal environment and external insults (1). Interactions between the nervous and immune systems occur through the hypothalamic–pituitary axis and through sympathetic/parasympathetic innervations of primary and secondary lymphoid organs. Remarkably, several neurotransmitter receptors, including cholinergic receptors are expressed by human peripheral blood lymphocytes (2). Among the critical transmitters involved in neuroimmunological connections are catecholamines produced by sympathetic-adrenergic termini, which can release both dopamine and/or norepinephrine.

Several studies have shown that immune system cells can be regulated by dopamine acting on immune cells expressing dopamine receptors (DARs) present on the surface of T cells, dendritic cells (DCs), B cells, NK cells, neutrophils, eosinophils, and monocytes (3, 4). The presence of these receptors on immune cells suggests that dopamine plays a physiological role in the regulation of the immune response and that its deregulation could be involved in the development of autoimmunity and, even, cancer (4, 5).

Furthermore, it implies that different physiological or pathological processes in the nervous system could be involved in the regulation of immune response. On the other hand, several studies show that certain immune cells can synthesize and store dopamine in intracellular vesicles and, upon specific stimuli, release it (6), suggesting that dopamine operates as a bidirectional mediator between nervous cells and immune cells. Here, we discuss the involvement of the dopaminergic system in the pathogenesis of autoimmune diseases.

NEUROIMMUNE INTERACTIONS IN CENTRAL NERVOUS SYSTEM HOMEOSTASIS

Immune cells can be exposed to dopamine from several sources. Plasma dopamine levels, which fluctuate depending on autonomic nervous system activity and which are altered in various diseases, are probably the primary source of dopamine available to immune cells present in the blood stream (6). In this regard, chromaffin cells in suprarenal glands may be the main source of plasma dopamine when stimulated by the autonomic nervous system (7). Another peripheral source of dopamine is the gut (8). Gastrointestinal (GI) dopamine is produced by aromatic amino acid decarboxylase (AAADC) expressed in epithelial cells in the gut lumen and luminal L-DOPA (9). There are three additional sources of dopamine: immune cells synthesizing and releasing dopamine, the peripheral nervous system (PNS), and the central nervous system (CNS).

The CNS parenchyma and cerebrospinal fluid (CSF) can be important sources of dopamine for immune cells. Entrance of immune system cells in the CNS parenchyma is restricted primarily by the brain–blood barrier (BBB) that surrounds parenchymal venules. The BBB is comprised of a first layer of endothelial cells interconnected by tight junctions, which are surrounded by a basement membrane, and an outer layer constituted by astroglial endfeet (10). Under physiological conditions, no immune cells are found in the CNS parenchyma. However, some immune cells may infiltrate into the CSF, which is produced by choroid plexus epithelia and flows into the subarachnoid space. Importantly, the CSF drains into cervical lymph nodes, enabling peripheral immune cells to recognize and respond to CNS antigens in the absence or presence of inflammation (11, 12). Accordingly, the subarachnoid space and choroid plexus of healthy mice contain substantial numbers of T cells and are heavily populated by myeloid cells, including DCs (13). Importantly, under normal conditions, immune cells found in the subarachnoid space are involved in surveillance of the CNS. When activated by environmental/psychological stimuli, they can regulate nervous system processes, such as memory consolidation, hippocampal long-term potentiation (LTP), neurogenesis, and psychological stress (14). In this regard, recent studies have demonstrated that spatial memory and learning in healthy animals are regulated by the presence of CD4⁺ T cells in the subarachnoid space (15, 16). Whereas recombination-activating gene 2 (RAG2) knockout mice (RAG2KO), devoid of mature T cells and B cells, show impaired performance in Morris-water maze (MWM) tasks, μMT mice (deficient in B cells) display normal learning and spatial memory, indicating that T cells, but not B cells, are required to acquire memory (16). Moreover, class II MHC-deficient mice, which are deficient in CD4⁺ T cells, but whose CD8⁺ T cell compartment remains functionally intact, display significant impairment in MWM tasks (16). Along the same lines, RAG1KO mice, which are, like RAG2KO mice, deficient in T and B cells, improve their learning capability in MWM tasks upon transfer of wild-type (WT) CD4⁺ T cells (16), further indicating the important contribution of the CD4⁺ T cell compartment to the acquisition of spatial memory. Importantly, pharmacological interventions of WT mice with drugs that avoid entrance of T cells into the meningeal compartment, such as FTY720 or anti-VLA4 antibody, impair acquisition of spatial memory (15). In particular, IL-4-producing T cells accumulate in the subarachnoid space during cognitive tasks. In turn, IL-4 produced by Th2 cells stimulates hippocampal astrocytes to produce and release brain-derived-neurotrophic-factor (BDNF) and probably other as yet unidentified factors, which act subsequently on hippocampal neurons, favoring spatial memory and learning (15). Such studies demonstrated an impaired learning in mice lacking CD4⁺ T cells or in mice bearing IL-4-deficient CD4⁺ T cells. In both cases, normal learning is recovered when WT CD4⁺ T cells were transferred, thus linking immune activity to steady-state cognitive functions. More recently, it has been shown that transfer of monoclonal transgenic CD4⁺ T cells bearing T cell receptors (TCRs) specific for CNS antigens into RAG1KO mice, but not CD4⁺ T cells bearing an ovalbumin-specific TCR, leads to improvement of the animal's capacity to acquire normal learning and spatial memory (16, 17). Similar to the contribution of CD4⁺ T cells to acquisition of spatial

memory, there is growing evidence indicating that immunity to self-antigens contributes to psychological stress resilience (18). Thus, the evidence suggests that T cells recognizing self-antigens from the CNS are necessary to CNS homeostasis. The importance of T cells in cognitive functions is also supported by observations made during aging, HIV infection, and chemotherapy, conditions associated with decreased or impaired T cell functions, and cognitive impairments (14, 17). Potential dopaminergic regulation of T cells and myeloid cells in the meningeal compartment remains to be explored.

IMMUNE REACTIONS DURING NEUROINFLAMMATION AND NEURODEGENERATION

As noted above, neuroimmune interactions in the CNS take place in steady-state conditions. Additionally, the presence of infiltrating immune cells in the CNS parenchyma has been detected in most neurodegenerative diseases studied (19). In a pathological scenario involving the CNS, such as neurodegeneration or imbalance of glial homeostasis, initial neuroinflammatory processes induce brain endothelial cells to express a specialized pattern of adhesion molecules on the cell surface. Adhesion molecules induced by inflammatory processes subsequently allow activated T cells to adhere to vessel walls and to be recruited into the CNS parenchyma. Presumably, T cells that infiltrate the CNS have previously been activated in the periphery, in cervical lymph nodes (12, 16). Once in the CNS parenchyma, infiltrating T cells can contribute to regulate the neurodegenerative process by the secretion of different cytokines and the recruitment of innate immune cells. Additionally, molecules derived from immune cells can act over glial cells, modulating microglia phenotype and function, including M1-like microglia, which mediates neurotoxicity, and M2-like microglia, which promotes neuroprotection (20). In this regard, recent studies have shown that peripheral T cells infiltrate into the brain parenchyma at the site of neuronal injury in Parkinson's disease, where they play a fundamental role in neurodegeneration (21–23). This T cell-mediated immune response contributes significantly to the destruction of dopaminergic neurons, through a CD4⁺ T cell-dependent cytotoxic mechanism. These studies support the involvement of pathogenic CD4⁺ T cell populations in the acquisition of an M1-like pro-inflammatory phenotype by microglia characterized by the secretion of inflammatory factors, such as TNF-α, IL-1β, and superoxide (20). Importantly, dopamine receptor D3 (DAR3) expressed in CD4⁺ T cells is fundamental for promoting destruction of dopaminergic neurons in the substantia nigra in a mouse model of Parkinson's disease (22). As a result, DAR3-deficient (DAR3KO) mice are resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease. However, when WT CD4⁺ T cells were transferred into DAR3KO mice, they acquired the capability to respond to MPTP-induced neurodegeneration. On the other hand, RAG1KO mice, which are resistant to MPTP-induced Parkinson's disease, acquire the capacity to respond to MPTP-induced neurodegeneration when WT, but not DAR3KO, CD4⁺ T cells were transferred (22). Furthermore, CD4⁺ T cells that infiltrate in the substantia nigra during MPTP-induced Parkinson's disease produced high levels of IFN-γ and TNF-α, two cytokines that act synergistically in microglia to promote the inflammatory M1-like phenotype (24).

Thus, these findings point toward an important role of CNS-derived dopamine in the regulation of T cell-mediated immunity during neuroinflammation. Conversely, other T cell subsets, i.e., regulatory T cells (Tregs) and Th2 cells, could contribute to microglial acquisition of an M2-like anti-inflammatory phenotype, releasing neurotrophic factors, such as IGF-1, and promoting neuronal protection (20, 23). In contrast to the role of CNS-infiltrating T cells in Parkinson's disease, studies carried out in amyotrophic lateral sclerosis (ALS) have shown that absence of T cells accelerates motoneuron disease while adoptive transfer of T cells ameliorates disease severity (19). These results could be explained by the contribution of T cells to the acquisition of M2-like phenotype by microglia. Consistently, both Treg numbers and FoxP3 protein expression were reduced in rapidly progressing ALS patients and inversely correlated with progression (25). Interestingly, expression of two Th2 molecular markers, GATA3 and IL-4, was also decreased in peripheral T cells in rapidly progressing patients and inversely correlated with progression rates (25). Thus, an imbalance of neuroimmune interactions may constitute an important component of the pathogenic mechanisms involved in neurodegenerative disorders regulating the onset and progression, and dopamine may be a key mediator in these neuroimmune interactions, such as demonstrated for Parkinson's disease (22).

SYMPATHETIC NERVOUS SYSTEM-MEDIATED REGULATION OF IMMUNITY

An important peripheral source of dopamine and other neurotransmitters is the innervation of primary and secondary organs by the sympathetic nervous system (SNS). For example, dopaminergic terminals have been detected in thymus, spleen, and lymph nodes where the SNS seems to play a role in regulation of T cell-mediated responses (26). The relevance of SNS-mediated regulation of immunity has been demonstrated by several studies that were mainly carried out in mice that received 6-hydroxydopamine, a neurotoxic drug that selectively ablates noradrenergic and dopaminergic neurons. This latter molecule is captured specifically through dopamine transporters (DAT) or norepinephrine transporters (NET), and, when administered systemically, it cannot cross the BBB. As a result, 6-hydroxydopamine depletes the SNS without affecting the CNS. NE is the main neurotransmitter released by the SNS and, thereby, this is probably the main neurotransmitter responsible for SNS-mediated regulation of immunity, although dopamine has also been involved in SNS-mediated regulation of T cell responses. The evidence points to a dual role of the SNS in T cell responses. First, the SNS inhibits TGF- β production in the spleen and lymph nodes, thereby attenuating generation and function of Tregs. Thus, sympathectomy attenuates disease severity of mice with collagen-induced arthritis (CIA) or experimental autoimmune encephalomyelitis (EAE) as compared with animals bearing intact SNS (27, 28). Second, by stimulating β 2-adrenergic receptors, the SNS attenuates immunogenicity of antigen-presenting cells (APCs) in secondary lymphoid organs, decreasing the potency of both Th1 response and CD8 $^{+}$ T cell-mediated cytotoxicity. This attenuation seems to alter the immune response during infection with influenza virus (29) or *Listeria monocytogenes* (30). In both cases, sympathectomized mice mount a stronger antiviral or antibacterial response than control

mice (29, 30). Thus, the SNS displays a dual role, potentiating autoimmune responses and attenuating antiviral and antibacterial responses. This dual function could be explained by a mechanism in which SNS-mediated attenuation of Th1 responses concomitantly favors Th17 responses. Another mechanism could involve dopamine. Indeed, SNS neurons express the enzyme dopamine- β -hydroxylase (D β H), which catalyzes the synthesis of NE from dopamine, a neurotransmitter normally present in low amounts in mouse SNS neurons. However, in D β H-deficient mice (D β HKO), noradrenergic neurons become exclusively dopaminergic, and the mice develop an attenuated antibacterial response against *L. monocytogenes* (31). Thus, exacerbated dopamine signaling by the SNS results in a decreased Th1 response.

IMMUNE CELLS AS A SOURCE OF DOPAMINE

An increasing number of studies revealed that cells involved in both adaptive and innate immune responses, such as DCs, T cells, B cells, and macrophages are capable of synthesizing neurotransmitters (32). Under specific stimuli, these cells may release neurotransmitters into the extracellular compartment, thus enabling communications with other different cell types. These interactions not only suggest that neurotransmitters can mediate communication between immune cells, but also that these molecules may be involved in a bidirectional cross-talk between the immune and the nervous system. With regard to dopamine, early studies showed that *in vitro* activation of human peripheral blood mononuclear cells (PBMCs) with mitogens induces production of intracellular dopamine and other catecholamines, probably involving both T and B lymphocytes (33, 34). Currently, several studies performed in human and mouse cells indicate that DCs and Tregs constitute dopamine sources. DCs express tyrosine hydroxylase (TH), which catalyzes the first step required for dopamine biosynthesis (Figure 1). However, these cells do not express dopamine- β -hydroxylase, the enzyme required to metabolize dopamine and to transform it into epinephrine and NE (35). In addition, DCs do not express DAT, required to take up dopamine from the extracellular compartment. Thus, DCs synthesize dopamine, but not other catecholamines, and they cannot capture dopamine from the extracellular space. These cells also express enzymes necessary to degrade dopamine in the cytoplasm: monoaminoxidases A and B (MAO-A, MAO-B) and vesicular monoamine transporter 2 (VMAT-2) required to store dopamine in vesicular compartments. In addition, human DCs contain intracellular dopamine, which is released upon antigen presentation to T cells (36). On the other hand, human Tregs constitutively express TH and contain substantial amounts of dopamine and other catecholamines, while effector T cells only contain trace amounts (37). Tregs also express VMAT-1 and VMAT-2, which allows them to accumulate catecholamines in vesicular stores (37). Interestingly, physiologically relevant amounts of dopamine are released by Tregs when stimulated by reserpine, a natural drug used to deplete monoamines (37). Addressing the physiological stimuli evoking release of intracellular catecholamines from lymphocytes, *in vitro* treatment of mitogen-stimulated PBMCs with IFN- β induces a stronger production of catecholamines and the release of these mediators into the culture supernatant (38), thus suggesting that IFN- β is an endogenous stimulus for secretion of

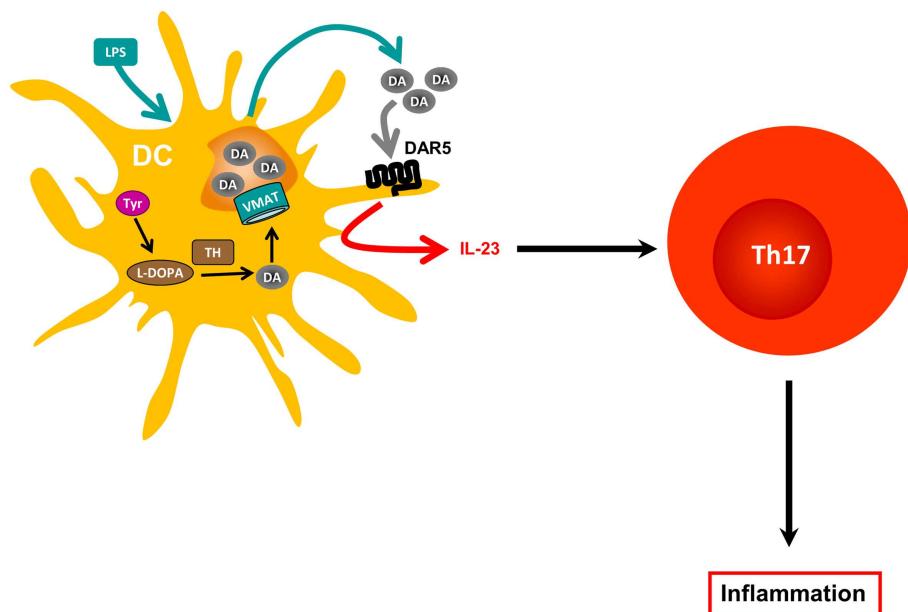


FIGURE 1 | Dopamine produced by dendritic cells and amplification of inflammation. Dendritic cells (DCs) express tyrosine hydroxylase (TH), which catalyzes the first step required for dopamine (DA) biosynthesis. However, since these cells do not express dopamine β -hydroxylase, the enzyme required to metabolize DA and to transform it into epinephrine and norepinephrine, they accumulate DA. DCs also express vesicular monoamine transporters 1 and 2 (VMAT) required to

store DA in vesicular compartments. In response to antigen presentation or to LPS stimulation, DCs release DA from intracellular stores, which can modulate both DC physiology in an autocrine manner and CD4 $^{+}$ T cell responses in a paracrine fashion (not depicted). At certain concentrations, DC-derived DA interacts with DAR5 expressed by DCs, which promotes IL-23 production in response to LPS, and, thereby, enhances Th17 responses.

catecholamines from lymphocytes. Other immune cells have been described to store dopamine in intracellular compartments. In this regard, stimulation of B cells with mitogens induces up-regulation of TH mRNA expression followed by production of intracellular dopamine and other catecholamines by a PKC-dependent mechanism (34). In addition, it has been shown that intracellular vesicles containing dopamine in B cells can be released by Ca $^{2+}$ -dependent mechanisms (39). Similarly, other studies suggest the existence of dopamine-containing vesicles in monocytes/macrophages (39, 40). Neutrophils and mast cells have been also suggested to contain dopamine (39, 41). Further experimental work is necessary to understand the relevance of these immune cells as sources of dopamine in neuroimmune interactions and in leukocyte–leukocyte communications.

DOPAMINE RECEPTOR EXPRESSION IN THE IMMUNE SYSTEM

Dopamine receptors have been found not only in cells of the innate immune response such as DCs, NK cells, macrophages/monocytes, and granulocytes (35), but also in cells of the adaptive immune response, such as B cells, CD8 $^{+}$ T cells, and CD4 $^{+}$ T cells (36, 42–45). DARs expression has also been described in murine T cells (45, 46). Pharmacological evidence obtained from studies performed with human T cells has suggested that, among the five known DARs (DAR1–DAR5), both type I (DAR1 and DAR5) and type II (DAR2, DAR3, and DAR4) receptors contribute to the regulation of T cell functions. It has been suggested that stimulation of type I

DARs expressed on human naive CD4 $^{+}$ T cells potentiates the production of Th2 cytokines (36). Other investigators have suggested that stimulation of type I DARs on human Tregs can decrease IL-10 and TGF- β production and their suppressive activity (37), and that DAR4 stimulation on human T cells promotes quiescence (44). On the other hand, there is evidence that stimulation of DAR2 and DAR3 in normal human resting T cells favors production of IL-10 and TNF- α , respectively (42), and that stimulation of DAR3 in resting T cells favors activation of β 1-integrins and adhesion to fibronectin, two critical events required for cell migration (45). Importantly, DAR3 stimulation in human activated CD4 $^{+}$ T cells decreases IL-4 and IL-10 synthesis and potentiates IFN- γ production, a key cytokine for Th1 cells; and pharmacologic DAR3 stimulation in human T cells potentiates expression of surface activation markers (47). In contrast, other studies indicated that dopamine, at concentrations that selectively stimulate DAR3, inhibits human T cell proliferation (43, 48). In addition to the different stimulatory effects of DARs in T cell physiology, it is important to consider that each DAR displays different affinities for dopamine: DAR3 > DAR5 > DAR4 > DAR2 > DAR1 [Ki (nM) = 27, 228, 450, 1705, 2340, respectively] (49–51). Thus, low levels of dopamine, e.g., 50 nM, would stimulate mainly DAR3 in T cells, favoring Th1-like responses and T cell migration, whereas moderate dopamine levels, e.g., 300 nM, would stimulate DAR5 as well, inhibiting T cell function. It is likely that, by stimulating multiple DARs, higher dopamine levels promote complex effects in T cells, probably inhibiting T cell-mediated immunity (4).

Regarding DCs, DAR1 and DAR5 are expressed at higher levels on the cell surface, whereas DAR3 and DAR2 are poorly represented on DCs (35). It has recently been demonstrated that stimulation of DAR5 on DCs strongly potentiates production of IL-23, a regulatory cytokine that favors polarization of naive CD4⁺ T cells toward the inflammatory Th17 phenotype (Figure 2). Specifically, stimulation of DAR5 on DCs potentiates Th17 responses *in vitro* and *in vivo* (35). Other pharmacological evidence indicates that selective stimulation of DAR2/DAR3 or selective inhibition of DAR1/DAR5 on DCs favors polarization of CD4⁺ T cell responses toward Th1, but impairs the Th17 fate (52). Thus, depending on the concentration of dopamine, the specific DARs expressed and the type of immune cell bearing DARs, this neurotransmitter may induce different effects in the immune response.

Emerging evidence has shown association of some autoimmune disorders with abnormal dopamine levels and deregulation of dopaminergic components expressed in immune cells. For example, dopamine levels are altered in the brain of mouse models of multiple sclerosis (MS) and systemic lupus erythematosus (SLE), and in inflamed tissues of patients with inflammatory bowel diseases (IBDs) or rheumatoid arthritis (RA). The distorted expression of DARs in peripheral lymphocytes of SLE and MS patients supports the importance of dopaminergic regulations in autoimmunity. Here, we critically discuss the role of dopaminergic regulation of the immune response and its implications with regard to autoimmune disorders.

CELL SUBSETS INVOLVED IN MULTIPLE SCLEROSIS PATHOGENESIS

Multiple sclerosis is a chronic demyelinating disease resulting from an autoimmune response against constituents of the CNS. With approximately 2.4 million individuals affected world wide, the disease is characterized by CD4⁺ T cell-mediated progressive loss of neurological function due to the destruction of axonal myelin sheath in several areas of the brain and spinal cord. Loss of myelin is manifested in clinical symptoms such as paralysis, muscle spasms, optic neuritis, and neuropathic pain (53). Pathological features of MS lesions include BBB permeability, myelin sheath destruction, axonal damage, glial scar formation, and presence of lymphocytes and inflammatory cells infiltrated into the CNS (54). Despite intense investigation, the etiology of MS is still unclear; but genetic and environmental factors have been suggested to be important in disease development.

Several cell types are present in the CSF and microvasculature. An increased number of DCs with an exacerbated state of maturation have been found in the CSF and lesions of patients with MS (55). It is thought that, in healthy individuals, CNS-resident DCs play a role in the induction of peripheral tolerance to CNS-derived self-constituents, including myelin. However, due to the abnormal mature phenotype of DCs and their proximity with infiltrated T cells in the CNS of MS patients, DCs could play an important role in presenting self-antigens and in re-stimulating self-reactive T cells infiltrated into the CNS. In addition, peripheral

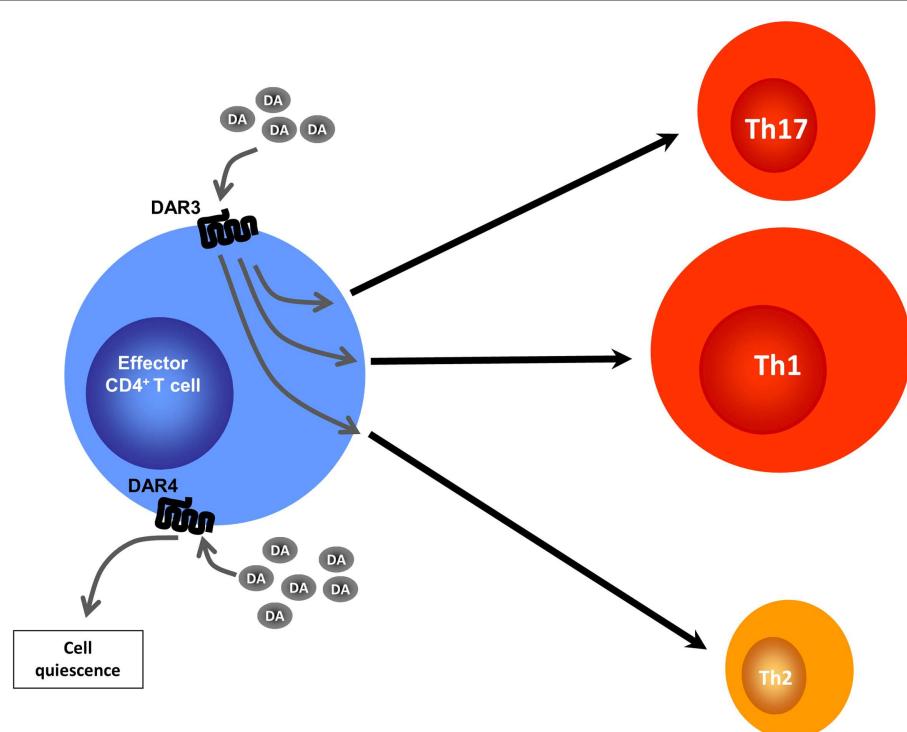


FIGURE 2 | Altered CD4⁺ T cell programming by different dopamine concentrations. Effector CD4⁺ T cells lack the ability to synthesize DA, but may be exposed to DA produced by DCs, Tregs, or by other sources (not depicted). At intermediate DA concentrations, stimulation of DAR4 expressed by effector CD4⁺ T cells leads to cell quiescence by inducing

expression of KLF2, a transcription factor that regulates T cell quiescence. At lower DA concentrations, stimulation of DAR3 expressed on DCs results, by means of an undefined mechanism, in heightened Th1 responses along with a reduction in Th17 immunity and a reduction of Th2-related cytokines.

blood DCs from MS patients produce increased amounts of IL-6, IFN- γ , and TNF- α (56). Importantly, these DCs are capable of promoting differentiation of CD4 $^+$ T cells toward the inflammatory Th17 phenotype (57). These observations support the notion that abnormal function of DCs from MS patients can promote an inappropriate functional phenotype in self-reactive T cells, contributing to the development of autoimmunity. In addition to CD4 $^+$ T cells, CD8 $^+$ T cells have also been described as an important cell subset in the disease (58). In fact, the frequency of CD8 $^+$ T cells is greater than that of CD4 $^+$ T cells in inflamed MS plaques, and CD8 $^+$ T cells show oligoclonal expansion in plaques, CSF, and blood, suggesting an important role of these cells in MS. In this regard, a group of studies performed in mice and in humanized mice showed participation of CD8 $^+$ T cells in EAE (58). B cells are also important by promoting the autoimmune response involved in MS; and it has been suggested that IL-6-producing B cells are the pathogenic cell subset that promotes functions of self-reactive T cells (59). Importantly, treatment with rituximab, an anti-CD20 antibody that alleviates symptoms of MS patients (60), induces preferential depletion of IL-6-producing B cells (59).

DOPAMINERGIC REGULATION IN MULTIPLE SCLEROSIS

Experimental autoimmune encephalomyelitis is a widely used mouse model of MS. It can be induced by injection of a peptide derived from the myelin-oligodendrocyte glycoprotein (MOG), MOG_{35–55} (pMOG), emulsified with adjuvant (35). Administration of myelin-derived antigens in an immunogenic context promotes activation of self-reactive T cells specific for myelin antigens, which mediate myelin destruction characterized by focal areas of demyelination along the CNS, with axonal loss that results in ascending paralysis, affecting first the tail and then the hind limbs. During EAE onset and at the peak of disease manifestations, there is an important increase of dopamine levels in the striatum nucleus (61). Interestingly, levels of IL-1 β and TNF- α mRNAs increase in the striatum nucleus with the same kinetics followed by dopamine (61). On the other hand, there is decreased expression of DAR5 in PBMCs obtained from untreated MS patients when compared with PBMCs obtained from healthy donors (62). Furthermore, when patients are treated with IFN- β , there is a progressive increase of the expression of DAR5 in PBMCs during the treatment period (63). It is possible that decreased expression of DAR5 on PBMCs could be a pathogenic factor favoring disease manifestations. Alternatively, DAR5 expressed on these cells could represent a detrimental factor in MS; and, somehow, a compensatory down-regulation of this receptor could be promoted in MS patients. Moreover, altered production of catecholamines by PBMCs, which is modulated through type I DARs (34), seems also to occur in PBMCs from MS patients (33). Importantly, IFN- β , which has been shown to be therapeutic for the treatment of MS, promotes a progressive increase of TH expression with a consequent increase of catecholamines production in PBMCs (63). It is noteworthy that IFN- β is capable to induce release of catecholamines from PBMCs *in vitro* (38); however, the physiological relevance of this IFN- β function remains unclear. These results suggest a relationship between expression of components of the dopaminergic system in immune cells and MS development (see Table 1).

Of late, it was demonstrated that DCs express the whole machinery to synthesize and store dopamine (35). The dopamine released from DCs subsequently acts in an autocrine manner to stimulate DAR5 expressed on DCs, promoting a potent production of IL-23 by DCs, thus conferring these cells with the ability to favor polarization of naïve CD4 $^+$ T cells toward the inflammatory Th17 phenotype (35). Since IL-23 can also stimulate $\gamma\delta$ T cell functions (64) and GM-CSF-producing CD4 $^+$ T cells (65), two cell subpopulations involved in EAE pathogenesis, the DAR5 expressed on DCs could play a role in EAE by regulating the activity of various target T cell populations. This autocrine loop mechanism mediated by dopamine and stimulation of DAR5 on DCs could represent a driving force in the autoimmune response during EAE development. Supporting this notion, DAR5KO mice manifest EAE with significantly lower severity than WT mice (35). In addition, prophylactic transfer of DAR5KO DCs loaded with pMOG into WT recipient mice significantly reduces EAE severity when compared with WT recipient mice transferred with pMOG-loaded WT DCs (35). In the same direction, another study has shown that systemic administration of a selective antagonist for type I DARs decreased significantly EAE severity (52). Furthermore, attenuated disease severity correlates with decreased numbers of Th17 and IFN- γ $^+$ IL-17 $^+$ CD4 $^+$ T cells infiltrated into the CNS of WT mice transferred with DAR5KO DCs when compared with those transferred with WT DCs. Conversely, there are no differences in the frequencies of Tregs or Th1 cells infiltrated into the CNS when compared to WT mice transferred with DAR5KO DCs versus those transferred with WT DCs. However, the participation of $\gamma\delta$ T cells, GM-CSF-producing CD4 $^+$ T cell, and CD8 $^+$ Tregs has not been explored.

Regarding dopaminergic regulation of T cells, the physiologic stimulus that induces dopamine release from Tregs remains unknown. Yet, when treated with reserpine, a drug used to deplete dopamine from dopaminergic cells, Tregs release high amounts of dopamine, which acts in an autocrine manner to inhibit their suppressive activity. Pharmacological evidence suggests that this inhibition of Treg suppressive activity is mediated through type I DARs, including DAR1 and/or DAR5 (37). Accordingly, a recent study that analyzed the suppressive function and expression of the dopaminergic machinery in Tregs obtained from MS patients or from healthy controls found that DAR5, as well as TH, were up-regulated in Tregs from untreated MS patients when compared with those from healthy controls; however, both TH and DAR5 were down-regulated when Tregs were obtained from IFN- β -treated MS patients (66). Whereas the suppressive function was partially or completely inhibited by dopamine when Tregs were obtained from healthy controls or untreated MS patients, respectively, dopamine-mediated inhibition of Treg function was abolished when Tregs were obtained from IFN- β -treated MS patients (66). Interestingly, DAR3 expression in Tregs was unaltered in untreated MS patients, but significantly decreased in IFN- β -treated MS patients when compared with healthy controls (66). Importantly, inhibition of Treg function could be achieved by 1 μ M dopamine (66). Considering the affinity of dopamine for DARs expressed on human Tregs (DAR1, DAR3, and DAR5) and the pharmacological evidence obtained using dopamine analogs in these cells (37), the results suggest that the inhibitory effect exerted on Treg suppressive functions is mediated by DAR5, rather

Table 1 | Observations linking the dopaminergic system with the development and progression of autoimmune disease.

Disease	Experimental mouse models	Reference	Patients	Reference
Multiple sclerosis	Increased dopamine levels in the striatum nucleus during EAE peak	(61)	Decreased expression of DAR5 in PBMCs obtained from untreated MS patients compared with healthy donors	(62)
	Administration of the DAR1-like antagonist SCH-23390 prevents the development of EAE	(52)	Increased expression of DAR5 and TH, along with elevated catecholamine content in PBMCs of MS patients treated with IFN- β	(63)
	Stimulation of DAR5 on DCs promotes Th17-driven EAE	(35)	Treatment of MS patients with IFN- β reduces high levels of DAR5 and TH expressed on Tregs and abolishes dopamine-mediated inhibition of suppressive activity of Tregs	(66)
Inflammatory bowel diseases	TNBS-induced colitis is associated with reduced tissue levels of dopamine	(96)	Inflamed gut mucosa from CD and UC patients shows a marked reduction of dopamine content	(95)
	6-Hydroxydopamine-induced sympathectomy increases gut inflammation in chronically DSS-treated and IL-10-deficient mice	(97)	CD patients have reduced numbers of sympathetic fibers interacting with the intestinal wall	(97)
	Dopamine acts via DAR2 to suppress both increased motility and ulcer development induced by chemical insult	(102)	A genetic polymorphism of DAR2 gene, which results in reduced receptor expression, has been reported as a risk factor to develop refractory CD	(103)
Rheumatoid arthritis	Treatment with the selective DAR1-like antagonist SCH-23390 suppresses collagen-induced arthritis severity, probably due to inhibition of macrophage differentiation into osteoclasts	(108)	Dopamine is significantly increased in the synovial tissue of RA patients	(107)
	Selective DAR2-like receptor antagonist haloperidol significantly exacerbated cartilage destruction, whereas DAR1-like receptor antagonist SCH-23390 strongly suppresses RA development	(107)	Administration of bromocriptine, a DAR2/3 agonist, suppresses immune parameters and reduces RA disease activity	(112)
	Adoptive transfer of TH ⁺ cells generated from mesenchymal stem cells reduces collagen-induced arthritis severity	(111)	Treatment of active RA with cabergoline, a DAR2/3 agonist, significantly reduces disease activity	(113)
Systemic lupus erythematosus	Lupus-prone MRL-lpr mice have impaired coordination and neurological deficits, with imbalanced dopamine function and neurodegeneration in dopamine-rich brain regions	(120)	Dopamine-producing TH ⁺ leukocytes with anti-inflammatory properties are found in synovial tissue of RA patients, but not in healthy controls	(110)
	Brains of MRL-lpr mice show elevated levels of dopamine and increased sensitivity to the DAR2/3 receptor agonist quinpirole, suggesting a neurotoxic role for dopamine	(122)	Autoantibodies targeting dopaminergic neurons are associated with rapidly progressing Parkinsonian symptomatology in a SLE patient	(119)
			SLE patients show reduced expression of DAR2 and increased DAR4 levels on PBMC-derived T cells compared to healthy individuals	(123)

(Continued)

Table 1 | Continued

Disease	Experimental mouse models	Reference	Patients	Reference
	Chronic administration of the selective DAR2/3 agonist quinpirole induces self-injurious behavior in lupic mice	(121)	Treatment with bromocriptine, a DAR2/3 agonist, is beneficial in SLE patients with mild to moderately active disease, leading to decreased serum immunoglobulin and anti-DNA antibody levels	(112)
	Bromocriptine, a DAR2/3 agonist, slows the course of SLE in (NZB × NZW) F1 mice and is effective in treating established disease in this model	(125)		
	Combined treatment with estrogen and bromocriptine, a DAR2/3 agonist, prevents development of a lupus-like syndrome in BALB/c mice expressing a transgenic anti-dsDNA antibody	(126)		

DC, dendritic cell; CD, Crohn's disease; EAE, experimental autoimmune encephalomyelitis; DAR, dopamine receptor; MS, multiple sclerosis; TH, tyrosine hydroxylase; UC, ulcerative colitis.

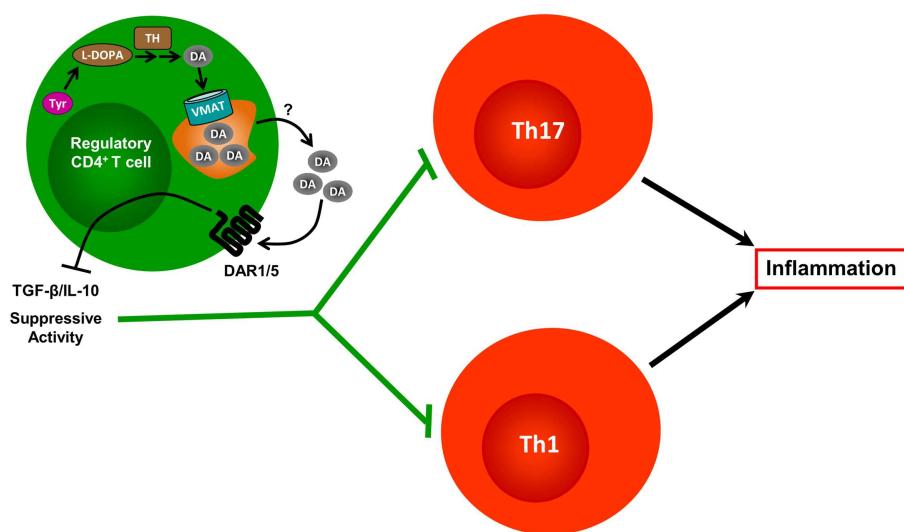


FIGURE 3 | Contrasted effects of dopamine produced by regulatory T cells. Tregs constitutively express TH and contain substantial amounts of DA. They also express VMAT-1 and VMAT-2, which allows them to accumulate DA in vesicular stores. In response to yet unknown physiological stimuli, Tregs release DA, which can

interact with DARs expressed on the Treg cell surface, but also with DARs present on DCs and effector CD4⁺ T cells (not depicted). Treg-derived DA interaction with DAR1/5 expressed by Tregs, reduces the expression of IL-10 and TGF-β, and weakens the Treg's suppressive activity exerted over effector CD4⁺ T cells.

than DAR1. Thus, these results support the notion that DAR stimulation could be involved in inhibition of Treg suppressive activity (Figure 3). On the other hand, because DAR5 expression is altered in MS patients, these findings also suggest the involvement of DAR5 expressed on Tregs in the physiopathology of MS (see Table 1). Since CD8⁺ T cells and B cells are also involved in MS pathogenesis, and because DARs are expressed in these cell populations, further efforts are necessary to understand the role of dopaminergic regulation of these cells in MS. Moreover, further experimental work is necessary to provide an integrated view of

the role of dopaminergic regulation in the autoimmune response during MS. Further understanding of the precise mechanisms involving dopaminergic-mediated regulation of the immune system and their alterations in MS could lead to development of drugs useful for MS treatment.

MUCOSAL IMMUNE SYSTEM IN INFLAMMATORY BOWEL DISEASES

Inflammatory bowel diseases form a group of chronic remittent inflammatory affections of the GI tract, among which Crohn's

disease (CD) and ulcerative colitis (UC) are the most common. The overall IBD prevalence approximates 500–900 cases per 100,000 individuals, and has shown a marked increase during the last decades, mainly in westernized nations (67, 68). In terms of clinical manifestations, both CD and UC share several symptoms such as abdominal pain, anemia, weight loss, and diarrhea, but they also differ in a number of pathological features. Lesions in CD may occur anywhere from the mouth to the anus, with a preference for the ileum, while UC patients typically present lesions restricted to the colon. UC involves mostly mucosal inflammation, while CD is characterized by discontinuous transmural inflammation. In addition, UC may evolve to toxic mega-colon or predispose to colorectal cancer, while CD may progress into perianal fistulas, abscesses, and strictures, which may lead to intestinal obstructions (69). Evidence from IBD mouse models has associated different CD4⁺ T helper lineages as the main drivers of inflammation, linking CD development to Th1/Th17 responses, and UC to Th2 responses (70). More recently, however, both gene and protein expression studies have confirmed that inflamed samples from both UC and CD patients are dominated by Th1/Th17 markers with little or no signs of Th2 contribution, suggesting that established inflammation in IBD is mainly driven by Th1/Th17 cell populations (71, 72). Treatment of these conditions relies on classical anti-inflammatory and immunosuppressive drugs such as mesalamine compounds, corticosteroids, and azathioprine. However, these agents are known to vary in their ability to control the symptoms and to be toxic. Novel therapies rely on neutralizing antibodies raised against inflammatory cytokines such as TNF- α and the p40 subunit of IL-12 and IL-23, which are important in the innate response that polarizes adaptive immunity and perpetuates inflammation (69, 73).

Current understanding of IBD pathogenesis indicates that these disorders are driven by an excessive immune response against normal constituents of the mucosal microbiota in genetically predisposed individuals. Specifically, intestinal microbiota is able to modulate the mucosal immune response and seems to play a key role in IBD pathology (74). Several observations suggest that inflammation *per se* may cause dysbiosis, and severe inflammation might be associated with profound changes in the intestinal microflora, acting as a perpetuating factor in IBD by increasing the contribution of commensals with pathogenic tendencies among the community (75). Additionally, the epithelial barrier plays a crucial role in maintaining mucosal homeostasis as it orchestrates the communication between the intestinal mucosa and luminal contents, and as it establishes an appropriate substrate for commensal microbiota. These various players enable the mucosal immune system to continuously sample luminal contents and to be able to decide between tolerating commensal microflora and dietary antigens, or activating and responding to invading pathogens. Through several mechanisms, sampled antigens are transported to APCs, like DCs and macrophages. Several studies have regarded DCs and macrophages as inducers of intestinal inflammation (76, 77). In contrast to DCs and macrophages, NK cells attenuate intestinal inflammation induced by transfer of IL-10-deficient CD4⁺ T cells into immunodeficient hosts by a mechanism dependent on perforin (78).

Activation of innate immunity leads to recruitment and activation of several components of the adaptive immune response, which mediate damage of intestinal tissues in IBDs and their mouse models. Th1 cells were initially credited as inflammation inducers in experimental colitis. Further studies drew attention to the involvement of Th17 cells in IBDs. Thus, IL-17 expression is increased in both CD and UC, with a higher frequency of IL-17-producing CD4⁺ T cells in colonic mucosa of CD patients (79, 80). In addition, Tregs seem to play a crucial role in maintaining intestinal homeostasis. These cells can suppress inflammation induced by CD4⁺ CD45RB^{hi} T cells in a T cell transfer model of colitis (81); and one of the main suppressive mechanisms relies on IL-10 secretion by these cells. Thus, specific IL-10 deletion of Foxp3⁺ Tregs results in spontaneous colitis, highlighting the fact that IL-10 produced by Tregs is instrumental in maintaining tolerance particularly at intestinal tissues (82). In humans, CD4⁺CD25⁺Foxp3⁺ Treg cells are increased in the inflamed lamina propria of CD and UC patients compared to uninflamed mucosa and mucosa from healthy controls, and they retain their ability to suppress CD4⁺CD25⁻ T cells, suggesting that Treg function is not altered during the course of intestinal inflammation (83, 84). In accordance with these data, T cells from IBD patients were found to be resistant to suppressive molecules, such as TGF- β , resulting in excessive T cell-mediated inflammation (85). Alterations in additional regulatory populations of T cells have been reported in IBDs, such as CD8⁺ CD28⁻ T cells, which are reduced or absent in the lamina propria of IBD patients. Regulatory B cells have also been associated with these diseases, as they are detected in inflamed sites and can suppress the progression, but not the initiation phase of colitis (86, 87). Finally, it is important to note that NKT cells, which respond to phospho- or glycolipids rapidly produce Th1-, Th2-, and Th17-associated cytokines, major drivers of UC pathology (88).

DOPAMINE AND MUCOSAL IMMUNITY IN INFLAMMATORY BOWEL DISEASES

Cells residing in the GI tract may encounter the neuromodulator dopamine, which can be produced from different sources, including the enteric nervous system, the intestinal epithelial layer, and certain immune cells, as described above. The intestine receives extrinsic signals from the CNS by means of sympathetic and parasympathetic nerve fibers belonging to the autonomic nervous system. While sympathetic fibers arise from the corresponding spinal nerves, parasympathetic fibers derive from the vagus nerve. Both vagal and spinal fibers connect within the gut wall at different points, interacting with the “intrinsic” enteric nervous system. The latter is organized in three layers, which include the myenteric plexus, located between the circular and longitudinal muscle layers, the submucosal plexus, localized at the submucosa, and the mucosal plexus, which contains nerve endings in close proximity to immune and epithelial cells. This system includes sensory neurons, interneurons, motor neurons, and enteric glial cells, and secretes more than 30 different neurotransmitters, with most neurons expressing multiple neurotransmitters (89).

Dopamine is detectable in the intestine, but it remains unclear if this neuromodulator comes from extrinsic or intrinsic innervations (90). Several lines of evidence support the view that dopamine is produced by intrinsic enteric neurons, since gastric fibers are immunoreactive with dopamine, and because nerve stimulation of the guinea pig stomach results in dopamine release (91). Furthermore DAT immunoreactivity has been detected in enteric nerves (92). In addition, the specific dopamine-derived metabolite dihydroxyphenylacetic acid (DOPAC) has been found in the mouse intestine, and treatment with 6-hydroxydopamine, an agent that ablates neurons expressing DAT or NET, results in depletion of enteric dopamine (90). Enteric dopamine has also been reported in human myenteric neurons, which are depleted in Parkinson's disease patients (93). Additional studies have shown that TH, dopamine, and DAT immunoreactivities colocalize in subsets of neurons from mouse intestines, known to be resistant to extrinsic denervation, thus strongly suggesting that enteric dopaminergic neurons are intrinsic (94). On the other hand, dopamine may be synthesized by epithelial cells of the intestinal mucosa, which show high AADC activity and are exposed both to circulating or luminal L-DOPA (9). Interestingly, inflamed mucosa from CD and UC patients shows a marked reduction of dopamine content (95), which may alter the activation or differentiation status of DAR-expressing immune cells, such as inflammatory T cells, Tregs, B cells, macrophages, NK cells, and DCs. Consistently, 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis has been associated with reduced tissue levels of dopamine and no changes in AADC activity (96). The authors hypothesized that the mechanism behind dopamine reduction under inflammatory conditions might be explained by the reduced uptake of L-DOPA by cultured epithelial cells upon IFN- γ treatment (96). However, the *in vitro* model of intestinal epithelial cells is an over-simplified system that lacks many cellular and molecular mediators. An alternative explanation comes from work showing that CD patients have reduced numbers of sympathetic fibers interacting with the intestinal wall, and that 6-hydroxydopamine-induced sympathectomy increases the inflammation in chronically DSS-treated and IL-10-deficient mice, strongly supporting an anti-inflammatory role of sympathetic nerves in IBDs (97). As previously observed for the cholinergic anti-inflammatory reflex, autonomic circuits may relay signals with the use of different neurotransmitters, and, in that way, a cholinergic pathway, i.e., the vagus nerve, may stimulate NE release into the target tissue, i.e., the splenic nerve (98). This could also be the case in the enteric nervous system, where neurons from the enteric nervous system could respond to vagus nerve stimulation by secreting locally relevant neuromodulators, such as dopamine (99). Thus, the reduced sympathetic innervation observed in CD patients could account for reduced levels of dopamine, also observed in mouse models of colitis.

As mentioned above, development of IBD results from an excessive mucosal immune response in genetically predisposed individuals, who may have alterations in one or various processes that ensure mucosal homeostasis. For example, deregulated intestinal motility may lead to lesion induction, and, eventually, to ulcer development, which may predispose to inflammation. In this context, dopamine or its agonists have been reported to act as protective agents in various rat ulcer models (100, 101). More recently,

it was shown that dopamine acts via DAR2 to suppress both increased motility and ulcer development induced by chemical insult (102). These findings suggest that, by controlling intestinal motility, dopamine may reduce the propensity to develop ulcers upon intestinal injury. In line with this notion, a genetic polymorphism of DAR2 gene, which results in a reduced receptor expression, has been reported as a risk factor to develop refractory CD (103). Another study showed participation of dopamine acting via DAR1 in smooth muscle inhibition of acute cold/restraint stress-induced contraction in the distal colon of the rat (104). Moreover, DAR5 exhibits immunoreactivity at apical and basolateral sides of intestinal crypts, where its pharmacological stimulation promotes epithelial K⁺ secretion via a cAMP-dependent mechanism, thus ensuring the correct function of the intestinal mucosa (105).

Regarding the expression of DARs by immune cells, DAR5 expressed on DCs seems to favor the expression of both IL-12 and IL-23 in response to LPS, potentiating CD4⁺ T cell activation (35). Considering the crucial role of IL-12 and IL-23 produced by DCs in intestinal inflammation, it is possible that dopamine acting via DAR5 on DCs may promote Th17 differentiation in the gut and contribute to IBDs. Previous work showed that DAR3-deficient CD4⁺ T cells mount impaired Th1 responses and fail to induce neurodegeneration in a murine Parkinson's disease model (22). Taking into account the reduction in intestinal dopamine levels [≈140 pg/ml in healthy individuals; ≈45 pg/ml in CD and UC patients (95)] and the fact that DAR3 and DAR5 may be stimulated at low dopamine concentrations, it is tempting to speculate that low dopamine levels present in the inflamed gut may drive an inflammatory phenotype in CD4⁺ T cells, thus perpetuating chronic inflammation. Conversely, high dopamine concentrations in the gut of healthy individuals would stimulate DAR2, promoting the production of the anti-inflammatory cytokine IL-10 by CD4⁺ T cells (42) and suppressing both increased motility and ulcer development (102) (**Table 1**).

Taken together, the data discussed here support the hypothesis that dopamine has an overall protective role in the intestinal environment by acting over muscles and promoting the relaxation of the gut and preventing ulcer and lesion development, which can predispose individuals to develop intestinal inflammation. This protective role is most probably lost during colitis onset, since several lines of evidence indicate that dopamine levels decrease upon intestinal inflammation; and under these conditions, low dopamine levels may stimulate both the innate and adaptive compartments to produce highly inflammatory cytokines, favoring the development of colitis. Exploration of this hypothesis could lead to the development of novel therapies directed to dopaminergic targets that may be useful in the control of intestinal inflammation in IBD patients.

DOPAMINE AND SYNOVIAL INFLAMMATION IN RHEUMATOID ARTHRITIS

The etiology of RA remains under investigation, but accumulating evidence suggests that the disease develops in genetically predisposed individuals after exposure to environmental triggers. In addition to the presence of autoantibodies such as rheumatoid factor and cytokines, there is an impaired expression of several B and T lymphocyte subsets. Initial indications that dopamine signaling

pathways are involved in the pathogenesis of RA come from the negative association between schizophrenia, thought to be dependent on excessive response of DAR2, and RA. Thus, in patients with schizophrenia, the median incidence rate of RA approximates 0.09%, a rate that is only one-tenth of that seen in RA in the general population (106). This negative association may suggest that excessive stimulation of DAR2-like receptors by dopamine may prevent the onset of RA.

In *ex vivo* studies, DAR1 was expressed at high levels in human naive CD4⁺ T cells, and dopamine could increase the cAMP concentrations in T cells via DAR1-like receptors, and subsequently induce the secretion of IL-4 and IL-5 (107). Additionally, dopamine increases IL-6-dependent IL-17 secretion from human T cells. Importantly, DAR1-like receptor antagonists inhibit dopamine-mediated IL-6 and IL-17 secretion from human T cells. This inhibitory effect by antagonizing DAR1-like receptors, which was demonstrated with SCH-23390 and LE300 DAR1-like receptor antagonists, involves the dopamine-mediated IL-6–Th17 axis (107).

Because dopamine is detectable in the inflamed synovial tissue of RA patients (107), it was of interest to investigate the effects of DAR-like receptor antagonists on RA development in animal models. DBA/1 mice immunized with type II collagen develop CIA, and treatment of the arthritic mice with the selective DAR1 antagonist SCH-23390 suppressed CIA severity (108). However, the treatment did not affect serum levels of antibodies to type II collagen or the Th1/Th17 differentiation of splenic T cells in the treated animals. When bone marrow-derived macrophages were stimulated *in vitro* in the presence of the DAR1 antagonist SCH23390, alteration of inflammatory cytokine expression was not observed, but their *in vitro* differentiation to osteoclasts was inhibited. Importantly, co-administration of the selective DAR1 agonist A68930 abrogated the *in vivo* anti-arthritis effect and the *in vitro* suppression of osteoclastogenesis by the DAR1 antagonist. The results suggest that DAR1 blockade could represent a potentially novel approach for RA treatment. Its effect could be partly attributable to the inhibition of osteoclastogenesis. DCs could synthesize and store dopamine, then release it to naive CD4⁺ T cells upon DC–T cell interactions, and, thereby, affect helper T cell differentiation. Such model is plausible because DCs have been proposed to play a pivotal role in the initiation and perpetuation of RA by presentation of arthritogenic antigens to T cells.

The effect of DAR-like receptor antagonists was also tested in rheumatoid synovitis of SCID mice engrafted with human RA synovium (107). Macroscopically, remarkable retraction of synovial tissue was observed in mice that received a DAR2-like receptor antagonist. In contrast, vascular proliferation and tissue enlargement were observed in mice that received the DAR2-like receptor antagonist. In the RA synovial/SCID mouse chimera model, although the selective DAR2-like receptor antagonist haloperidol significantly induced accumulation of IL-6⁺ and IL-17⁺ T cells, and exacerbated cartilage destruction, SCH-23390 strongly suppressed these responses. Taken together, the findings suggest that dopamine released by DCs acts on the IL-6–Th17 axis and causes aggravation of RA synovial inflammation.

During the last decade, the SNS has been proposed to be involved in the pathogenesis of RA. In experimental studies, CIA

developed with less severity during early manifestations of disease in sympathectomized mice than in animals bearing intact SNS (27), suggesting a pro-inflammatory role exerted by SNS. *In vivo*, adoptive transfer of Tregs showed that an intact SNS attenuates suppressive functions of Tregs during CIA, and results in a significant increase of disease severity (27). In addition, recent studies revealed that sympathetic nerve fibers are lost in inflamed synovial tissue and, importantly, dopamine and NE have been described to induce repulsion of sympathetic nerve fibers (109). Of note, dopamine and NE are actively produced by synovial TH⁺ leukocytes during RA, independently of SNS function (110). Importantly, TH⁺ cells have been found in synovial tissue of RA patients, but not in healthy controls. Moreover, those TH⁺ cells start to replace sympathetic nerve fibers around the onset of disease, and modulation of locally produced catecholamines has strong anti-inflammatory effects *in vivo* in CIA and *in vitro* in samples obtained from synovial tissues of RA patients (110). More recently, *in vitro* experiments have shown that hypoxia stimulates expression of TH in synovial cells with a consequent synthesis of catecholamines and inhibition of TNF- α production in samples obtained from RA patients (111). Furthermore, the therapeutic relevance of these findings has been probed in a mouse model wherein adoptive transfer of TH⁺ neuronal cells generated from mouse mesenchymal stem cells into arthritic mice decreased disease severity (111). When TH⁺ neuronal cells were previously depleted of catecholamines by treatment with 6-hydroxydopamine, the therapeutic effect in CIA was lost (111). Thus, these data indicate that dopamine and other catecholamines produced by leukocytes play an important regulatory role in the physiopathology of RA.

In human naive CD4⁺ T cells, dopamine increases IL-6-dependent IL-17 production via DAR-like receptors, in response to anti-CD3 plus anti-CD28 antibodies. Furthermore, dopamine localizes with DCs in the synovial tissue of RA patients and is significantly increased in RA synovial fluid (107). Such observations, together with the preclinical studies of dopamine receptor antagonists prompted studies in patients. This resulted in several clinical trials that evaluated the effect of bromocriptine – a dopaminergic agonist that exerts a therapeutic activity through action on peripheral DARs – on RA disease activity. Clinical therapeutic trials using 2.5–30 mg of bromocriptine per day in a single or divided dose have shown efficacy with minimal side effects in the treatment of rheumatic diseases (112). Bromocriptine administration induced immunosuppression of several immune parameters and was associated with improvements in morning stiffness, grip strength, numbers of swollen/painful joints, and the Health Assessment Questionnaire disability index. Even though double-blind, placebo-controlled studies are limited, the clinical observations and trials support the use of bromocriptine, which is relatively safe, as a non-standard primary or adjunctive therapy in the treatment of recalcitrant RA, and associated conditions unresponsive to traditional approaches.

Cabergoline is another potent dopamine receptor agonist of DAR2 receptors. It exhibits a higher affinity for DAR2s; it also has less severe side effects and more convenient dosing schedule than bromocriptine. Its administration once or twice a week has much less tendency to cause nausea than bromocriptine. A pilot

randomized double-blind clinical trial carried out in 10 patients with active RA reported improvement of tender and swollen joints (113). In addition, patient assessment of pain and global assessment of disease activity were significant when patients were treated with the long-acting cabergoline, a drug whose potential side-effects and cost are acceptable (**Table 1**).

LUPUS NEUROPSYCHIATRIC DYSFUNCTIONS AND THE DOPAMINERGIC SYSTEM

Systemic lupus erythematosus is an autoimmune disease characterized by the involvement of multiple organs, including the kidneys and the brain. The disease course is variable and unpredictable, and often difficult to treat. It is characterized by immune deregulation and production of autoantibodies directed toward a variety of nuclear antigens, such as chromatin, RNP, Ro, La, Sm, Ku, and DNA. Despite considerable interest, the primary disturbances that lead to the production of pathogenic autoantibodies in SLE remain to be elucidated. It is, however, clear that the disease is multifactorial, with genetic and environmental factors contributing to its pathogenesis (114, 115). Several observations suggest that hormones are crucial regulators of SLE activity, including its preponderance in women, the fluctuations of disease activity with the menstrual cycle, the tendency of the disease to flare during pregnancy, and the remissions after menopause or cyclophosphamide-induced ovarian failure (116).

Neurologic manifestations of unknown etiology are common in SLE and have been proposed to represent a more severe form of the disease, occurring in up to 75% of patients (117). They range from diffuse CNS disorders, i.e., acute confusional state, psychosis, anxiety, and depressive disorders, clinical to subclinical cognitive disorders of variable functional significance, to CNS syndromes, i.e., seizures, cerebrovascular disease, chorea and myopathy, transverse myelitis, demyelinating syndrome and aseptic meningitis, headaches, and PNS disorders, i.e., polyneuropathies and mononeuropathies, autonomic disorders, plexopathy, and myasthenia gravis. The neurologic manifestations may also include Parkinsonian-like deficits and changes in the basal ganglia. Although a pathologic neuro-immuno-endocrine circuitry has not yet been elucidated, significant loss of central neurons seems to underlie changes in sensorimotor function and behavior in many SLE patients (117). Support for the notion that behavioral impairments and neuronal demise are a consequence of autoimmunity comes from several case studies in which cyclophosphamide effectively reversed Parkinsonian-manifestations in SLE patients.

It is possible that disruption of the BBB and anti-neuronal autoantibodies account for CNS manifestations of the disease. Indeed, antibodies reacting with neuronal cell lines and brain tissue have been reported in the sera and CSF of patients with CNS-lupus, but they are also found in lupus patients with no clinical evidence of CNS involvement. Yet, specific autoantibodies may account for certain behavioral impairments (118). For example, a single injection of anti-dopamine antibodies reduces motor activity in healthy mice. In parallel, autoantibodies targeting dopaminergic neurons were associated with rapidly progressing Parkinsonian symptomatology in a SLE patient (119). Consistently, deposition of antigen–antibody complexes in choroidal blood vessels has been associated with neuropsychiatric dysfunction.

Systemic lupus erythematosus is also frequently accompanied by psychiatric manifestations of unknown origin. Although damage of central neurons had been documented, little is known about the neurotransmitter systems affected by the autoimmune/inflammatory process. Studies in lupus-prone MRL-lpr mice point to imbalanced dopamine function and neurodegeneration in dopamine-rich brain regions (120). Reminiscent of symptoms seen in patients, diseased MRL-lpr mice have impaired coordination in a beam-walking task and neurological deficits, and the accelerated autoimmune manifestations coincide with a deviation in their behavioral performance from congenic controls.

The series of performance deficits in motivated behavior, emotional reactivity, and learning/memory capacity in MRL-lpr mice have been operationally defined as “autoimmunity-associated behavioral syndrome” or AABS (121), and their physiopathology has been the focus of investigation. Significant changes in brain morphology have been observed at the onset of lupus-like disease in the MRL-lpr substrain, and post-mortem analysis revealed impaired catabolism of neurotransmitters in brains of lupic mice. Interestingly, autoimmune MRL-lpr mice exhibit behaviors reminiscent of stressed animals in a number of performance tests. The fact that the dopaminergic system of the mesencephalon is important in the control of movements, emotion, and motivated behavior, raises the issue of whether autoimmunity and inflammation are associated with midbrain degeneration, and whether anhedonic- and depressive-like behaviors may reflect an outcome of autoimmunity-induced damage on the mesolimbic dopaminergic system.

MRL-lpr brains show increased dopamine levels in the paraventricular nucleus (PVN) and median eminence, decreased concentrations of serotonin in the PVN, enhanced levels of serotonin in the hippocampus, and decreased NE levels in the prefrontal cortex (121). Importantly, the behavioral deficits correlate with changes in PVN and median eminence, suggesting that an imbalanced neurotransmitter regulation of the hypothalamus–pituitary axis plays a role in the etiology of behavioral dysfunctions induced by the autoimmune disease in MRL-lpr mice.

The causative role of autoimmunity and inflammation in the pathogenesis of AABS is supported by studies using the immunosuppressive drug cyclophosphamide, which prevents some behavioral deficits in lupus animals (122). More specifically, this drug prevents anxiety- and depressive-like behaviors as indicated by the restoration of novel object exploration, increased responsiveness to a sweet palatable solution, and reduced floating in the forced swimming test (FST).

Deficits in neurotransmitter catabolism are known to often be a consequence of aberrant synthesis and/or enzymatic activity, and excessive levels of dopamine can be neurotoxic. Accumulation of dopamine catabolism metabolites could contribute to the reduced density of dopaminergic cells. Regarding experimental lupus, elevated levels of dopamine in the brains of MRL-lpr mice and their increased sensitivity to the dopamine receptor agonist quinpirole are observations consistent with this notion (122).

There is now evidence that damage to central dopaminergic circuits in MRL-lpr brains accounts for some behavioral deficits. For example, chronic injection with the selective DAR2/DAR3 agonist quinpirole induced self-injurious behavior in lupus mice

(121). Similarly, rotational behavior increased in MRL-lpr mice following acute injection with the selective DAR2/DAR3 dopamine agonist apomorphine. In the sucrose preference test, acute injection with the indirect dopamine agonist D-amphetamine failed to alter the response rates of diseased animals to sucrose solutions (120), while chronic treatment increased their mobility in the FST. Immunosuppressive treatment, suppressing autoimmunity and preventing hippocampal damage, circumvented an age-related decline in spatial memory and retrieval. Taken together, these results link neuropathological findings of dopaminergic cell death in nigrostriatal, mesolimbic, and mesocortical pathways to certain behavioral deficits (e.g., locomotor, motivated, and learning behaviors) in lupus-prone animals. Although the contribution of peripheral disease manifestations to behavioral performance cannot be excluded, these pharmacological results indicate that anhedonic- and depressive-like behaviors are a consequence of disease-driven damage to several dopamine systems in MRL-lpr brains.

As discussed above, human lymphocytes express all subtypes of DARs (DAR1–DAR5), each of which exerts specific actions on the regulation of lymphocyte functions. Since there are indications that neuromediators are involved in SLE pathogenesis, it was important to assess expression of dopamine receptor genes in this disease. A study of a cohort of SLE patients by sybergreen-based real-time PCR revealed that all receptors are expressed in lupus PBMCs. In addition, DAR2 was underexpressed, and DAR4 was overexpressed, as compared to control individuals (123). Cell sorting experiments of peripheral T and B lymphocytes disclosed that the altered DAR2 and DAR4 expressions were borne by T cells. These data support the view that these distorted expressions of DAR2 and DAR4 play a role in the pathophysiology of SLE. This assumption converges with the observation that lupus-prone MRL-*lpr* mice exhibit increased dopamine levels in specific regions of the brain, e.g., the PVN and median eminence (121).

It should be emphasized that changes in mRNA levels are often not as pronounced as the resulting differences in protein amounts. Therefore, comparison of the expression of DARs at the protein level could reveal additional distortions in SLE patients and in other lymphocytes subsets. A multicolor flow cytometric analysis of these receptors with highly specific antibodies or fluorochrome-labeled ligands and additional staining for different leukocyte populations will represent a fast, accurate, and easy method to assess DAR expression in blood samples. Such an approach would allow easy screening of a high number of patients and testing if the expression levels of DAR2 and DAR4 on T cells can be used as a diagnostic marker.

The observed changes in DAR expression in lymphocytes could influence immune functions in SLE patients through several mechanisms. First, stimulation of DAR2 in T cells has been shown to have functional consequences. Thus, DAR2 was found to be effective in regulating the activation and differentiation of naive CD4⁺ T cells by promoting polarization toward regulatory Treg cells (4, 42, 44, 47). In SLE patients, Treg cell function and/or numbers were reportedly diminished, which is consistent with the observation that expression of the Treg cell-promoting DAR2 is decreased in lupus (123). Second, DR4 is expressed in unstimulated human T cells, and is effective in regulating the activation and

differentiation of naive CD4⁺ cells by triggering T cell quiescence (4, 42, 44, 47). Its activation is associated with the expression of KLF2, a transcription factor that regulates T cell quiescence (124) and permits a functional link between the nervous system and T cells. In SLE, there is an uncontrolled T cell proliferation that is thought to play an important pathogenic role. Together with the overexpression of DAR4 on lupus T cells (123), the observations (44) suggest that inducing quiescence using specific DAR4 agonists may represent a useful strategy in the treatment of this disease.

It has been hypothesized that the dopamine analog bromocriptine has the potential to suppress autoimmune disease. This rationale was applied to the treatment of SLE and in its (NZB × NZW) F₁ mouse model. Treatment with bromocriptine was effective in treating the autoimmune disease in this experimental model. When treatment was started before the appearance of clinical disease, bromocriptine could slow the course of SLE in (NZB × NZW) F₁ mice. In addition, this drug was effective in treating established disease in this model (125). In another animal model of SLE, simultaneous treatment with estrogen and the dopaminergic agent bromocriptine prevented the development of a lupus-like syndrome in BALB/c mice that express a transgene encoding a pathogenic anti-dsDNA antibody (126).

In clinical trials, bromocriptine showed evidence that it had a therapeutic effect in treating human lupus. For example, treatment with bromocriptine was found to be beneficial in SLE patients with mild to moderately active disease, leading to decreased serum immunoglobulin and anti-DNA antibody levels (112). Discontinuation of bromocriptine was followed by a flare of disease activity. In another clinical trial, the therapeutic effect of bromocriptine was found to be comparable to that of hydroxychloroquine, a well-accepted treatment for cutaneous and articular manifestations of SLE (125). The fact that bromocriptine was effective in treating (NZB × NZW) F₁ mice, the beneficial therapeutic effects in human trials, and the low toxicity of the drug represent a solid rationale for undertaking further therapeutic trials. Thus, these observations suggest that alterations in dopamine and/or its receptors could be associated with the physiopathology of SLE (**Table 1**).

CONCLUSIONS

Accumulating pharmacological and genetic evidence has indicated a crucial role of dopamine in the regulation of inflammatory responses involved in autoimmune disorders. Altered levels of dopamine in inflamed tissues and deregulated expression of the dopaminergic machinery in immune cells have been consistently associated with the physiopathology of autoimmune diseases in patients and in animal models, including MS, IBDs, SLE, and RA. Accordingly, drugs targeting the dopaminergic machinery have proven to be anti-inflammatory *in vitro* and *in vivo* in animal models and, in some cases, also in human patients. Understanding the precise mechanisms involving dopaminergic-mediated regulation of the immune system and their alterations in autoimmune disorders would allow development of drugs that could have beneficial effects for the treatment of autoimmunity. Despite current efforts in this field focusing in pharmacologically targeting components of dopaminergic machinery as a therapeutic approach *in vivo* in animal models of autoimmune diseases, there is a lack of

available specific drugs, i.e., targeting DAR1 and DAR5. Therefore, future efforts should focus on evaluation of the targeting potential of these dopaminergic components in a receptor-specific manner (for example, by developing new agonists/antagonists specific for a particular receptor, or, alternatively, targeting specific receptor expression, i.e., by inducing ectopic up- or down-regulation with viral vectors). Furthermore, the complexity of dopaminergic regulation of the immune response, which involves several different DARs expressed in diverse leukocyte types, suggests that future therapies should point toward the development of strategies to target specific DARs or enzymes in precise cell populations. In this regard, recent studies have shown that adoptive transfer of cells with an altered dopaminergic machinery may decrease disease severity in some animal models of autoimmunity. Finally, not only targeting of neurotransmitter receptors should be assessed in isolation as therapeutic approaches in immune-related diseases, but also targeting different receptors in combination should be considered.

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Genome-wide analysis of DNA methylation, copy number variation, and gene expression in monozygotic twins discordant for primary biliary cirrhosis

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Primary biliary cirrhosis (PBC) is an uncommon autoimmune disease with a homogeneous clinical phenotype that reflects incomplete disease concordance in monozygotic (MZ) twins. We have taken advantage of a unique collection consisting of genomic DNA and mRNA from peripheral blood cells of female MZ twins ($n=3$ sets) and sisters of similar age ($n=8$ pairs) discordant for disease. We performed a genome-wide study to investigate differences in (i) DNA methylation (using a custom tiled four-plex array containing tiled 50-mers 19,084 randomly chosen methylation sites), (ii) copy number variation (CNV) (with a chip including markers derived from the 1000 Genomes Project, all three HapMap phases, and recently published studies), and/or (iii) gene expression (by whole-genome expression arrays). Based on the results obtained from these three approaches we utilized quantitative PCR to compare the expression of candidate genes. Importantly, our data support consistent differences in discordant twins and siblings for the (i) methylation profiles of 60 gene regions, (ii) CNV of 10 genes, and (iii) the expression of 2 interferon-dependent genes. Quantitative PCR analysis showed that 17 of these genes are differentially expressed in discordant sibling pairs. In conclusion, we report that MZ twins and sisters discordant for PBC manifest particular epigenetic differences and highlight the value of the epigenetic study of twins.

Keywords: autoimmune cholangitis, epigenetics, environment

INTRODUCTION

Primary biliary cirrhosis (PBC) is a female-predominant autoimmune liver disease affecting the small interlobular bile ducts, ultimately leading to periportal fibrosis and cirrhosis (1). Similar to most autoimmune diseases, PBC onset results from the interplay of genomic predisposition and environmental factors (2–5) with a possible role for sex factors (6). Recent genome-wide association studies (GWAS) have reported consistent associations with polymorphisms of genes such as *IL12RA* and *HLA class II* in subgroups of patients with PBC (7–13) and a pathway analysis was recently performed (13). PBC concordance rates in dizygotic

(DZ) and monozygotic (MZ) twins are significantly different being 0 and 63%, respectively, thus supporting the role of both genetic and environmental factors (14) with the latter supported also by epidemiology (15, 16).

Promoter methylation influences gene expression (GEX) and our group recently reported differences in the DNA methylation and expression of two X-linked genes (*PIN4* and *CLIC2*) in MZ twins discordant for PBC (17). On the other hand, copy number variations (CNV) are the result of duplications and other rearrangements (18) occur *de novo* at much higher rates than single nucleotide variants, and may regulate GEX (19). While

sharing their genomic sequence, MZ twins may develop different phenotypes over the years because of increasing differences in DNA methylation (20) and CNV (21, 22).

We have taken advantage of a unique DNA collection of identical and non-identical twins with PBC and performed a genome-wide investigation to determine differences in DNA methylation, CNV, and GEX. Our data identify 17 candidate genes that are significantly under- or up-regulated in affected individuals and we suggest that these might constitute new candidates as disease markers of genetic determinants. The value of this approach is highlighted and suggests the need for the study of a large number of patients and cell subpopulations (23) to support this thesis.

MATERIALS AND METHODS

SUBJECTS

Blood samples from three MZ twin pairs discordant for PBC whose zygosity had been determined using microsatellite analysis (Ballestar) and eight sister pairs of similar age (within 5 years) discordant for PBC studied (**Table 1**). Serum antimitochondrial antibodies (AMA) were positive at indirect immunofluorescence in all patients with PBC and none of the healthy twins and sisters. In these subjects, PBC was excluded when serum AMA was negative and serum alkaline phosphatase was within normal range on two different occasions. Genomic DNA was isolated from peripheral blood mononuclear cells (PBMCs) using the QIAamp Blood Midi Kit (Qiagen, Valencia, CA, USA) and stored at -20°C until used. Additional blood samples were obtained using TempusTM Blood RNA Tubes (Applied Biosystems, Foster City, CA, USA) that were stored at -20°C until mRNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and then stored at -80°C . This study was performed in compliance with the ethical standards of medicine and, following approval by the local IRB, informed consents were obtained from all patients and controls in accordance with the Declaration of Helsinki.

METHYLATED DNA IMMUNOPRECIPITATION AND METHYLATION ANALYSIS

DNA samples of three MZ twin sets (#1/2, 9/52, and 24/57; see **Table 1**) were sonicated and then immunoprecipitated with a monoclonal antibody that specifically recognizes 5-methylcytidine (Roche NimbleGen, Madison, WI, USA). DNA fragments were converted into PCR-amplifiable OmniPlexTM Library molecules flanked by universal primer sites and the library amplified by PCR using universal primers and a limited number of cycles. Immunoprecipitated and reference DNA were tagged, respectively, with cyanine-5 (Cy5) and cyanine-3 (Cy3)-labeled random 9-mers and then hybridized by the NimbleGen Array Hybridization Kit (Roche NimbleGen, Madison, WI, USA).

A four-plex array was custom-designed to include 998 X chromosome and 18,086 randomly selected autosomal chromosome promoter sites (Roche NimbleGen, Madison, WI, USA) and samples analyzed following the manufacturers protocols. First, NimbleScan software (Roche NimbleGen, Madison, WI, USA) was utilized for DNA methylation data analysis using a threshold *p*-value of 0.05 equivalent to 1.31 based on the Gaussian distribution of data. Second, exclusive elements corresponding to

Table 1 | Summary of the patients with PBC and the corresponding healthy sibling and twin sisters utilized in the study.

PBC case #	Age (years)	Serum AMA	Control # (twin/sibling)	Age	Serum AMA
2	60	Pos	1 (MZ twin)	60	Neg
9	60	Pos	52 (MZ twin)	60	Neg
24	64	Pos	57 (MZ twin)	64	Neg
4	62	Pos	10 (Sister)	59	Neg
5	55	Pos	14 (Sister)	59	Neg
6	52	Pos	11 (Sister)	55	Neg
12	61	Pos	7 (Sister)	64	Neg
13	70	Pos	8 (Sister)	68	Neg
15	54	Pos	16 (Sister)	57	Neg
27	45	Pos	26 (Sister)	43	Neg
34	41	Pos	33 (Sister)	45	Neg
35	64	Pos	50 (Sister)	60	Neg

specific microarray probes were identified in affected and healthy subjects and peaks found only in either group were selected for further analysis. Third, elements of interest were inserted into the UCSC Genome Browser (GRCh36/hg19) to identify corresponding genes.

COPY NUMBER VARIATION ANALYSIS

Copy number variation analysis was performed on genomic DNA from one MZ twin set (#1/2; see **Table 1**) using the Infinium R HD Assay Super platform (Illumina, San Diego, CA, USA): in particular, we utilized the HumanOmni1-Quad BeadChip that includes markers derived from the 1000 Genomes Project, all three HapMap phases, and recently published studies (7, 9, 24, 25) as well as adequate tools for quality control, CNV calling, and validation. The protocol included the initial DNA preamplification, fragmentation, and precipitation. Data obtained from four-plex chips were analyzed using iScan and Illumina BeadArray system (Illumina, San Diego, CA, USA) followed by the GenomeStudio software (Illumina, San Diego, CA, USA). The position of each probe and the number of copies for each probe were determined using the PennCNV platform based on a hidden Markov model algorithm (26). The UCSC Genome Browser was then used to determine the genes involved and the number of CNV.

MICROARRAY GENE EXPRESSION ANALYSIS

We utilized RNA samples from eight pairs of sisters of similar age (**Table 1**) discordant for PBC. In the first part, we performed a whole-genome microarray comparison of transcripts to detect consistently up- or down-regulated genes in affected subjects. We obtained biotin-labeled cRNA using the Illumina R TotalPrep RNA Amplification Kit (Illumina, San Diego, CA, USA) and used the whole-genome Gene Expression Direct Hybridization Assay (Illumina, San Diego, CA, USA) including 24,500 transcripts. Microarrays were scanned using the BeadArray Reader (Illumina Inc., San Diego, CA, USA) and data were processed using BeadStudio software (Illumina Inc.). Expression data were quantified using a cut-off for significant gene differences of *p* < 0.05 with a twofold difference in expression as described elsewhere (27).

RT-PCR EXPRESSION ANALYSIS

Real-time PCR was utilized to analyze samples prepared from 1 µg total RNA according to high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) in seven pairs of sisters of similar age (#15/16, 5/14, 6/11, 7/12, 8/13, 26/27, 33/34; see Table 1). Micro-fluidic real-time quantitative PCR cards were customized to include single-plex assays for all candidate genes obtained with DNA methylation, CNV, and GEX analyses. Genes reported by GWAS studies were also included among the candidates (7, 9, 24, 25). All samples were analyzed in duplicate, and included 94 candidate genes and the 18S and β-actin housekeeping genes. Analyses were performed using the ABI Prism 7900HT Sequence Detection System (SDS 2.2.2 software, Applied Biosystems, Foster City, CA, USA). PCR cycle conditions included 50°C for 2 min, 94.5°C for 10 min, and 40 cycles of 97°C for 30 s followed by 59.7°C for 1 min. The preliminary study of all 10 samples defined the maximum allowable cycle threshold (CT) that was set at 38 while outliers exceeding this threshold were excluded from the statistical analysis and no adjustment of *p*-value was performed. Internal controls for calculating expression levels of candidate genes were 18S and ACTB (beta-actin). The analysis has been performed with Data Assist version 3 statistical software (Applied Biosystems). The software exports data from real-time PCR and performs relative quantification analysis. The data assist analysis contains: C_t data, sample design, assay design, average of C_t values of replicates, ΔC_t , normalized versus endogenous controls C_t values ± SD and fold change (RQ) files, which displays RQ min and RQ max for each sample. *p*-Value is calculated from ΔC_t files.

A heat map is used to visualize the data and illustrates, for all case/control sibling pairs, GEX in red/green color based on ΔC_t values using Pearson's correlation. The neutral/middle expression was set as the median of all the ΔC_t values from all samples, the red indicated an increase with a ΔC_t value below the middle level and the green indicated a decrease with ΔC_t above the middle level.

PATHWAY ANALYSIS

Gene networks were generated through the use of Ingenuity Pathways Analysis software 8.0. Edition (Ingenuity Systems, <http://www.ingenuity.com>). Each gene identified was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base and overlaid onto a global molecular network. The SDS 2.2.2 software (Applied Biosystems, Foster City, CA, USA) was used to determine changes in expression of a target in an experimental sample relative to the same target in a reference sample with the Student's *t*-test and *p*-value <0.05 were considered statistically significant. We utilized Data Assist Software version 3 statistical software (Applied Biosystems) and Stata 8.0 for MacIntosh (Stata Corp, College Station, TX, USA) for statistical analyses.

RESULTS

DNA METHYLATION

DNA methylation comparison showed 60 differentially methylated regions (DMR) in affected compared to the non-affected twin (*p* < 0.05 for each of the three discordant twin pairs). These DMR corresponded to 51 genes on the X chromosome and 9

genes on autosomal chromosomes, listed in Table 2. For each DMR, the PBC proband was hypermethylated compared with the non-affected twin.

COPY NUMBER VARIATIONS

Ten CNV were discordant between affected and the non-affected twin in one twin set. The healthy twin had four CNVs that were missing in the affected twin and six CNVs were present only in the affected twin. The CNVs were found in the following genes: *RYBP* ring 1, YY1 binding protein, *HERV-V2* envelope glycoprotein *ENVV2*, *POTEK* Pankirin domain family member K pseudogene, *THSD7A* thrombospondin type 1 domain containing 7A = *KIAA0960*, *GOLGA8A* golgin A8 family member A, *BPTF* bromodomain PHD finger transcription factor, and *C17orf58* open reading frame. Two additional CNV did not correspond to known genes.

MICROARRAY GENE EXPRESSION

Gene expression analysis using the genome-wide microarray showed two genes significantly down-regulated in PBC compared to the healthy sister in each of the eight discordant sister pairs. These genes were *IFIT1* (interferon-induced protein with tetratricopeptide repeats; chromosome 10q23.31) and *IFI44L* (interferon-induced protein 44-like; chromosome 1p31.1) and both are interferon-induced (28).

RT-PCR ANALYSIS

To provide additional support for our initial findings, we used RT-PCR to evaluate expression of each of the candidates that emerged from the DNA methylation (60), CNV (10), and expression studies (2), as well as previously reported GWAS in seven pairs of discordant sisters of similar age (Table 1) (7–9, 12, 13, 24, 25). Our data assist analysis contained: C_t data, sample design, assay design, average of C_t values of replicates, ΔC_t , normalized versus endogenous controls C_t values ± SD and fold change (RQ) files, which displays RQ min and RQ max for each sample. *p*-Value was calculated from ΔC_t files. Data assist v3.0 software was used with results exported from real-time PCR and for relative quantification analysis. Graphic result in heat map visualized analyzed data (Figure 1). Heat map showed, for all case/control sibling pairs, genes expression in red/green color based on ΔC_t values using Pearson's correlation. The neutral/middle expression was set as the median of all the ΔC_t values from all samples, the red indicated an increase with a ΔC_t value below the middle level and the green indicated a decrease with ΔC_t value above the middle level. The heat map from all samples is represented in Figure 1. Among the entire set of candidate genes, we found five genes that were underexpressed in at least three of seven sibling pairs with FC < 0.5 (*CXCR5*, *HLA-B*, *IFI44L*, *IFIT1*, *SMARCA1*) and one overexpressed gene in at least three of seven pairs with an FC > 2 (*IL6*). Additional 11 genes showed a widely variable expression profile in each sibling pair (*CD80*, *FAM104B*, *HLA-DQB1*, *HLA-DRB1*, *HLA-G*, *MTCP1*, *NHS*, *PIN4*, *PRPF38A*, *THSD7A*, and *TNFAIP2*) (Table 3; Figure 2).

PATHWAY ANALYSIS

Pathway analyses were performed using the 17 resulting genes from our study and demonstrated that the most representative functions

Table 2 | Differentially methylated genes in PBC-discordant MZ twins.

Gene	Chr/base pair (bp) ^a	Description/function	Localization ^b
ABCD1	chrX:152989993–152991024	ATP-binding cassette, sub-family D (ALD), member 1	PM
ATP12A	chr13:25254828–25254890	ATPase, H+/K+ transporting, non-gastric, alpha polypeptide	PM
ATP5A1	chr18:43678161–43678731	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	C
BCAP31	chrX:152989493–152990063	B cell receptor-associated protein 31	C
BGN	chrX:152760629–152761596	Biglycan	E
BRCC3	chrX:154299261–154299925	BRCA1/BRCA2-containing complex, subunit 3	N
CFP	chrX:47483418–47483642	Complement factor properdin	E
CHST7	chrX:46434647–46434858	Carbohydrate (<i>N</i> -acetylglucosamine 6- <i>O</i>) sulfotransferase 7	C
CTAG1A, CTAG1B	chrX:153813591–153814161	Cancer/testis antigen 1A, B	C
DDX41	chr5:176943911–176944481	DEAD (Asp–Glu–Ala–Asp) box polypeptide 41	N
FAM104B	chrX:55187570–55188140	Family with sequence similarity 104, member B	U
FGD1	chrX:54521696–54522266	FYVE, RhoGEF, and PH domain containing 1	C
FUNDC2	chrX:154255133–154255703	FUN14 domain containing 2	C
GAGE12B, 12I, 2A, 5, 7, 8	chrX:49315376–49315946	G antigen 1, 5, 7	U
GTPBP6	chrX:230686–231256	GTP-binding protein 6 (putative)	U
HCCS	chrX:11129525–11129638	Holoxyochrome c synthase	C
HOXD4	chr2:177016716–177017157	Homeobox D4	N
IDH3G	chrX:153059742–153059944	Isocitrate dehydrogenase 3 (NAD+) gamma	C
IDS	chrX:148586616–148587185	Iduronate 2-sulfatase	C
IRAK1	chrX:153285317–153285887	Interleukin-1 receptor-associated kinase 1	PM
KBTBD6	chr13:41706829–41707399	Kelch repeat and BTB (POZ) domain containing 6	U
MAGEA3	chrX:151938154–151938356	Melanoma antigen family A, 3	U
MAGEA6	chrX:151867135–151867705	Melanoma antigen family A, 6	U
MAGEA9	chrX:148793401–148793568	Melanoma antigen family A, 9	U
MAGED4B	chrX:51928209–51929228	Melanoma antigen family D, 4B	U
MTCP1	chrX:154299410–154299612	Mature T cell proliferation 1	C
MTM1	chrX:149737348–149737918	Myotubularin 1	C
MTMR8	chrX:63614954–63615524	Myotubularin-related protein 8	U
NHS	chrX:17393481–17393959	Nance–Horan syndrome (congenital cataracts and dental anomalies)	N
ORC1L	chr1:52869831–52870401	Origin recognition complex, subunit 1	N
CDK16	chrX:47078470–47079428	Cyclin-dependent kinase 16	C
PDZD4	chrX:153095693–153096406	PDZ domain containing 4	C
PHF16	chrX:46772444–46773014	PHD finger protein 16	N
PRKX	chrX:3631431–3632001	Protein kinase, X-linked	C

(Continued)

Table 2 | Continued

Gene	Chr/base pair (bp) ^a	Description/function	Localization ^b
PRPF38A	chr1:52869831–52870401	PRPF38 pre-mRNA processing factor 38 (yeast) domain containing A	N
RIBC1	chrX:53449681–53450600	RIB43A domain with coiled-coils 1	U
RNF128	chrX:105970276–105970478	Ring finger protein 128	C
SCLY	chr2:238969783–238970252	Selenocysteine lyase	C
SHROOM4	chrX:50557007–50557209	Shroom family member 4	PM
SLC10A3	chrX:153718280–153718749	Solute carrier family 10 (sodium/bile acid cotransporter family), member 3	PM
SLC9A6	chrX:135067977–135068547	Solute carrier family 9 (sodium/hydrogen exchanger), member 6	PM
SLITRK2	chrX:144903417–144903908	SLIT and NTRK-like family, member 2	U
SLITRK4	chrX:142722571–142723141	SLIT and NTRK-like family, member 4	U
SMARCA1	chrX:128657308–128657936	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, sub-family A, member 1	N
SSR4	chrX:153060191–153060761	Signal sequence receptor, delta (translocon-associated protein delta)	C
TAF9B	chrX:77394695–77395265	TAF9B RNA polymerase II, TATA box-binding protein-associated factor, 31 kDa	N
TCEAL6	chrX:101397122–101397692	Transcription elongation factor A (SII)-like 6	U
TUSC3	chr8:15397909–15398479	Tumor suppressor candidate 3	PM
UBL4A	chrX:153714886–153715456	Ubiquitin-like 4°	U
VCX2, VCX3A	chrX:6451316–6452154	Variable charge, X-linked 2, X-linked 3A	U, N
YIPF6	chrX:67718891–67718965	Yip1 domain family, member 6	C
ZIC3	chrX:136649002–136649910	Zic family member 3 (odd-paired homolog, <i>Drosophila</i>)	N
ZNF182	chrX:47862911–47863428	Zinc finger protein 182	N

Of note, all regions were hypermethylated in the PBC proband and only SMARCA1 was differentially expressed in RT-PCR.

^aPositions of each gene based on GRCh37/hg19.

^bFor each gene product the localization is specified as nuclear (N), cytoplasmic (C), plasma membrane (PM), extracellular (E), unknown (U).

and diseases were inflammatory, immunological, and connective tissue disorders. Furthermore the top canonical pathways involved were: T helper cell differentiation ($p = 3.98E-19$), dendritic cell maturation ($p = 1.39E-13$), altered T and B cell signaling in rheumatoid arthritis ($p = 1.02E-12$), type I diabetes mellitus signaling ($p = 1.04E-11$), and the crosstalk between dendritic cells and natural killer cells ($p = 5.98E-11$) (Table 4).

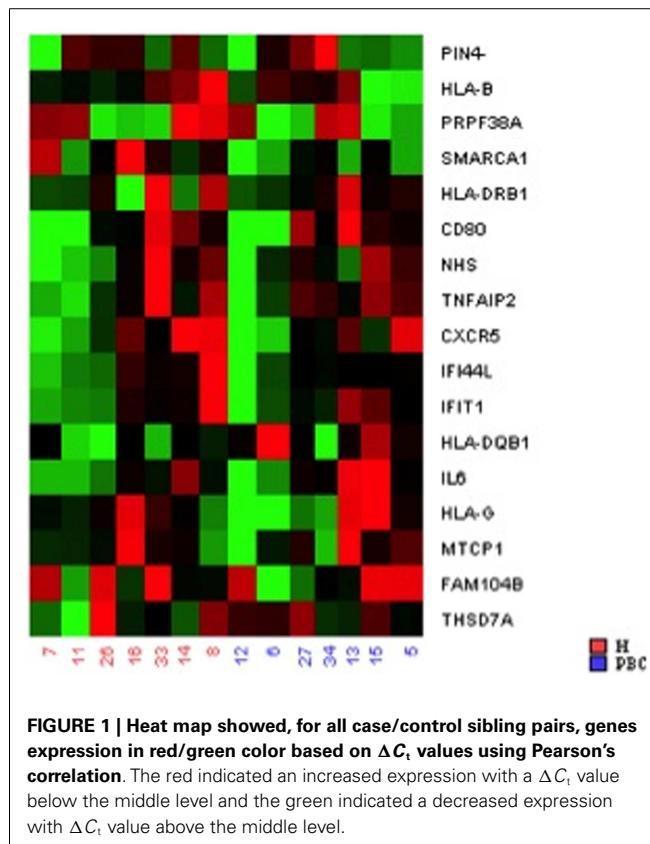
DISCUSSION

Primary biliary cirrhosis is considered a prototypic autoimmune disease because of the clinical homogeneity between patients and the relative consistency in natural history and pathology. Although relatively uncommon, several independent GWAS (7–13) have identified associations with transcription factors that further suggest a potential role for epigenetic shifts and thus our approach using this unique collection of DNA is a particularly important resource. We are aware of the numerous limitations of our study and that the observed changes in GEX may be stochastic rather than secondary to disease progression or involved in pathways involved in PBC pathogenesis, as suggested for other

autoimmune diseases (29–32). The latter includes the possibility of portal hypertension and resulting leukopenia.

We identified 60 DMR and 10 CNV between discordant MZ twins with 14 (20%) also differently expressed between PBC cases and control sisters, thus being stronger candidates as PBC biomarkers or determinants. One of the strengths of our study is the confirmation of identified genes by quantitative PCR and that this approach was extended also to genes identified in recent GWAS allowing identification of six genes differently expressed in PBC mononuclear cells. First, these genes support a down-modulation of Th2-cytokines such as *IFIT1*, an interferon type I signature represented by *IFI44L*, in favor of a fibrogenic phenotype as represented by the *IL6* up-regulation (33). Regarding this last observation, we note the apparent discrepancy between DNA methylation and GEX of *IL6* but we recognize that methylation does not fully correlate with GEX, and the difference could be explained by different mechanisms such as allele-specific methylation (34, 35) (Table 4). Second, a single DMR-associated gene, i.e., hypermethylated SMARCA1, manifested a reduced GEX confirmed in our RT-PCR study of sibling pairs. SMARCA1 is a transcription

regulator that modulates the chromatin structure and is involved in apoptosis, DNA damage, and differentiation. Moreover, the gene encodes for a member of the SWI/SNF family of proteins, which



are master regulators of GEX, regulating expression among others *FOS*, *CSF-1*, *CRYAB*, *MIM-1*, *p21* (also known as *CDKN1A*), *HSP70*, *VIM*, and *CCNA2*; SWI/SNF has also been reported to modulate alternative splicing (36). Third, 5/7 sibling pairs had consistent dysregulation of *CXCR5* being down-regulated in PBC lymphocytes, which may reflect a compartmentalization of *CXCR5*+ cells within the liver or may reflect the chronic activation of B cells, as reported in rheumatoid arthritis (37). In fact, the chemokine receptor *CXCR5* is expressed by B and T cells and controls their migration within lymph nodes while its ligand *BCA-1/CXCL13* is present in lymph nodes and spleen and also in the liver. A down-regulation of *CXRC5* is correlated with an increased production of *IL-2*, which may cause the production of immunoglobulins by B cells; *IL-2* is normally produced by T cells during an immune response. *IL-2* is also necessary during T cell differentiation in Treg, which are involved in self antigens recognition, which could result

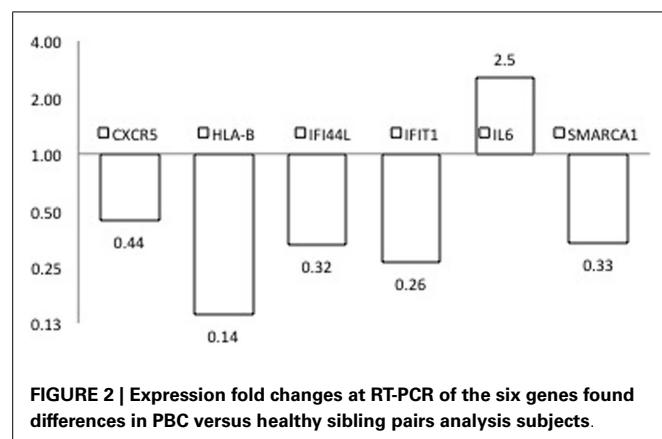


Table 3 | Genes showing consistent differences in DNA methylation, CNV, or expression^a.

Analysis	ID	Status ^b	Entrez gene name	Chr/base pair (bp)	Localization ^c
GWAS	CXCR5	Down-regulated in three sibling pairs 0.44	Chemokine (C-X-C motif) receptor 5	chr11: 118764101–118766980	PM
GWAS	HLA-B	Down-regulated in three sibling pairs 0.14	Major histocompatibility complex, class I, B	chr6: 31321649–31324989	PM
GEX	IFI44L	Down-regulated in four sibling pairs 0.32	Interferon-induced protein 44-like	chr1: 79086088–79111830	U
GEX	IFIT1	Down-regulated in three sibling pairs 0.26	Interferon-induced protein with tetratricopeptide repeats 1	chr10: 91152303–91166244	C
GWAS	IL6	Up-regulated in three sibling pairs 2.5	Interleukin 6 (interferon, beta 2)	chr7: 22766798–22771620	E
MeDIP	SMARCA1	Down-regulated in three sibling pairs 0.33	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, sub-family a, member 1	chrX: 128484989–128485617	N

^aList of genes evaluated with RT-PCR.

Each of the tested genes showed consistent differences in methylation (MeDIP), copy number variation (CNV), or gene expression (GEX) (in the current study) or were candidate genes from genome-wide association studies (GWAS) studies.

^bStatus: Log(RQ) is the logarithm of fold change = , which identifies the expression ratio: a positive Log(RQ) implies that the gene is up-regulated.

^cFor each gene product the localization is specified as nuclear (N), cytoplasmic (C), plasma membrane (PM), extracellular (E), unknown (U).

Table 4 | List of PBC-associated genes analyzed with ingenuity pathways analysis software 8.0 IPA.

Genes	IPA findings
<i>IL6</i>	Up-regulation of human IL6 protein in serum is associated with human PBC
<i>IL4</i>	Up-regulation of human IL4 mRNA in liver is associated with human PBC
<i>IL17A</i>	Up-regulation of human IL-17 (IL17A) mRNA in liver is associated with human PBC
<i>IL13</i>	Up-regulation of human IL13 mRNA in liver is associated with human PBC
<i>IL12RB2</i>	Mutant human IL12RB2 gene (SNP substitution mutation (rs3790567) is associated with human PBC (<i>p</i> -value = 2.76E-11)
<i>IL12</i>	Mutant human IL12A gene [SNP substitution mutation, allelic variations: A/G (rs4679868)] is associated with human PBC Mutant human IL12A gene (SNP substitution mutation (rs574808) is associated with human PBC (<i>p</i> -value = 1.88E-13)
<i>HLA-DQB1</i>	Mutant human HLA-DQB1 gene (SNP substitution mutation (rs9275312) is associated with human PBC Mutant human HLA-DQB1 gene (SNP substitution mutation (rs2856683) is associated with human PBC (<i>p</i> -value = 1.78E-19) Mutant human HLA-DQB1 gene (SNP substitution mutation (rs7775228) is associated with human PBC Mutant human HLA-DQB1 gene (SNP substitution mutation (rs9275390) is associated with human PBC Mutant human HLA-DQB1 gene (SNP substitution mutation (rs9357152) is associated with human PBC
<i>HLA-DPB1</i>	Mutant human HLA-DPB1 gene (SNP substitution mutation (rs9277535) is associated with human PBC Mutant human HLA-DPB1 gene (SNP substitution mutation (rs2281389) is associated with human PBC Mutant human HLA-DPB1 gene (SNP substitution mutation (rs660895) is associated with human PBC Mutant human HLA-DPB1 gene (SNP substitution mutation (rs9277565) is associated with human PBC
<i>CTLA4</i>	Mutant human CTLA4 gene is associated with human PBC

Of these, *IL6* was found differentially expressed by RT-PCR in discordant sisters.

in autoimmunity (38). Of note, following B cell activation and differentiation into plasma cells and memory cells, CXCR5 becomes down-regulated while the same effect is induced *in vitro* following anti-CD40 stimulation (39) and CD40L methylation appears to be altered in PBC (40). Fourth, *HLA-B* is also down-regulated in PBC, similar to several types of cancer (41–43).

The majority of the identified genes map on the X chromosome, in agreement with the female predominance of the disease, and are involved in many cellular pathways. Our group in a previous

work assessed the expression of 125 genes with variable X inactivation status and found that two genes (*CLIC2* and *PIN4*) were consistently down-regulated in PBC affected twin of discordant pairs (17). Three genes are differentially methylated in lymphocytes of patients with PBC and systemic sclerosis (32) and may thus be representative of general autoimmunity or fibrosis development; these genes include *MTM1* hypermethylated in PBC and in systemic sclerosis while *SSR4* and *IGH3G* are hypomethylated in both diseases. Of note, a recent study reported the up-regulation of the X-linked costimulatory molecule *CD40L* (40) but our data failed to confirm such hypomethylation in our cohort. The CNV differences observed in our MZ twin set warrant some further observations as the *de novo* post-twinning CNV frequency was estimated to be as high as 5% on a per-individual basis or 10% per twinning event (21). While the impact of CNV on GEX can vary (44), it would be of great interest to obtain parental information to determine the origin and timing of CNV in the offspring. On the other hand, there are several limitations to our data. PBC is relatively uncommon and our DNA collection reflects a several-year worldwide search; it is nonetheless a limited dataset. In addition, there is only limited information available using PBMC. PBC is an organ-specific disease affecting small intrahepatic bile ducts and thus studies of the portal infiltrating lymphocytes will provide a more valuable resource as would a detailed and well-defined lymphoid cell populations. These comments notwithstanding, the data obtained are intriguing and consistent with our thesis that one explanation for discordant MZ twins is DNA changes on the critical genomic element involved in disease susceptibility and these observations should be recapitulated also in unrelated pairs of patients and controls. With the increased interest in the balance between genetic susceptibility, it becomes critical for research groups to combine resources and improve access to clinical material and data that permits more extensive studies and the potential for more powerful statistical analysis and interpretation.

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Corrigendum: Genome-wide analysis of DNA methylation, copy number variation, and gene expression in monozygotic twins discordant for primary biliary cirrhosis

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Regulation of immune reactivity by intercellular transfer

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It was recently proposed that T lymphocytes, which closely interact with APCs, can extract surface molecules from the presenting cells when they dissociate. These observations question the classical view of discrete interactions between phenotypically defined cell populations. In this review, we summarize some reports suggesting that membrane exchange at the immune synapse can be a vector for intercellular communication and envisage some consequences on the biology of T cells.

Keywords: T/APC interaction, trogocytosis, T cell activation, costimulation, immune regulation

INTRODUCTION

The first evidence that activation of T and B lymphocytes required a co-operation between distinct cell types was provided in the late 60s. In 1967, Mosier showed that both adherent and non-adherent cells were necessary for the induction of antibody formation to sheep red blood cells *in vitro* (1). A few years later, it was shown that recognition of soluble protein antigens by guinea pig T lymphocytes required the presentation of antigen on histocompatible macrophages (2). Since then, numerous observations have highlighted the multiple interactions, which occur at various steps of the immune response: in particular, antigen-presenting cells are likely to provide three signals to T lymphocytes, which in turn trigger antibody production by B cells. These cell populations are located at discrete sites in lymphoid organs and migrate to particular sites to interact with each other. The immune response appears therefore as an exchange of signals between cells displaying well-defined phenotypes, and the specificity of the interaction is ensured by receptor/ligand interaction or binding of soluble cytokines on their receptor.

However, recent observations may challenge this scenario. Indeed, there is increasing evidence that intercellular transfer of membrane fragments and molecules occurs frequently during cell–cell close contact, thereby modifying the phenotype and probably the function of immune cells. This process has been named “trogocytosis,” from the ancient Greek *trogo*, meaning “gnaw.” In this report, we will review recent observations illustrating membrane exchange between immune cells, focusing on T cells and antigen-presenting cells, and envisage the possible physiological consequences of this phenomenon.

TCR MHC/PEPTIDE COMPLEX

A number of old reports have documented the existence of T cells bearing IA antigens on their membrane, at a time when MHC restriction was unclear and the T cell receptor unidentified. In particular, Nepom et al. showed that I-A⁺ T cell blasts appeared in antigen-stimulated proliferative culture, and that this acquisition

was strictly antigen-dependent and required positive adherent antigen-presenting cells (3). Subsequent reports confirmed that T cells may acquire peptide/MHC complexes at the T cell–APC interface. Huang et al. showed that these complexes on APCs formed clusters at the site of T cell contact within minutes, and were subsequently acquired and internalized in T cells (4). The intercellular transfer of membrane molecules was also observed *in vivo* in several models: rat T cells transferred in irradiated SCID mice acquired MHC molecules as well as adhesion and costimulatory molecules (5), and encephalitogenic T cells were shown to express abundant surface MHC class II molecules in rat and mouse models of EAE (6). In addition, in the course of studies aimed at understanding the affinity maturation of secondary T cell responses, Kedl et al. (7) provided evidence for a mechanism of stripping of antigen/MHC complexes by T cells. The interaction of antigen-specific T cells with the APCs *in vivo* induced the selective loss of the antigen–MHC ligand from the surface of DCs. Another report describes the transfer of specific GFP–MHC–peptide complexes from transfected fibroblasts to T cells. Among T cells interacting with transfected fibroblasts, about 10% spontaneously dissociated within about 10 min and acquired GFP-labeled complexes from the immunological synapse. The intercellular transfer was peptide-specific and -correlated with the activation state of the T cell, as assessed by CD69 expression (8). Acquisition of membrane molecules from APCs seems to be an inherent feature of activated CD4⁺ T cells, and continues during cell cycle progression (9). Of note, T helper cells and regulatory T cells have a comparable capacity of trogocytosis *in vivo*, as demonstrated by the similar acquisition of MHC II by CD4⁺CD25⁻ (helper T cells) and CD4⁺CD25⁺ (regulatory T cells) cells from HA-transgenic mice adoptively transferred into Balb/c mice followed by immunization with HA (9). Finally, a recent report demonstrates that MHC II was displayed on the surface of TCR transgenic CD8 T cells activated *in vitro* with the cognate peptide. Notably, in mice infected with LCMV Arm i.v., up to 25% of viral peptide-specific CD8⁺ T cells displayed MHC II on their surface. Among the three

major populations of APCs, DCs transferred the most MHC-II onto CD8⁺ T cells (10).

COSTIMULATORY SIGNALS

In addition to the appropriate antigenic signal, APCs may provide costimulatory signals, which are required for optimal activation of naïve T cells. Several ligand/receptor pairs have been described, which potentize the signal induced via the TCR. In particular, signaling downstream of the CD28 receptor on T cells positively regulates proliferation and survival of T cells, as well as their cytokine production (11). The first evidence that B7 ligands could be taken up by T cells was provided by Hwang et al. who showed that rat T cells acquired murine CD80 and CD86, both *in vitro* when co-cultured with murine DCs or *in vivo* when transferred into irradiated SCID mice (5). This acquisition was under the control of either CD28–B7 or TCR–peptide–MHC interaction: indeed, CD28^{-/-} T cells cultured with DC displayed a 10-fold reduced expression of MHC II and CD80, as compared to CD28⁺ T cells. Subsequent studies confirmed these observations in mice, using cocultures of DCs and CD86/CD80 double knock-out T cells (12). The acquisition was directly related to the strength of signals 1 and 2. Interestingly, the observations suggest a different outcome in naïve versus memory T cells: naïve T cells became capable of acting as APCs, whereas memory T cells underwent increased apoptosis.

PHYSIOLOGICAL CONSEQUENCES

POSITIVE REGULATION

Although the physiological consequences of the intercellular transfer are still questionable, several observations suggest an active role in the immune responses. (i) Kedl et al. concluded that T cells may compete with each other by lowering the amount of antigen–MHC complexes on the APCs, and showed that their ability to compete was affected by their affinity for the MHC/antigen complexes, thereby driving the affinity maturation of memory T cell responses (7). These data provided a mechanism for their previous observation that competition between T cells of the same peptide–MHC specificity occurred efficiently *in vivo* (13); (ii) The transferred antigen–MHC complexes appeared associated with molecules that imply continuous signaling, namely the src family kinase p56lck and tyrosine-phosphorylated proteins. The sustained signaling may be required for full activation of T cells even when contacts with DCs are of short duration (8); (iii) CD8⁺ T cells have been shown to acquire MHC class II molecules *in vitro* and *in vivo* in response to viral infection, a transfer which conferred to them the capacity to directly activate CD4⁺ T cells. The direct CD4/CD8 T cell interaction may contribute to help for CD8⁺ T cells and provide an alternative model to the DC licensing or the three cell cluster (10); (iv) the intercellular transfer of antigen–MHC complexes may expand the repertoire of cells that can function as APCs, and regulate an ongoing immune response. This hypothesis would be consistent with a recent report (14) showing that differentiation of CD8⁺ T cells required not only T cell–APC interactions but also T cell–T cell synapses. The authors showed that these T cell interactions promoted critical synaptic cytokine exchange, allowing CD8⁺ T cells to share IFN- γ for example, and interpret their data as a collective decision-making resulting to positive reinforcement.

However, it is possible that, in addition, these synapses could mediate antigen-specific signaling through the captured peptide–MHC complexes.

NEGATIVE REGULATION

Conversely, intercellular transfer may downregulate immune responses. There is some evidence that the presence of APC-derived peptide/MHC complexes on T cells may render them susceptible to fratricide lysis. Huang et al. have indeed shown that T cells cultured with APCs for 1 h were susceptible to lysis provided a high density of peptide/MHC complexes was transferred (4). Another report confirms that triggering of fratricide required extremely high levels of antigenic peptides (15), suggesting that this mechanism of exhaustion would occur in the presence of high antigen concentration, i.e., in certain viral infections.

A few studies revealed an interesting correlation between anergy induction and T cell-mediated APC activity (16–18). Adoptive transfer of MBP-pulsed transformed T cells (expressing high levels of MHC II, CD80, and CD86) resulted in reduced severity of EAE in naïve rats (17), whereas mouse CD4⁺ T cells, which have acquired MHCI/peptide complexes were susceptible to apoptosis and hyporesponsive to the antigen pulsed on mature dendritic cells (18). These observations suggest that T cells may present peptide–MHC complexes in a tolerogenic manner.

It is likely that the nature of the cell that has acquired antigen/MHC would determine the consequence of trogocytosis. In particular, double-negative Tregs have been shown to acquire alloantigen *in vivo*, allowing them to specifically kill syngeneic CD8⁺ T cells that can interact with the alloantigen (19). The outcome of trogocytosis by T helper versus regulatory T cells (following coculture with antigen-pulsed A20 cells) was different, with T helper cells able to drive activation of naïve CD4⁺ T cells and Treg displaying an enhanced suppressive activity (9).

A few reports suggest a cross-regulation between a receptor and its ligand, which could involve intercellular transfer of either molecule. The analysis of ICOS-Tg mice revealed unexpectedly a phenotype resembling ICOS-deficient mice, i.e., reduced titers of IgG1 and IgE in serum and attenuation of germinal center formation. The defect of ICOS-Tg mice in antibody production was not due to an intrinsic defect of T or B lymphocytes but rather to a defect in the *in vivo* environment. It was further shown that APCs displayed reduced ICOSL expression (at the protein but not the mRNA level), suggesting a negative feedback regulation by ICOSL downregulation in response to ICOS expression (20). Similarly, Kuka et al. studied mice deficient in either CD27 or CD70 and found that CD27 and CD70 cell-surface expression was reciprocally regulated. When CD27 was blocked, CD70 transcripts increased more than 300-fold, indicating that the interaction of CD27 with CD70 inhibits CD70 transcription (21). Our own studies revealed a distinct mechanism of regulation, as we found that thymus-derived Tregs and activated T cells inhibited CD70 expression on DCs at the protein level, by a mechanism that involves transfer of intact CD27 from the T cell to the DCs (Dhainaut et al., submitted). Collectively, these observations highlight a reciprocal regulation of a unique ligand/receptor pair, which may provide rapid fine-tuning of ongoing T cell responses.

HUMAN STUDIES

A few reports suggest that a similar acquisition of membrane molecules may occur in humans. Human T cells cultured with DCs acquired CD80, and the level of “expression” was related to the level of CD80 expression on APCs and was enhanced upon TCR engagement (by anti-CD3 mAb or alloMHC recognition). The transfer of CD80 to T cells was mediated by its receptor, as blockade with soluble fusion proteins (sCD28, sCTLA-4, and sCD80) prevented its acquisition, and resulted in T cells able to provide costimulatory signals (22). Another report confirmed these observations and showed that T cells could acquire HLA-DR and B7 molecules from DCs during an alloresponse and then acted as APCs to resting autologous T cells (23). In addition to CD28/CTLA-4 and their ligands, other receptor ligand pairs can provide costimulatory signals to T cells. Baba et al. showed in humans that the intact OX40L molecule was transferred from APC to T cell, in various cell combinations, in a contact dependent manner. The transferred OX40L was functional and displayed as discrete punctate pattern on the T cell surface (24).

T cells can also be imprinted by tumor antigen. A high proportion (ranging from 10 to 70%) of melanoma specific T cell clones were shown to acquire tumor antigens *in vitro* and this transfer could be used to identify tumor antigen-specific T cells in patients. Thus, freshly isolated tumor-infiltrating lymphocytes expressed melanoma antigens and the tumor antigen imprinting correlated with antitumor T cell function. Indeed, tumor antigen-imprinted CTL exhibited superior killing activity, suggesting that the antigen acquisition may enhance their effector function (25, 26).

MOLECULAR MECHANISM OF ACQUISITION

Martinez-Martin et al. have examined the mechanism of TCR internalization at the immunological synapse and showed that it was coupled to the TCR-triggered acquisition of membrane fragments from the antigen-presenting cell (27). They further showed that two Ras family GTPases, TC21 and RhoG, which colocalize

with the TCR at the immune synapse (28), mediated internalization of the TCR via a clathrin-independent endocytosis. The authors interpret the process as an incomplete phagocytosis of the whole APC by the T cell, which results in the removal of an APC fragment. Whether the TCR and the trogocytosed APC membrane fragments that include MHC complexes are recycled or degraded is an important question, as it would have opposite impact on the immune response. TC21- and RhoG-deficient T cells showed increased responsiveness to TCR stimulation, suggesting that TCR downregulation (and possibly acquisition of APC fragments) could be involved in the termination of the response.

As peptide/MHC complexes, costimulatory, and adhesion molecules appear to be co-transferred to T cells (5), it is likely that the mechanism described by Martinez-Martin et al. could be a common mechanism for membrane exchange at the immune synapse. The strength of the interaction, which results from several ligand/receptor interaction, seems to determine the amount of membrane fragments transferred. Accordingly, CD28-deficient T cells exhibited less stable interactions with APCs in cocultures and absorbed less MHC molecules than CD28 competent T cells (5), and the acquisition of CD80 was directly correlated to the strength of signal 1, i.e., the concentration of antigenic peptide (12). In addition to trogocytosis (which allows rapid transfer of intact surface molecules by phagocytosis probably at the immune synapse), other mechanisms exist which involve transfer of various types of vesicles with a slower kinetics [for review, see (29, 30)]. The respective contribution of both mechanisms remains to be determined but could be dependent on the nature of the cells, their state of activation, and the microenvironment.

CONCLUSION

The outcome of the process of membrane exchange remains elusive but could lead to an enhancement of the resulting immune response (Figure 1). In particular, T cells which have

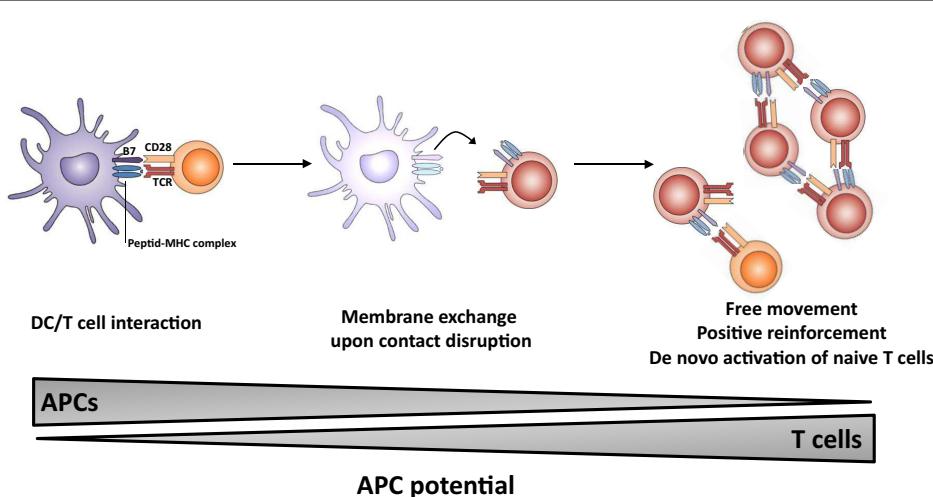


FIGURE 1 | Proposed model for the role of membrane exchange in T cell activation. The first step involves close interaction between APC and T cell (left panel) and acquisition of MHC and costimulatory molecules by T cells

upon dissociation (middle panel). The second step involves presentation of antigen and costimulatory molecules by T cells, leading to sustained activation (and possibly naïve T cell priming) in the absence of conventional APCs.

acquired molecules from APCs may gain some capacity of antigen presentation, thereby (i) multiplying the number of cells presenting the antigen, (ii) prolonging the presentation step in the absence of DC/T interaction possibly outside lymphoid organs, i.e., in peripheral tissue; (iii) allowing T cells to move freely and interact with effector lymphocytes (B cells and CTL). It is of note that T lymphocytes do probably display a higher lifespan than dendritic cells. Thus, the membrane exchange would result in sustained autonomous activation without requirement for prolonged T-cell interaction between DC, CD4 T helper, and effector cell. Collectively, these observations highlight the multiple roles of the immunological synapse, which appears to trigger membrane-bound receptor-ligand interactions, cytokine release as well as membrane exchange.

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Primary immunodeficiency diseases: an update on the classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency

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We report the updated classification of primary immunodeficiencies (PIDs) compiled by the Expert Committee of the International Union of Immunological Societies. In comparison to the previous version, more than 30 new gene defects are reported in this updated version. In addition, we have added a table of acquired defects that are phenocopies of PIDs. For each disorder, the key clinical and laboratory features are provided. This classification is the most up-to-date catalog of all known PIDs and acts as a current reference of the knowledge of these conditions and is an important aid for the molecular diagnosis of patients with these rare diseases.

Keywords: primary immunodeficiencies, IUIS, classification, genetic defects, genotype

BACKGROUND

The International Union of Immunological Societies (IUIS) Expert Committee on Primary Immunodeficiency met in New York on 19th–21st April 2013 to update the classification of human primary immunodeficiencies (PIDs). This report represents the most current and complete catalog of known PIDs. It serves as a reference for these conditions and provides a framework to help in the diagnostic approach to patients suspected to have PID.

As in previous reports, we have classified the conditions into major groups of PIDs and these are now represented in nine different tables. In each table, we list the condition, its genetic defect if known, and the major immunological and in some conditions the non-immunological abnormalities associated with the disease. The classification this year differs slightly from the previous edition in that **Table 1** lists combined immunodeficiencies without non-immunologic phenotypes, whereas **Table 2** refers to combined

Table 1 | Combined immunodeficiencies.

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
1. T ⁺ -B ⁺ severe combined immunodeficiency (SCID)							
(a) γc deficiency	Mutation of <i>IL2RG</i> Defect in γ chain of receptors for IL-2, -4, -7, -9, -15, -21	XL	Markedly decreased	Normal or increased	Decreased	Markedly decreased NK cells	300400
(b) JAK3 deficiency	Mutation of <i>JAK3</i> Defect in Janus-activating kinase 3	AR	Markedly decreased	Normal or increased	Decreased	Markedly decreased NK cells	600173
(c) IL7R α deficiency	Mutation of <i>IL7RA</i> Defect in IL7 receptor α chain	AR	Markedly decreased	Normal or increased	Decreased	Normal NK cells	146661
(d) CD45 deficiency ^a	Mutation of <i>PTPRC</i> Defect in CD45	AR	Markedly decreased	Normal	Decreased	Normal γ/δ T cells	151460
(e) CD3 δ deficiency	Mutation of <i>CD3D</i> Defect in CD3 δ chain of T cell antigen receptor complex	AR	Markedly decreased	Normal	Decreased	Normal NK cells No γ/δ T cells	186790
(f) CD3 ϵ deficiency ^a	Mutation of <i>CD3E</i> Defect in CD3 ϵ chain of T cell antigen receptor complex	AR	Markedly decreased	Normal	Decreased	Normal NK cells No γ/δ T cells	186830
(g) CD3 ζ deficiency ^a	Mutation of <i>CD3Z</i> Defect in CD3 ζ chain of T cell antigen receptor complex	AR	Markedly decreased	Normal	Decreased	Normal NK cells No γ/δ T cells	186740
(h) Coronin-1A deficiency ^a	Mutation of <i>CORO1A</i> defective thymic egress of T cells and defective T cell locomotion	AR	Markedly decreased	Normal	Decreased	Detectable thymus EBV associated B cell lymphoproliferation	605000
2. T ⁻ -B ⁻ SCID							
(i) DNA recombination defects							
(a) RAG 1 deficiency	Mutation of <i>RAG1</i> Defective VDJ recombination; defect of recombinase activating gene (RAG) 1	AR	Markedly decreased	Markedly decreased	Decreased		601457
(a) RAG 2 deficiency	Mutation of <i>RAG2</i> Defective VDJ recombination; defect of recombinase activating gene (RAG) 2	AR	Markedly decreased	Markedly decreased	Decreased		601457
(b) DCLRE1C (artemis) deficiency	Mutation of <i>ARTEMIS</i> Defective VDJ recombination; defect in artemis DNA recombinase repair protein	AR	Markedly decreased	Markedly decreased	Decreased	Radiation sensitivity	602450
(c) DNA PKcs deficiency ^a	Mutation of <i>PRKDC</i> - Defective VDJ recombination; defect in DNA PKcs Recombinase repair protein	AR	Markedly decreased	Markedly decreased	Decreased	Radiation sensitivity, microcephaly, and developmental defects	600899
(ii) Reticular dysgenesis, AK2 deficiency	Mutation of <i>AK2</i> Defective maturation of lymphoid and myeloid cells (stem cell defect) Defect in mitochondrial adenylate kinase 2	AR	Markedly decreased	Decreased or normal	Decreased	Granulocytopenia and deafness	103020

(Continued)

Table 1 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
(iii) Adenosine deaminase deficiency (ADA) deficiency	Mutation of ADA absent ADA activity, elevated lymphotoxic metabolites (dATP, S-adenosyl homocysteine)	AR	Absent from birth (null mutations) or progressive decrease	Absent from birth of progressive decrease	Progressive decrease	Decreased NK cells, often with costochondral junction flaring, neurological features, hearing impairment, lung and liver manifestations; partial ADA deficiency may lead to delayed or milder presentation	102700
Combined immunodeficiencies generally less profound than severe combined immunodeficiency							
3. CD40 ligand deficiency	Mutation of <i>CD40LG</i> defects in CD40 ligand (CD40L; also called TNFSF5 or CD154) cause defective isotype switching and impaired dendritic cell signaling	XL	Normal; may progressively decrease	slgM ⁺ and slgD ⁺ B cells present, other surface isotype positive B cells absent	IgM increased or normal, other isotypes decreased	Neutropenia, thrombocytopenia; hemolytic anemia, biliary tract and liver disease, opportunistic infections	300386
4. CD40 deficiency ^a	Mutation of <i>CD40</i> (also called TNFRSF5) defects in CD40 cause defective isotype switching and impaired dendritic cell signaling	AR	Normal	IgM ⁺ and IgD ⁺ B cells present, other isotypes absent	IgM increased or normal, other isotypes decreased	Neutropenia, gastrointestinal and liver/biliary tract disease, opportunistic infections	109535
5. Purine nucleoside phosphorylase (PNP) deficiency	Mutation of <i>PNP</i> , absent PNP and T cell and neurologic defects from elevated toxic metabolites, especially dGTP	AR	Progressive decrease	Normal	Normal or decreased	Autoimmune hemolytic anemia, neurological impairment	164050
6. CD3γ deficiency ^a	Mutation of <i>CD3G</i> defect in CD3 γ – component of the T cell antigen receptor complex	AR	Normal, but reduced TCR expression	Normal	Normal		186740
7. CD8 deficiency ^a	Mutation of <i>CD8A</i> , defects of CD8 α chain – important for maturation and function of CD8 T cells	AR	Absent CD8, normal CD4 cells	Normal	Normal		186910
8. ZAP70 deficiency	Mutation in ZAP70 intracellular signaling kinase, acts downstream of TCR	AR	Decreased CD8, normal CD4 cells	Normal	Normal	Autoimmunity in some cases	269840
9. MHC class I deficiency	Mutations in <i>TAP1</i> , <i>TAP2</i> , or <i>TAPBP</i> (tapasin) genes giving MHC class I deficiency	AR	Decreased CD8, normal CD4	Normal	Normal	Vasculitis; pyoderma gangrenosum	604571
10. MHC class II deficiency	Mutation in transcription factors for MHC class II proteins (<i>CIITA</i> , <i>RFX5</i> , <i>RFXAP</i> , <i>RFXANK</i> genes)	AR	Normal number, decreased CD4 cells	Normal	Normal or decreased	Failure to thrive, diarrhea, respiratory tract infections, liver/biliary tract disease	209920
11. ITK deficiency ^a	Mutations in <i>ITK</i> encoding IL-2-inducible T cell kinase required for TCR-mediated activation	AR	Progressive decrease	Normal	Normal or decreased	EBV-associated B cell lymphoproliferation, lymphoma Normal or decreased IgG	613011

(Continued)

Table 1 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
12. SH2D1A deficiency (XLP1)	Mutations in <i>SH2D1A</i> encoding an adaptor protein regulating intracellular signals	XL	Normal or increased activated T cells	Reduced memory B cells	Partially defective NK cell and CTL cytotoxic activity	Clinical and immunologic features triggered by EBV infection: HLH, lymphoproliferation, aplastic anemia, lymphoma Hypogamma globulinemia Absent iNKT cells	308240
13. Cartilage hair hypoplasia	Mutations in <i>RMRP</i> (RNase MRP RNA) involved in processing of mitochondrial RNA and cell cycle control	AR	Varies from severely decreased (SCID) to normal; impaired lymphocyte proliferation	Normal	Normal or reduced antibodies variably decreased	Can present just as combined immunodeficiency without other features of short-limbed dwarfism Also see Table 2	250250
14. MAGT1 deficiency ^a	Mutations in <i>MAGT1</i> , impaired Mg ⁺⁺ flux leading to impaired TCR signaling	XL	Decreased CD4 cells reduced numbers of RTE, impaired T cell proliferation in response to CD3	Normal	Normal	EBV infection, lymphoma; viral infections, respiratory, and GI infections	300715
15. DOCK8 deficiency	Mutations in <i>DOCK8</i> – regulator of intracellular actin reorganization	AR	Decreased impaired T lymphocyte proliferation	Decreased, low CD27+ memory B cells	Low IgM, increased IgE	Low NK cells with impaired function, hypereosinophilia, recurrent infections; severe atopy, extensive cutaneous viral and bacterial (staph.) infections, susceptibility to cancer	243700
16. RhoH deficiency ^a	Mutations in <i>RHOH</i> – an atypical Rho GTPase transducing signals downstream of various membrane receptors	AR	Normal Low naïve T cells and RTE, restricted T cell repertoire and impaired T cells proliferation in response to CD3 stimulation	Normal	Normal	HPV infection, lymphoma, lung granulomas, molluscum contagiosum	602037
17. MST1 deficiency	Mutations in <i>STK4</i> – a serine/threonine kinase	AR	Decreased/increased proportion of terminal differentiated effector memory cells (TEMRA), low naïve T cells, restricted T cell repertoire in the TEMRA population, and impaired T cells proliferation	Decreased	High	Recurrent bacterial, viral, and candidal infections; intermittent neutropenia; EBV-driven lymphoproliferation; lymphoma; congenital heart disease, autoimmune cytopenias; HPV infection	614868

(Continued)

Table 1 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
18. TCR α deficiency ^a	Mutations in <i>TRAC</i> – essential component of the T cell receptor	AR	Normal all CD3 T cells expressed TCR $\gamma\delta$ (or may be better to say: TCR $\alpha\beta$ T cell deficiency), impaired T cells proliferation	Normal	Normal	Recurrent viral, bacterial, and fungal infections, immune dysregulation autoimmunity, and diarrhea	615387
19. LCK deficiency ^a	Defects in <i>LCK</i> – a proximal tyrosine kinase that interacts with TCR	AR	Normal total numbers but CD4+ T cell lymphopenia, low Treg numbers, restricted T cell repertoire, and impaired TCR signaling	Normal	Normal IgG and IgA and increased IgM	Diarrhea, recurrent infections, immune dysregulation autoimmunity	153390
20. MALT1 deficiency ^a	Mutations in <i>MALT1</i> – a caspase-like cysteine protease that is essential for nuclear factor kappa B activation	AR	Normal impaired T cells proliferation	Normal	Normal Impaired antibody response	Bacterial, fungal, and viral infections	604860
21. IL-21R deficiency ^a	Defects in <i>IL-21R</i> – together with common gamma chain binds IL-21	AR	Abnormal T cell cytokine production; abnormal T cell proliferation to specific stimuli	Normal	Normal but impaired specific responses	Susceptibility to cryptosporidium and pneumocystis and cholangitis	605383
22. UNC119 deficiency ^a	Defects in <i>UNC119</i> – an activator of src tyrosine kinases	AD	Low T cells CD4+ T cell lymphopenia, impaired TCR signaling	Mostly low	Normal	Recurrent bacterial, fungal, and viral infections	604011
23. CARD11 deficiency ^a	Defects in <i>CARD11</i> – acts as a scaffold for NF- κ B activity in the adaptive immune response	AR	Normal predominance of naive T lymphocyte, impaired T cells proliferation	Normal predominance of transitional B lymphocytes	Absent/low	<i>Pneumocystis jiroveci</i> pneumonia, bacterial infections	615206
24. OX40 deficiency ^a	Defects in <i>OX40</i> – a co-stimulatory molecule expressed on activated T cells	AR	Normal T cell numbers Low levels of antigen-specific memory CD4+ cells	Normal B cell numbers Lower frequency of memory B cells	Normal	Kaposi's sarcoma; impaired immunity to HHV8	615593
25. IKBKB deficiency ^a	Defects in <i>IKBKB</i> – encodes I κ B kinase 2 a component of the NF- κ B pathway	AR	Normal total T cells; absent regulatory and gdT cells; impaired TCR activation	Normal B cell numbers; impaired BCR activation	Decreased	Recurrent bacterial, viral, and fungal infections; clinical phenotype of SCID	615592
26. Activated PI3K- δ	Mutation in <i>PIK3CD</i> , PI3K- δ gain-of-function	AD	Decreased total numbers of T cells	Decreased total peripheral B cell and switched memory B cells; increased transitional B cells	Reduced IgG2 and impaired antibody to pneumococci and hemophilus	Respiratory infections, bronchiectasis; autoimmunity; chronic EBV, and CMV infection	602839

(Continued)

Table 1 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
27. LRBA deficiency	Mutations in <i>LRBA</i> (lipopolysaccharide responsive beige-like anchor protein)	AR	Normal or decreased CD4 numbers; T cell dysregulation	Low or normal numbers of B cells	Reduced IgG and IgA in most	Recurrent infections, inflammatory bowel disease, autoimmunity; EBV infections	606453
28. CD27 deficiency ^a	Mutations in <i>CD27</i> , encoding TNF-R member superfamily required for generation and long-term maintenance of T cell immunity	AR	Normal	No memory B cells	Hypogamma globulinemia following EBV infection	Clinical and immunologic features triggered by EBV infection, HLH Aplastic anemia, lymphoma Hypogammaglobulinemia Low iNKT cells	615122
29. Omenn syndrome	Hypomorphic mutations in <i>RAG1</i> , <i>RAG2</i> , <i>artemis</i> , <i>IL7RA</i> , <i>RMRP</i> , <i>ADA</i> , <i>DNA ligase IV</i> , <i>IL-2RG</i> , <i>AK2</i> , or associated with DiGeorge syndrome; some cases have no defined gene mutation		Present; restricted T cell repertoire, and impaired function	Normal or decreased	Decreased, except increased IgE	Erythroderma, eosinophilia, adenopathies, hepatosplenomegaly	603554

XL, X-linked inheritance; AR, autosomal recessive inheritance; AD, autosomal dominant inheritance; SCID, severe combined immune deficiencies; EBV, Epstein–Barr virus; Ca⁺⁺, calcium; MHC, major histocompatibility complex, RTE, recent thymic emigrants, HPV, human papillomavirus.

^aTen or fewer unrelated cases reported in the literature.

Infants with SCID who have maternal T cells engraftment may have T cells that do not function normally; these cells may cause autoimmune cytopenias or graft versus host disease. Hypomorphic mutations in several of the genes that cause SCID may result in Omenn syndrome (OS), or “leaky” SCID or a less profound CID phenotype. Both OS and leaky SCID can be associated with higher numbers of T cells and reduced rather than absent activation responses when compared with typical SCID caused by null mutations. A spectrum of clinical findings including typical SCID, OS, leaky SCID, granulomas with T lymphopenia, autoimmunity, and CD4+ T lymphopenia can be found with RAG gene defects. RAC2 deficiency is a disorder of leukocyte motility and is reported in Table 5; however, one patient with RAC2 deficiency was found to have absent T cell receptor excision circles (TRECs) by newborn screening, but T cell numbers and mitogen responses were not impaired. For additional syndromic conditions with T cell lymphopenia, such as DNA repair defects, cartilage hair hypoplasia, IKAROS deficiency, and NEMO syndrome, see Tables 2 and 6; however, it should be noted that individuals with the most severe manifestations of these disorders could have clinical signs and symptoms of SCID. Severe folate deficiency (such as with malabsorption due to defects in folate carrier or transporter genes *SLC10A1* or *PCFT*) and some metabolic disorders, such as methylmalonic aciduria, may present with reversible profound lymphopenia in addition to their characteristic presenting features.

immunodeficiencies with syndromic features, as increasing numbers of these are being identified. The title and classification of Tables 3–8 present the same major PID groups as in the previous report.

In this updated version, we have added a new category in Table 9 in which “Phenocopies of PID” are listed. This has resulted from our understanding and study of conditions that present as inherited immunodeficiencies, but which are not due to germline mutations and instead arise from acquired mechanisms. Examples include somatic mutations in specific immune cell populations that give rise to the phenotype of autoimmune lymphoproliferative syndrome (ALPS), and also autoantibodies against specific cytokines or immunological factors, with depletion of these factors leading to immunodeficiency. It is likely that increasing numbers of PID phenocopies will be identified in the future, and this may be the start of a much longer table.

As with all complex diseases, any classification cannot be strictly adhered to. Certain conditions fall into more than one category

and so appear in more than one table. For example, CD40L ligand deficiency is reported in both Tables 1 and 3 as it was initially identified as a defect of B cell isotype switching but is now known to be a defect of co-stimulatory T cell help and function. Similarly, XLP1 due to defects in SH2D1A is listed in Table 1 – combined immunodeficiencies, due to defects of T cell cytotoxicity, T cell help, and B cell maturation, but also in Table 4 – diseases of immune dysregulation, due to the susceptibility to hemophagocytosis. There is a growing appreciation that there can be wide phenotypic variability within a specific genotype that is a product of varied specific mutations between different patients as well as other host and/or environmental factors. The complexities of these conditions in terms of clinical and immunological presentation and heterogeneity cannot be easily captured in the limited space of a table format. For this reason, the furthest left column contains the Online Mendelian Inheritance in Man (OMIM) reference for each condition to allow access to greater detail and updated information.

Table 2 | Combined immunodeficiencies with associated or syndromic features.

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
1. Congenital thrombocytopenia							
(a) Wiskott– Aldrich syndrome (WAS)	Mutations in <i>WAS</i> ; cytoskeletal, and immunologic synapse defect affecting hematopoietic stem cell derivatives	XL	Progressive decrease, abnormal lymphocyte responses to anti-CD3	Normal	Decreased IgM: antibody to polysaccharides particularly decreased; often increased IgA and IgE	Thrombocytopenia with small platelets; eczema; lymphoma; autoimmune disease; IgA nephropathy; bacterial and viral infections. XL thrombocytopenia is a mild form of WAS, and XL neutropenia is caused by missense mutations in the GTPase binding domain of WASP	301000
(b) WIP deficiency ^a							
	Mutations in <i>WIPF1</i> ; cytoskeletal and immunologic synapse defect affecting hematopoietic stem cell derivatives	AR	Reduced, defective lymphocyte responses to anti-CD3	Low	Normal, except for increased IgE	Recurrent infections; eczema; thrombocytopenia. WAS-like phenotype	614493
2. DNA repair defects (other than those in Table 1)							
(a) Ataxia– telangiectasia	Mutations in <i>ATM</i> ; disorder of cell cycle checkpoint; and DNA double-strand break repair	AR	Progressive decrease	Normal	Often decreased IgA, IgE, and IgG subclasses; increased IgM monomers; antibodies variably decreased	Ataxia; telangiectasia; pulmonary infections; lymphoreticular and other malignancies; increased alpha fetoprotein and increased radiosensitivity; chromosomal instability	208900
(b) Ataxia– telangiectasia– like disease (ATLD) ^a	Hypomorphic mutations in <i>MRE11</i> ; disorder of cell cycle checkpoint and DNA double-strand break repair	AR	Progressive decrease	Normal	Antibodies variably decreased	Moderate ataxia; pulmonary infections; severely increased radiosensitivity	604391
(c) Nijmegen breakage syndrome	Hypomorphic mutations in <i>NBS1</i> (<i>Nibrin</i>); disorder of cell cycle checkpoint and DNA double-strand break repair	AR	Progressive decrease	Variably reduced	Often decreased IgA, IgE, and IgG subclasses; increased IgM; antibodies variably decreased	Microcephaly; bird-like face; lymphomas; solid tumors; increased radiosensitivity; chromosomal instability	251260
(d) Bloom syndrome	Mutations in <i>BLM</i> ; RecQ-like helicase	AR	Normal	Normal	Reduced	Short stature; bird-like face; sun-sensitive erythema; marrow failure; leukemia; lymphoma; chromosomal instability	210900

(Continued)

Table 2 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
(e) Immunodeficiency with centromeric instability and facial anomalies (ICF)	Mutations in DNA methyltransferase <i>DNMT3B</i> (ICF1) resulting in defective DNA methylation	AR	Decreased or normal; responses to PHA may be decreased	Decreased or normal	Hypogamma globulinemia; variable antibody deficiency	Facial dysmorphic features; macroglossia; bacterial/opportunistic infections; malabsorption; cytopenias; malignancies; multiradial configurations of chromosomes 1, 9, 16; no DNA breaks	242860
(f) Immunodeficiency with centromeric instability and facial anomalies (ICF)	Mutations in <i>ZBTB24</i> (ICF2)	AR	Decreased or normal; responses to PHA may be decreased	Decreased or normal	Hypogamma globulinemia; variable antibody deficiency	Facial dysmorphic features; macroglossia; bacterial/opportunistic infections; malabsorption; cytopenias; malignancies; multiradial configurations of chromosomes 1, 9, 16	242860
(g) PMS2 deficiency	Mutations in <i>PMS2</i> , resulting in class switch recombination deficiency due to impaired mismatch repair	AR	Normal	Switched and non-switched B cells are reduced	Low IgG and IgA, elevated IgM, abnormal antibody responses	Recurrent infections; café-au-lait spots; lymphoma, colorectal carcinoma, brain tumor	600259
(h) RNF168 deficiency ^a	Mutations in <i>RNF168</i> , resulting in defective DNA double-strand break repair	AR	Normal	Normal	Low IgG or low IgA	Short stature; mild motor control to ataxia and normal intelligence to learning difficulties; mild facial dysmorphism to microcephaly; increased radiosensitivity	611943
(i) MCM4 deficiency	Mutations in <i>MCM4</i> (minichromosome maintenance complex component 4) gene involved in DNA replication and repair	AR	Normal	Normal	Normal	Viral infections (EBV, HSV, VZV) Adrenal failure Short stature	609981
3. Thymic defects with additional congenital anomalies							
(a) DiGeorge anomaly	Contiguous gene defect in 90% affecting thymic development; may also be due to heterozygous mutation in <i>TBX1</i> (chromosome 22q11.2 deletion or <i>TBX1</i> haploinsufficient syndrome)	<i>De novo</i> defect (majority) or AD	Decreased or normal; 5% have <1500 CD3 T cells/µL	Normal	Normal or decreased	Hypoparathyroidism, conotruncal malformation; abnormal facies; large deletion (3 Mb) in 22q11.2 (or rarely a deletion in 10p)	188400

(Continued)

Table 2 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
(b) CHARGE syndrome	Variable defects of the thymus and associated T cell abnormalities often due to deletions or mutations in <i>CHD7</i> , <i>SEMA3E</i> , or as yet unknown genes	<i>De novo</i> defect (majority) or AD	Decreased or normal; some have <1500 CD3 T cells/ μ L	Normal	Normal or decreased	Coloboma, heart anomaly, choanal atresia, retardation, genital and ear anomalies	214800 608892
4. Immune-osseous dysplasias							
(a) Cartilage hair hypoplasia	Mutations in <i>RMRP</i> (RNase MRP RNA) involved in processing of mitochondrial RNA and cell cycle control	AR	Varies from severely decreased (SCID) to normal; impaired lymphocyte proliferation	Normal	Normal or reduced. Antibodies variably decreased	Short-limbed dwarfism with metaphyseal dysostosis, sparse hair, bone marrow failure, autoimmunity, susceptibility to lymphoma and other cancers, impaired spermatogenesis, neuronal dysplasia of the intestine	250250
(b) Schimke syndrome	Mutations in <i>SMARCAL1</i> involved in chromatin remodeling	AR	Decreased	Normal	Normal	Short stature, spondyloepiphyseal dysplasia, intrauterine growth retardation, nephropathy; bacterial, viral, and fungal infections; may present as SCID; bone marrow failure	242900
5. Hyper-IgE syndromes (HIES)							
(a) AD-HIES (Job's syndrome)	Dominant-negative heterozygous mutations in <i>STAT3</i>	AD Often <i>de novo</i> defect	Normal Th-17 and T follicular helper cells decreased	Normal Switched and non-switched memory B cells are reduced; BAFF level increased	Elevated IgE; specific antibody production decreased	Distinctive facial features (broad nasal bridge), eczema, osteoporosis, and fractures, scoliosis, delay of shedding primary teeth, hyperextensible joints, bacterial infections (skin and pulmonary abscesses, pneumatoceles) due to <i>Staphylococcus aureus</i> , candidiasis, aneurysm formation	147060
(i) Tyk2 deficiency ^a	Mutation in <i>TYK2</i>	AR	Normal, but multiple cytokine signaling defect	Normal	(\pm) Elevated IgE	Susceptibility to intracellular bacteria (<i>Mycobacteria</i> , <i>Salmonella</i>), fungi, and viruses	611521

(Continued)

Table 2 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
(ii) DOCK8 deficiency	Mutations in <i>DOCK8</i> – regulator of intracellular actin reorganization	AR	Decreased impaired T lymphocyte proliferation	Decreased, low CD27+ memory B cells	Low IgM, increased IgE	Low NK cells with impaired function, hypereosinophilia, recurrent infections; severe atopy, extensive cutaneous viral and bacterial (staph.) infections, susceptibility to cancer	243700
6. Dyskeratosis congenital (DKC)							
(a) XL-DKC	Mutations in dyskerin (<i>DKC1</i>) (Hoyeraal–Hreidarsson syndrome)	XL	Progressive decrease	Progressive decrease	Variable	Intrauterine growth retardation, microcephaly, nail dystrophy, recurrent infections, digestive tract involvement, pancytopenia, reduced number and function of NK cells	305000
(b) AR-DKC due to NHP2 deficiency	Mutation in <i>NOLA2 (NHP2)</i>	AR	Decreased	Variable	Variable	Pancytopenia, sparse scalp hair and eyelashes, prominent periorbital telangiectasia, and hypoplastic/dysplastic nails	613987
(c) AR-DKC due to NOP10 deficiency	Mutation in <i>NOLA3 (NOP10 PCFT)</i>	AR	Decreased	Variable	Variable	Pancytopenia, sparse scalp hair and eyelashes, prominent periorbital telangiectasia, and hypoplastic/dysplastic nails	224230
(d) AR-DKC due to RTEL1 deficiency	Mutation in <i>(RTEL1)</i>	AR	Decreased	Variable	Variable	Pancytopenia, sparse scalp hair and eyelashes, prominent periorbital telangiectasia, and hypoplastic/dysplastic nails	608833
(e) AD-DKC due to TERC deficiency	Mutation in <i>TERC</i>	AD	Variable	Variable	Variable	Reticular hyperpigmentation of the skin, dystrophic nails, osteoporosis premalignant leukokeratosis of the mouth mucosa, palmar hyperkeratosis, anemia, pancytopenia	127550

(Continued)

Table 2 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
(f) AD-DKC due to TERT deficiency	Mutation in <i>TERT</i>	AD	Variable	Variable	Variable	Reticular hyperpigmentation of the skin, dystrophic nails, osteoporosis premalignant leukokeratosis of the mouth mucosa, palmar hyperkeratosis, anemia, pancytopenia	614742
(g) AD-DKC due to TINF2 deficiency	Mutation in <i>TINF2</i>	AD	Variable	Variable	Variable	Reticular hyperpigmentation of the skin, dystrophic nails, osteoporosis premalignant leukokeratosis of the mouth mucosa, palmar hyperkeratosis, anemia, pancytopenia	613990
7. Defects of vitamin B12 and folate metabolism							
(a) TCN2 deficiency	Mutation in <i>TCN2</i> ; encodes transcobalamin, a transporter of cobalamin into blood cells	AR	Normal	Variable	Decreased	Megaloblastic anemia, pancytopenia, untreated for prolonged periods results in mental retardation	275350
(b) SLC46A1 deficiency	Mutation in <i>SLC46A1</i> ; a proton coupled folate transporter	AR	Variable numbers and activation profile	Variable	Decreased	Megaloblastic anemia, failure to thrive untreated for prolonged periods results in mental retardation	229050
(c) MTHFD1 ^a deficiency	Mutations in <i>MTHFD1</i> ; essential for processing of single-carbon folate derivatives	AR	Low	Low	Decreased	Megaloblastic anemia, failure to thrive neutropenia, seizures, mental retardation	
8. Cornel-Netherton syndrome	Mutations in <i>SPINK5</i> resulting in lack of the serine protease inhibitor LEKTI, expressed in epithelial cells	AR	Normal	Switched and non-switched B cells are reduced	Elevated IgE and IgA Antibody variably decreased	Congenital ichthyosis, bamboo hair, atopic diathesis, increased bacterial infections, failure to thrive	256500
9. Winged helix deficiency (Nude) ^a	Defects in forkhead box N1 transcription factor encoded by <i>FOXN1</i>	AR	Markedly decreased	Normal	Decreased	Alopecia, abnormal thymic epithelium, impaired T cell maturation	600838
10. ORAI-I deficiency ^a	Mutation in <i>ORAI1</i> , a Ca ⁺⁺ release-activated channel (CRAC) modulatory component	AR	Normal number, but defective TCR-mediated activation	Normal	Normal	Autoimmunity, anhydrotic ectoderm dysplasia, non-progressive myopathy defective TCR-mediated activation	610277

(Continued)

Table 2 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Serum Ig	Associated features	OMIM number
11. STIM1 deficiency ^a	Mutations in <i>STIM1</i> , a stromal interaction molecule 1	AR	Normal number, but defective TCR-mediated activation	Normal	Normal	Autoimmunity, anhydrotic ectodermal dysplasia, non-progressive myopathy defective TCR-mediated activation	605921
12. STAT5b deficiency ^a	Mutations in <i>STAT5B</i> , signal transducer, and transcription factor, essential for normal signaling from IL-2 and 15, key growth factors for T and NK cells	AR	Modestly decreased	Normal	Normal	Growth-hormone insensitive dwarfism Dysmorphic features Eczema Lymphocytic interstitial pneumonitis, autoimmunity	245590
13. Hepatic veno-occlusive disease with immunodeficiency (VODI)	Mutations in <i>SP110</i>	AR	Normal (decreased memory T cells)	Normal (decreased memory B cells)	Decreased IgG, IgA, IgM, absent germinal centers, absent tissue plasma cells	Hepatic veno-occlusive disease; <i>Pneumocystis jiroveci</i> pneumonia; susceptibility to CMV, <i>Candida</i> ; thrombocytopenia; hepatosplenomegaly	235550
14. IKAROS deficiency ^a	Mutation in <i>IKAROS</i>	AD <i>de novo</i>	Normal, but impaired lymphocyte proliferation	Absent	Presumably decreased	Anemia, neutropenia, thrombocytopenia	Not assigned
15. FILS syndrome ^a	Mutation in <i>POLE1</i> ; defective DNA replication	AR	Low naïve T cells; decreased T cell proliferation	Low memory B cells	Decreased IgM and IgG; lack of antibodies to polysaccharide antigens	Mild facial dysmorphism (malar hypoplasia, high forehead), livedo, short stature; recurrent upper and lower respiratory tract infections, recurrent pulmonary infections, and recurrent meningitis	615139
16. Immunodeficiency with multiple intestinal atresias	Mutation in <i>TTC7A</i> [tetrastricopeptide repeat (TPR) domain 7A] protein of unknown function	AR	Variable, but sometimes absent	Normal	Decreased	Multiple intestinal atresias, often with intrauterine polyhydramnios and early demise; some with SCID phenotype	243150

SCID, severe combined immune deficiencies; XL, X-linked inheritance; AR, autosomal recessive inheritance; AD, autosomal dominant inheritance; MSMD, Mendelian susceptibility of mycobacterial disease.

^aTen or fewer unrelated cases reported in the literature.

T and B cell number and function in these disorders exhibit a wide range of abnormality; the most severely affected cases meet diagnostic criteria for SCID or leaky SCID and require immune system restoring therapy such as allogeneic hematopoietic cell transplantation. While not all *DOCK8*-deficient patients have elevated serum IgE, most have recurrent viral infections and malignancies as a result of combined immunodeficiency. AR-HIES due to *Tyk2* deficiency is also listed in **Table 6**, because of its association with atypical mycobacterial disease resulting in MSMD. Riddle syndrome is caused by mutations in a gene involved in DNA double-strand break repair and is associated with hypogammaglobulinemia. Autosomal dominant and autosomal recessive forms of dyskeratosis congenita are included in this table. IKAROS-deficiency represents a single prematurely born infant who died at the age of 87 days and who had absent B and NK cells and non-functional T cells.

Table 3 | Predominantly antibody deficiencies.

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Serum Ig	Associated features	OMIM number
1. Severe reduction in all serum immunoglobulin isotypes with profoundly decreased or absent B cells					
(a) BTK deficiency	Mutations in <i>BTK</i> , a cytoplasmic tyrosine kinase activated by crosslinking of the BCR	XL	All isotypes decreased in majority of patients; some patients have detectable immunoglobulins	Severe bacterial infections; normal numbers of pro-B cells	300300
(b) μ Heavy chain deficiency	Mutations in μ heavy chain; essential component of the pre-BCR	AR	All isotypes decreased	Severe bacterial infections; normal numbers of pro-B cells	147020
(c) λ 5 Deficiency ^a	Mutations in <i>I5</i> ; part of the surrogate light chain in the pre-BCR	AR	All isotypes decreased	Severe bacterial infections; normal numbers of pro-B cells	146770
(d) Ig α deficiency ^a	Mutations in <i>Iga</i> (<i>CD79a</i>); part of the pre-BCR and BCR	AR	All isotypes decreased	Severe bacterial infections; normal numbers of pro-B cells	112205
(e) Ig β deficiency ^a	Mutations in <i>Igb</i> (<i>CD79b</i>); part of the pre-BCR and BCR	AR	All isotypes decreased	Severe bacterial infections; normal numbers of pro-B cells	147245
(f) BLNK deficiency ^a	Mutations in <i>BLNK</i> ; a scaffold protein that binds to BTK	AR	All isotypes decreased	Severe bacterial infections; normal numbers of pro-B cells	604615
(g) PI3 kinase deficiency ^a	Mutations in <i>PIK3R1</i> ; a kinase involved in signal transduction in multiple cell types	AR	All isotypes decreased	Severe bacterial infections; decreased or absent pro-B cells	171833
(h) E47 transcription factor deficiency ^a	Mutations in <i>TCF3</i> ; a transcription factor required for control of B cell development	AD	All isotypes decreased	Recurrent bacterial infections	147141
(i) Myelodysplasia with hypogamma-globulinemia	May have monosomy 7, trisomy 8, or dyskeratosis congenita	Variable	One or more isotypes may be decreased	Infections; decreased number of pro-B cells	Not assigned
(j) Thymoma with immunodeficiency	Unknown	None	One or more isotypes may be decreased	Bacterial and opportunistic infections; autoimmunity; decreased number of pro-B cells	Not assigned
2. Severe reduction in at least two serum immunoglobulin isotypes with normal or low number of B cells					
(a) Common variable immunodeficiency disorders	Unknown	Variable	Low IgG and IgA and/or IgM	Clinical phenotypes vary: most have recurrent infections, some have polyclonal lymphoproliferation, autoimmune cytopenias, and/or granulomatous disease	Not assigned
(b) ICOS deficiency ^a	Mutations in <i>ICOS</i> ; a co-stimulatory molecule expressed on T cells	AR	Low IgG and IgA and/or IgM	Recurrent infections; autoimmunity, gastroenteritis, granuloma in some	604558
(c) CD19 deficiency ^a	Mutations in <i>CD19</i> ; transmembrane protein that amplifies signal through BCR	AR	Low IgG and IgA and/or IgM	Recurrent infections; may have glomerulonephritis	107265
(d) CD81 deficiency ^a	Mutations in <i>CD81</i> ; transmembrane protein that amplifies signal through BCR	AR	Low IgG, low or normal IgA and IgM	Recurrent infections; may have glomerulonephritis	186845

(Continued)

Table 3 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Serum Ig	Associated features	OMIM number
(e) CD20 deficiency ^a	Mutations in <i>CD20</i> ; a B cell surface receptor involved in B cell development and plasma cell differentiation	AR	Low IgG, normal or elevated IgM and IgA	Recurrent infections	112210
(f) CD21 deficiency ^a	Mutations in <i>CD21</i> ; also known as complement receptor 2 and forms part of the CD19 complex	AR	Low IgG; impaired anti-pneumococcal response	Recurrent infections	614699
(g) TACI deficiency	Mutations in <i>TNFRSF13B</i> (TACI); a TNF receptor family member found on B cells and is a receptor for BAFF and APRIL	AD or AR or complex	Low IgG and IgA and/or IgM	Variable clinical expression	604907
(h) LRBA deficiency	Mutations in <i>LRBA</i> (lipopolysaccharide responsive beige-like anchor protein)	AR	Reduced IgG and IgA in most	Recurrent infections, inflammatory bowel disease, autoimmunity; EBV infections	606453
(i) BAFF receptor deficiency ^a	Mutations in <i>TNFRSF13C</i> (BAFF-R); a TNF receptor family member found on B cells and is a receptor for BAFF	AR	Low IgG and IgM	Variable clinical expression	606269
(j) TWEAK ^a	Mutations in <i>TWEAK</i>	AD	Low IgM and IgA; lack of anti-pneumococcal antibody	Pneumonia, bacterial infections, warts; thrombocytopenia; neutropenia	602695
(k) NFKB2 deficiency ^a	Mutations in <i>NFKB2</i> ; an essential component of the non-canonical NF-κB pathway	AD	Low IgG and IgA and IgM	Recurrent infections	615577
(l) Warts, hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome	Gain-of-function mutations of <i>CXCR4</i> , the receptor for CXCL12	AD	Panhypogammaglobulinemia, Warts/human papilloma virus (HPV) infection Decreased B cells Neutropenia Reduced B cell number Hypogammaglobulinemia		193670
3. Severe reduction in serum IgG and IgA with normal/elevated IgM and normal numbers of B cells					
(a) CD40L deficiency	Mutations in <i>CD40LG</i> (also called <i>TNFSF5</i> or <i>CD154</i>)	XL	IgG and IgA decreased; IgM may be normal or increased; B cell numbers may be normal or increased	Bacterial and opportunistic infections, neutropenia, autoimmune disease	300386
(b) CD40 deficiency ^a	Mutations in <i>CD40</i> (also called <i>TNFRSF5</i>)	AR	Low IgG and IgA; normal or raised IgM	Bacterial and opportunistic infections, neutropenia, autoimmune disease	109535
(c) AID deficiency	Mutations in <i>AICDA</i> gene	AR	IgG and IgA decreased; IgM increased	Bacterial infections, enlarged lymph nodes, and germinal centers	605257
(d) UNG deficiency	Mutations in <i>UNG</i>	AR	IgG and IgA decreased; IgM increased	Enlarged lymph nodes and germinal centers	191525
4. Isotype or light chain deficiencies with generally normal numbers of B cells					
(a) Ig heavy chain mutations and deletions	Mutation or chromosomal deletion at 14q32	AR	One or more IgG and/or IgA subclasses as well as IgE may be absent	May be asymptomatic	Not assigned

(Continued)

Table 3 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Serum Ig	Associated features	OMIM number
(b) κ Chain deficiency ^a	Mutations in Kappa constant gene	AR	All immunoglobulins have lambda light chain	Asymptomatic	147200
(c) Isolated IgG subclass deficiency	Unknown	Variable	Reduction in one or more IgG subclass	Usually asymptomatic; a minority may have poor antibody response to specific antigens and recurrent viral/bacterial infections	Not assigned
(d) IgA with IgG subclass deficiency	Unknown	Variable	Reduced IgA with decrease in one or more IgG subclass	Recurrent bacterial infections	Not assigned
(e) PRKC δ deficiency ^a	Mutation in <i>PRKCD</i> ; encoding a member of the protein kinase C family critical for regulation of cell survival, proliferation, and apoptosis	AR	Low IgG levels; IgA and IgM above the normal range	Recurrent infections; EBV chronic infection Lymphoproliferation SLE-like autoimmunity (nephrotic and antiphospholipid syndromes)	615559
(f) Activated PI3K-δ	Mutation in <i>PIK3CD</i> , PI3K-δ	AD gain-of-function	Reduced IgG2 and impaired antibody to pneumococci and hemophilus	Respiratory infections, bronchiectasis; autoimmunity; chronic EBV, CMV infection	602839
(g) Selective IgA deficiency	Unknown	Variable	IgA decreased/absent	Usually asymptomatic; may have recurrent infections with poor antibody responses to carbohydrate antigens; may have allergies or autoimmune disease. A very few cases progress to CVID, others coexist with CVID in the family	137100
5. Specific antibody deficiency with normal Ig concentrations and normal numbers of B cells	Unknown	Variable	Normal	Reduced ability to produce antibodies to specific antigens	Not assigned
6. Transient hypogammaglobulinemia of infancy with normal numbers of B cells	Unknown	Variable	IgG and IgA decreased	Normal ability to produce antibodies to vaccine antigens, usually not associated with significant infections	Not assigned

XL, X-linked inheritance; AR, autosomal recessive inheritance; AD, autosomal dominant inheritance; BTK, Bruton tyrosine kinase; BLNK, B cell linker protein; AID, activation-induced cytidine deaminase; UNG, uracil-DNA glycosylase; ICOS, inducible costimulator; Ig(κ), immunoglobulin or κ light chain type.

^aTen or fewer unrelated cases reported in the literature.

Several autosomal recessive disorders that might previously have been called CVID have been added to **Table 3**. CD81 is normally co-expressed with CD19 on the surface of B cells. As for CD19 mutations, mutations in CD81 result in normal numbers of peripheral blood B cells, low serum IgG, and an increased incidence of glomerulonephritis. Single patient with a homozygous mutation in CD20 and CD21 has been reported.

Common variable immunodeficiency disorders (CVID) include several clinical and laboratory phenotypes that may be caused by distinct genetic and/or environmental factors. Some patients with CVID and no known genetic defect have markedly reduced numbers of B cells as well as hypogammaglobulinemia. Alterations in TNFRSF13B (TACI) and TNFRSF13C (BAFF-R) sequences may represent disease-modifying mutations rather than disease causing mutations. CD40L and CD40 deficiency are included in **Table 1** as well as this table. A small minority of patients with XLP (**Table 4**), WHIM syndrome (**Table 6**), ICF (**Table 2**), VOD1 (**Table 2**), thymoma with immunodeficiency (Good syndrome), or myelodysplasia are first seen by an immunologist because of recurrent infections, hypogammaglobulinemia, and normal or reduced numbers of B cells. Patients with GATA2 mutations (**Table 5**) may have markedly reduced numbers of B cells, as well as decreased monocytes and NK cells, and a predisposition to myelodysplasia but they do not usually have an antibody deficiency.

Table 4 | Diseases of immune dysregulation.

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Functional defect	Associated features	OMIM number
1. Familial hemophagocytic lymphohistiocytosis (FHL) syndromes							
1.1 FHL syndromes without hypopigmentation							
(a) Perforin deficiency (FHL2)	Mutations in <i>PRF1</i> ; perforin is a major cytolytic protein	AR	Increased activated T cells	Normal	Decreased to absent NK and CTL activities (cytotoxicity)	Fever, hepatosplenomegaly (HSMG), hemophagocytic lymphohistiocytosis (HLH), cytopenias	603553
(b) UNC13D/Munc13-4 deficiency (FHL3)	Mutations in <i>UNC13D</i> ^a ; required to prime vesicles for fusion	AR	Increased activated T cells	Normal	Decreased to absent NK and CTL activities (cytotoxicity and/or degranulation)	Fever, HSMG, HLH, cytopenias	608898
(c) Syntaxin 11 deficiency (FHL4)	Mutations in <i>STX11</i> , required for secretory vesicle fusion with the cell membrane	AR	Increased activated T cells	Normal	Decreased NK activity (cytotoxicity and/or degranulation)	Fever, HSMG, HLH, cytopenias	603552
(d) STXBP2/Munc18-2 deficiency (FHL5)	Mutations in <i>STXBP2</i> , required for secretory vesicle fusion with the cell membrane	AR	Increased activated T cells	Normal	Decreased NK and CTL activities (cytotoxicity and/or degranulation)	Fever, HSMG, HLH, cytopenias	613101
1.2. FHL syndromes with hypopigmentation							
(a) Chediak–Higashi syndrome	Mutations in <i>LYST</i> Impaired lysosomal trafficking	AR	Increased activated T cells	Normal	Decreased NK and CTL activities (cytotoxicity and/or degranulation)	Partial albinism Recurrent infections, fever HSMG, HLH Giant lysosomes, neutropenia, cytopenias Bleeding tendency Progressive neurological dysfunction	214500
(b) Griscelli syndrome, type 2	Mutations in <i>RAB27A</i> encoding a GTPase that promotes docking of secretory vesicles to the cell membrane	AR	Normal	Normal	Decreased NK and CTL activities (cytotoxicity and/or degranulation)	Partial albinism, fever, HSMG, HLH, cytopenias	607624
(c) Hermansky–Pudlak syndrome, type 2	Mutations in <i>AP3B1</i> gene, encoding for the b subunit of the AP-3 complex	AR	Normal	Normal	Decreased NK and CTL activities (cytotoxicity and/or degranulation)	Partial albinism Recurrent infections Pulmonary fibrosis Increased bleeding Neutropenia HLH	608233
2. Lymphoproliferative syndromes							
(a) SH2D1A deficiency (XLP1)	Mutations in <i>SH2D1A</i> encoding an adaptor protein regulating intracellular signaling	XL	Normal or increased activated T cells	Reduced memory B cells	Partially defective NK cell and CTL cytotoxic activity	Clinical and immunological features triggered by EBV infection: HLH Lymphoproliferation, aplastic anemia, lymphoma Hypogammaglobulinemia Absent iNKT cells	308240

(Continued)

Table 4 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Functional defect	Associated features	OMIM number
(b) XIAP deficiency (XLP2)	Mutations in <i>XIAP/BIRC4</i> encoding an inhibitor of apoptosis	XL	Normal or increased activated T cells; low/normal iNKT cells	Normal or reduced memory B cells	Increased T cells susceptibility to apoptosis to CD95 and enhanced activation-induced cell death (AICD)	EBV infection, splenomegaly, lymphoproliferation HLH, colitis, IBD, hepatitis Low iNKT cells	300635
(c) ITK deficiency ^a	Mutations in <i>ITK</i> encoding IL-2 inducible T cell kinase required for TCR-mediated activation	AR	Progressive decrease	Normal	Decreased T cell activations	EBV-associated B cell lymphoproliferation, lymphoma Normal or decreased IgG	613011
(d) CD27 deficiency ^a	Mutations in <i>CD27</i> , encoding TNF-R member superfamily required for generation and long-term maintenance of T cell immunity	AR	Normal	No memory B cells	Low T and NK cells functions	Clinical and immunological features triggered by EBV infection: HLH Aplastic anemia, lymphoma, hypogammaglobulinemia Low iNKT cells	615122
3. Genetic defects of regulatory T cells							
(a) IPEX, immune dysregulation, polyen-docrinopathy, enteropathy X-linked	Mutations in <i>FOXP3</i> , encoding a T cell transcription factor	XL	Normal	Normal	Lack of (and/or impaired function of) CD4+ CD25+ FOXP3+ regulatory T cells (Tregs)	Autoimmune enteropathy Early-onset diabetes Thyroiditis, hemolytic anemia, thrombocytopenia, eczema Elevated IgE, IgA	304790
(b) CD25 deficiency ^a	Mutations in <i>IL-2RA</i> , encoding IL-2R α chain	AR	Normal to decreased	Normal	No CD4+ C25+ cells with impaired function of Tregs cells	Lymphoproliferation, autoimmunity. Impaired T cell proliferation	606367
(c) STAT5b deficiency ^a	Mutations in <i>STAT5B</i> , signal transducer, and transcription factor, essential for normal signaling from IL-2 and 15, key growth factors for T and NK cells	AR	Modestly decreased	Normal	Impaired development and function of $\gamma\delta$ T cells, Tregs, and NK cells Low T cell proliferation	Growth-hormone insensitive dwarfism Dysmorphic features Eczema Lymphocytic interstitial pneumonitis, autoimmunity	245590
4. Autoimmunity without lymphoproliferation							
(a) APECED (APS-1), autoimmune polyen-docrinopathy with candidiasis and ectodermal dystrophy	Mutations in <i>AIRE</i> , encoding a transcription regulator needed to establish thymic self-tolerance	AR	Normal	Normal	AIRE-1 serves as checkpoint in the thymus for negative selection of autoreactive T cells and for generation of Tregs	Autoimmunity: hypoparathyroidism, hypothyroidism, adrenal insufficiency, diabetes, gonadal dysfunction, and other endocrine abnormalities Chronic mucocutaneous candidiasis Dental enamel hypoplasia Alopecia areata Enteropathy, pernicious anemia	240300

(Continued)

Table 4 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Functional defect	Associated features	OMIM number
(b) ITCH deficiency ^a	Mutations in <i>ITCH</i> , an E3 ubiquitin ligase catalyzes the transfer of ubiquitin to a signaling protein in the cell including phospholipase Cγ1 (PLCγ1)	AR	Not assessed	Not assessed	Itch deficiency may cause immune dysregulation by affecting both anergy induction in autoreactive effector T cells and generation of Tregs	Early-onset chronic lung disease (interstitial pneumonitis) Autoimmune disorder (thyroiditis, type I diabetes, chronic diarrhea/enteropathy, and hepatitis) Failure to thrive, developmental delay, dysmorphic facial features	613385
5. Autoimmune lymphoproliferative syndrome (ALPS)							
(a) ALPS-FAS	Germinal mutations in <i>TNFRSF6</i> , encoding CD95/Fas cell surface apoptosis receptor ^b	AD	Increased CD4 ⁻ CD8 ⁻ TCRα/β double negative (DN) T cells	Normal, low memory B cells	Apoptosis defect FAS mediated	Splenomegaly, adenopathies, autoimmune cytopenias Increased lymphoma risk IgG and A normal or increased Elevated FasL and IL-10, vitamin B12	601859
(b) ALPS-FASLG	Mutations in <i>TNFSF6</i> , Fas ligand for CD95 apoptosis	AR	Increased DN T cells	Normal	Apoptosis defect FAS mediated	Splenomegaly, adenopathies, autoimmune cytopenias, SLE Soluble FasL is not elevated	134638
(c) ALPS-caspase 10 ^a	Mutations in <i>CASP10</i> , intracellular apoptosis pathway	AD	Increased DN T cells	Normal	Defective lymphocyte apoptosis	Adenopathies, splenomegaly, autoimmunity	603909
(d) ALPS-caspase 8 ^a	Mutations in <i>CASP8</i> , intracellular apoptosis, and activation pathways	AR	Slightly increased DN T cells	Normal	Defective lymphocyte apoptosis and activation	Adenopathies, splenomegaly, bacterial and viral infections, hypogammaglobulinemia	607271
(e) FADD deficiency ^a	Mutations in <i>FADD</i> encoding an adaptor molecule interacting with FAS, and promoting apoptosis	AR	Increased DN T cells	Normal	Defective lymphocyte apoptosis	Functional hyposplenism, bacterial and viral infections Recurrent episodes of encephalopathy and liver dysfunction	613759
(f) CARD11 gain-of-function (GOF) mutations ^a	GOF mutations in <i>CARD11</i> , encoding a protein required for antigen receptor-induced NF-κB activation in B and T lymphocytes	AD	Normal	Increased M ⁺ D ⁺ CD19 ⁺ CD20 ⁺ B cells	Constitutive activation of NF-κB in B & T	Lymphoproliferation Bacterial and viral infections EBV chronic infection Autoimmune cytopenia Hypogammaglobulinemia	606445
(g) PRKCδ deficiency ^a	Mutations in <i>PRKCD</i> , encoding a member of the protein kinase C family critical for regulation of cell survival, proliferation, and apoptosis	AR	Normal	Low memory B cells and elevation of CD5 B cells	Apoptotic defect in B cells	Recurrent infections; EBV chronic infection Lymphoproliferation SLE-like autoimmunity (nephrotic and antiphospholipid syndromes) HypogG	615559

(Continued)

Table 4 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Functional defect	Associated features	OMIM number
6. Immune dysregulation with colitis							
(a) IL-10 deficiency ^a	Mutations in <i>IL-10</i> , encoding IL-10	AR	Normal	Normal	No functional IL-10 secretion	Inflammatory bowel disease (IBD) folliculitis Recurrent respiratory diseases Arthritis	Not assigned
(b) IL-10R α deficiency	Mutations in <i>IL-10RA</i> , encoding IL-10R1	AR	Normal	Normal	Leukocytes, no response to IL-10	IBD, folliculitis Recurrent respiratory diseases Arthritis, lymphoma	613148
(c) IL-10R β deficiency	Mutations in <i>IL-10RB</i> , encoding IL-10R2	AR	Normal	Normal	Leukocytes, no response to IL-10, IL22, IL26, IL28A, IL28B, and IL29	IBD, folliculitis Recurrent respiratory diseases Arthritis, lymphoma	612567
7. Type 1 interferonopathies							
(a) TREX1 deficiency, Aicardi-Goutieres syndrome 1 (AGS1)	Mutations in <i>TREX1</i> , encoding nuclease involves in clearing cellular nucleic debris	AR AD ^b	Not assessed	Not assessed	Intracellular accumulation of abnormal single-stranded (ss) DNA species leading to increased CSF alpha-IFN production	Progressive encephalopathy intracranial calcifications Cerebral atrophy, leukodystrophy HSMG, thrombocytopenia Elevated hepatic transaminases Chronic cerebrospinal fluid (CSF) lymphocytosis	606609
(b) RNASEH2B deficiency, AGS2	Mutations in <i>RNASEH2B</i> , encoding nuclease subunit involves in clearing cellular nucleic debris	AR	Not assessed	Not assessed	Intracellular accumulation of abnormal ss-DNA species leading to increased CSF alpha-IFN production	Progressive encephalopathy intracranial calcifications Cerebral atrophy, leukodystrophy HSMG, thrombocytopenia Elevated hepatic transaminases Chronic CSF lymphocytosis	610326
(c) RNASEH2C deficiency, AGS3	Mutations in <i>RNASEH2C</i> , encoding nuclease subunit involves in clearing cellular nucleic debris	AR	Not assessed	Not assessed	Intracellular accumulation of abnormal ss-DNA species leading to increased CSF alpha-IFN production	Progressive encephalopathy intracranial calcifications Cerebral atrophy, leukodystrophy HSMG, thrombocytopenia Elevated hepatic transaminases Chronic CSF lymphocytosis	610330
(d) RNASEH2A deficiency, AGS4 ^a	Mutations in <i>RNASEH2A</i> , encoding nuclease subunit involves in clearing cellular nucleic debris	AR	Not assessed	Not assessed	Intracellular accumulation of abnormal ss-DNA species leading to increased CSF alpha-IFN production	Progressive encephalopathy intracranial calcifications Cerebral atrophy, leukodystrophy HSMG, thrombocytopenia Elevated hepatic transaminases Chronic CSF lymphocytosis	606034

(Continued)

Table 4 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Circulating T cells	Circulating B cells	Functional defect	Associated features	OMIM number
(e) SAMHD1 deficiency, AGS5	Mutations in <i>SAMHD1</i> , encoding negative regulator of the immunostimulatory DNA response	AR	Not assessed	Not assessed	Induction of the cell intrinsic antiviral response, apoptosis, and mitochondrial DNA destruction leading to increased CSF alpha-IFN production	Progressive encephalopathy intracranial calcifications Cerebral atrophy, leukodystrophy HSMG, thrombocytopenia, anemia elevated lactates Chronic CSF lymphocytosis Skin vasculitis, mouth ulcers, arthropathy	612952
(f) ADAR1 deficiency, AGS6	Mutations in <i>ADAR1</i> , encoding an RNA-specific adenosine deaminase	AR	Not assessed	Not assessed	Catalyzes the deamination of adenosine to inosine in dsRNA substrates markedly elevated CSF IFN-alpha	Progressive encephalopathy intracranial calcification Severe developmental delay, leukodystrophy	615010
(g) Spondylo-enchondro-dysplasia with immune dysregulation (SPENCD)	Mutations in <i>ACP5</i> , encoding tartrate-resistant acid phosphatase (TRAP)	AR	Not assessed	Not assessed	Upregulation of IFN-alpha and type I IFN-stimulated genes	Recurrent bacterial and viral infections, intracranial calcification SLE-like autoimmunity (Sjögren's syndrome, hypothyroidism, inflammatory myositis, Raynaud's disease and vitiligo), hemolytic anemia, thrombocytopenia, skeletal dysplasia, short stature	607944

XL, X-linked inheritance; AR, autosomal recessive inheritance; AD, autosomal dominant inheritance; FHL, familial hemophagocytic lymphohistiocytosis; HLH, hemophagocytic lymphohistiocytosis; HSMG, hepatosplenomegaly; DN, double negative; SLE, systemic lupus erythematosus; IBD, inflammatory bowel disease; CSF, chronic cerebrospinal fluid.

^aTen or fewer unrelated cases reported in the literature.

^bSomatic mutations of *TNFRSF6* cause a similar phenotype (ALPS-sFAS), see **Table 9**. Germinal mutation and somatic mutation of *TNFRSF6* can be associated in some ALPS-FAS patients.

^cAR ALPS-FAS patients have a most severe clinical phenotype.

^dSomatic mutations in *KRAS* or *NRAS* can give this clinical phenotype associated autoimmune leukoproliferative disease (RALD) and are now included in **Table 9** entitled phenocopies of PID.

^eDe novo dominant *TREX1* mutations have been reported.

Fourteen new disorders have been added to **Table 4**. Two new entries have been added in the table, including immune dysregulation with colitis and Type 1 interferonopathies. EBV-driven lymphoproliferation is also observed in *MAGT1* deficiency (**Table 1**).

Table 5 | Congenital defects of phagocyte number, function, or both.

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Affected cells	Affected function	Associated features	OMIM number
1. Defects of neutrophil function						
(a) Severe congenital neutropenia 1 (ELANE deficiency)	Mutation in <i>ELANE</i> : misfolded protein response, increased apoptosis	AD	N	Myeloid differentiation	Susceptibility to MDS/leukemia	202700
(b) SCN2 ^a (GFI 1 deficiency)	Mutation in <i>GFI1</i> : loss of repression of <i>ELANE</i>	AD	N	Myeloid differentiation	B/T lymphopenia	613107

(Continued)

Table 5 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Affected cells	Affected function	Associated features	OMIM number
(c) SCN3 (Kostmann disease)	Mutation in <i>HAX1</i> : control of apoptosis	AR	N	Myeloid differentiation	Cognitive and neurological defects in patients with defects in both HAX1 isoforms, susceptibility to MDS/leukemia	610738
(d) SCN4 (G6PC3 deficiency)	Mutation in <i>G6PC3</i> : abolished enzymatic activity of glucose-6-phosphatase, aberrant glycosylation, and enhanced apoptosis of N and F	AR	N + F	Myeloid differentiation, chemotaxis, O ₂ ⁻ production	Structural heart defects, urogenital abnormalities, inner ear deafness, and venous angiectasias of trunks and limbs	612541
(e) SCN5	Mutation in <i>VPS45</i> controls vesicular trafficking	AR	N + F	Myeloid differentiation, migration	Extramedullary hematopoiesis, bone marrow fibrosis, nephromegaly	615285
(f) Glycogen storage disease type 1b	Mutation in <i>G6PT1</i> : glucose-6-phosphate transporter 1	AR	N + M	Myeloid differentiation, chemotaxis, O ₂ ⁻ production	Fasting hypoglycemia, lactic acidosis, hyperlipidemia, hepatomegaly	232220
(g) Cyclic neutropenia	Mutation in <i>ELANE</i> : misfolded protein response	AD	N	Differentiation	Oscillations of other leukocytes and platelets	162800
(h) X-linked neutropenia/ ^a myelodysplasia	Mutation in <i>WAS</i> : regulator of actin cytoskeleton (loss of auto-inhibition)	XL, gain-of-function	N + M	Mitosis	Monocytopenia	300299
(i) P14/LAMTOR2 deficiency ^a	Mutation in <i>ROBLD3/LAMTOR2</i> : endosomal adaptor protein 14	AR	N + L Mel	Endosome biogenesis	Neutropenia Hypogammaglobulinemia ↓ CD8 cytotoxicity Partial albinism Growth failure	610389
(j) Barth syndrome	Mutation in tafazzin (<i>TAZ</i>) gene: abnormal lipid structure of mitochondrial membrane, defective carnitine metabolism	XL	N	Myeloid differentiation	Cardiomyopathy, myopathy, growth retardation	302060
(k) Cohen syndrome	Mutation in <i>COH1</i> gene: Pg unknown	AR	N	Myeloid differentiation	Retinopathy, developmental delay, facial dysmorphisms	216550
(l) Clericuzio syndrome poikiloderma with neutropenia	Mutation in <i>C16ORF57</i> , affects genomic integrity	AR	N	Myeloid differentiation	Poikiloderma, neutropenia, MDS	613276
2. Defects of motility						
(a) Leukocyte adhesion deficiency type 1 (LAD1)	Mutation in <i>ITGB2</i> : adhesion protein (CD18)	AR	N + M + L + NK	Adherence, chemotaxis, endocytosis, T/NK cytotoxicity	Delayed cord separation, skin ulcers Periodontitis Leukocytosis	116920
(b) Leukocyte adhesion deficiency type 2 (LAD2) ^a	Mutation in <i>FUCT1</i> : GDP-fucose transporter	AR	N + M	Rolling, chemotaxis	Mild LAD type 1 features plus hh-blood group plus mental and growth retardation	266265
(c) Leukocyte adhesion deficiency type 3 (LAD3)	Mutation in <i>KINPLIN3</i> : Rap1-activation of β1-3 integrins	AR	N + M + L + NK	Adherence, chemotaxis	LAD type 1 plus bleeding tendency	612840
(d) Rac 2 deficiency ^a	Mutation in <i>RAC2</i> : regulation of actin cytoskeleton	AD	N	Adherence, chemotaxis, O ₂ ⁻ production	Poor wound healing, leukocytosis	602049

(Continued)

Table 5 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Affected cells	Affected function	Associated features	OMIM number
(e) β -Actin deficiency ^a	Mutation in <i>ACTB</i> : cytoplasmic actin	AD	N + M	Motility	Mental retardation, short stature	102630
(f) Localized juvenile periodontitis	Mutation in <i>FPR1</i> : chemokine receptor	AR	N	Formylpeptide induced chemotaxis	Periodontitis only	136537
(g) Papillon–Lefèvre syndrome	Mutation in <i>CTSC</i> : cathepsin C activation of serine proteases	AR	N + M	Chemotaxis	Periodontitis, palmoplantar hyperkeratosis in some patients	245000
(h) Specific granule deficiency ^a	Mutation in <i>C/EBPE</i> : myeloid transcription factor	AR	N	Chemotaxis	Neutrophils with bilobed nuclei; absent secondary granules and defensins	245480
(i) Shwachman–Diamond syndrome	Mutation in <i>SBDS</i> : defective ribosome synthesis	AR	N	Chemotaxis	Pancytopenia, exocrine pancreatic insufficiency, chondrodysplasia	260400
3. Defects of respiratory burst						
(a) X-linked chronic granulomatous disease (CGD)	Mutation in <i>CYBB</i> : electron transport protein (gp91phox)	XL	N + M	Killing (faulty O ₂ [−] production)	Recurrent bacterial infection, susceptibility to fungal infection, inflammatory gut manifestations McLeod phenotype in patients with deletions extending into the contiguous Kell locus	306400
(b) Autosomal recessive CGD – p22 phox deficiency	Mutation in <i>CYBA</i> : electron transport protein (p22phox)	AR	N + M	Killing (faulty O ₂ [−] production)	Recurrent bacterial infection, susceptibility to fungal infection, and inflammatory gut manifestations	233690
(c) Autosomal recessive CGD – p47 phox deficiency	Mutation in <i>NCF1</i> : adapter protein (p47phox)	AR	N + M	Killing (faulty O ₂ [−] production)	Recurrent bacterial infection, susceptibility to fungal infection, and inflammatory gut manifestations	233700
(d) Autosomal recessive CGD – p67 phox deficiency	Mutation in <i>NCF2</i> : activating protein (p67phox)	AR	N + M	Killing (faulty O ₂ [−] production)	Recurrent bacterial infection, susceptibility to fungal infection, inflammatory gut manifestations	233710
(e) Autosomal recessive CGD – p40 phox deficiency ^a	Mutation in <i>NCF4</i> : activating protein (p40phox)	AR	N + M	Killing (faulty O ₂ [−] production)	Inflammatory gut manifestations only	601488
4. Mendelian susceptibility to mycobacterial disease (MSMD)						
(a) IL-12 and IL-23 receptor β 1 chain deficiency	Mutation in <i>IL-12RB1</i> : IL-12 and IL-23 receptor β 1 chain	AR	L + NK	IFN- γ secretion	Susceptibility to <i>Mycobacteria</i> and <i>Salmonella</i>	209950
(b) IL-12p40 deficiency	Mutation in <i>IL-12B</i> : subunit p40 of IL-12/IL23	AR	M	IFN- γ secretion	Susceptibility to <i>Mycobacteria</i> and <i>Salmonella</i>	161561
(c) IFN- γ receptor 1 deficiency	Mutation in <i>IFNGR1</i> : IFN- γ R ligand binding chain	AR, AD	M + L	IFN- γ binding and signaling	Susceptibility to <i>Mycobacteria</i> and <i>Salmonella</i>	107470
(d) IFN- γ receptor 2 deficiency	Mutation in <i>IFNGR2</i> : IFN- γ R accessory chain	AR	M + L	IFN- γ signaling	Susceptibility to <i>Mycobacteria</i> and <i>Salmonella</i>	147569
(e) STAT1 deficiency (AD form) ^a	Mutation in <i>STAT1</i> (loss of function)	AD	M + L	IFN- γ signaling	Susceptibility to <i>Mycobacteria</i>	600555

(Continued)

Table 5 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Affected cells	Affected function	Associated features	OMIM number
(f) Macrophage gp91 phox deficiency ^a	Mutation in <i>CYBB</i> : electron transport protein (gp 91 phox)	XL	Mφ only	Killing (faulty O ₂ ⁻ production)	Isolated susceptibility to <i>Mycobacteria</i>	306400
(g) IRF8-deficiency (AD form) ^a	Mutation in <i>IRF8</i> : IL-12 production by CD1c ⁺ MDC	AD	CD1c ⁺ MDC	Differentiation of CD1c ⁺ MDC subgroup	Susceptibility to <i>Mycobacteria</i>	601565
(h) ISG15	Mutation in <i>ISG15</i> ; an interferon (IFN) α/β-inducible, ubiquitin-like intracellular protein	AR	M + N + L	IFN-γ secretion	Susceptibility to <i>Mycobacteria</i>	14751
5. Other defects						
(a) IRF 8-deficiency (AR form) ^a	Mutation in <i>IRF8</i> : IL-12 production	AR	Monocytes peripher- eral DC	Cytopenias	Susceptibility to <i>Mycobacteria</i> , <i>Candida</i> , myeloproliferation	614893
(b) GATA2 deficiency (Mono MAC syndrome)	Mutation in <i>GATA2</i> : loss of stem cells	AD	Monocytes peripher- eral DC + NK + B	Multilineage cytopenias	Susceptibility to <i>Mycobacteria</i> , papilloma viruses, histoplasmosis, alveolar proteinosis, MDS/AML/CMML	137295
(c) Pulmonary alveolar proteinosis ^a	Mutation in <i>CSF2RA</i>	Biallelic mutations in pseudo-autosomal gene	Alveolar macro- phages	GM-CSF signaling	Alveolar proteinosis	306250

XL, X-linked inheritance; AR, autosomal recessive inheritance; AD, autosomal dominant inheritance; ACTB, actin beta; B, B lymphocytes; CEBPE, CCAAT/enhancer-binding protein epsilon; CMML, chronic myelomonocytic leukemia; CTSC, cathepsin C; CYBA, cytochrome b alpha subunit; CYBB, cytochrome b beta subunit; DC, dendritic cells; ELANE, elastase neutrophil-expressed; GATA2, GATA binding protein 2; IFN, interferon; IFNGR1, interferon-gamma receptor subunit 1; IFNGR2, interferon-gamma receptor subunit 2; IL-12B, interleukin-12 beta subunit; IL-12RB1, interleukin-12 receptor beta 1; IFR8, interferon regulatory factor 8; F, fibroblasts; FPR1, formylpeptide receptor 1; FUCT1, fucose transporter 1; GFI1, growth factor independent 1; HAX1, HLCs1-associated protein X1; ITGB2, integrin beta-2; L, lymphocytes; M, monocytes–macrophages; MDC, myeloid dendritic cells; MDS, myelodysplasia; Mel, melanocytes; Mφ, macrophages; MSMD, Mendelian susceptibility to mycobacterial disease; N, neutrophils; NCF1, neutrophil cytosolic factor 1; NCF2, neutrophil cytosolic factor 2; NCF4, neutrophil cytosolic factor 4; NK, natural killer cells; ROBLD3: roadblock domain containing 3; SBDS, Shwachman–Bodian–Diamond syndrome; STAT, signal transducer and activator of transcription.

^aTen or fewer unrelated cases reported in the literature.

Table 5 includes seven newly described genetic defects of phagocyte number and/or function including Barth syndrome, Cohen syndrome, and poikiloderma with neutropenia. In these three clinically well-known diseases, the genetic defects have been elucidated, although their molecular pathogenesis remains ill-defined. A new cause of autosomal recessive chronic granulomatous disease, namely a deficiency of the cytosolic activating protein p40 phox, has now been found in two CGD patients and is included under defects of respiratory burst. Under the heading of Mendelian susceptibility of mycobacterial disease (MSMD), two new entities were added: (a) a subgroup of X-linked gp91 phox deficiency with isolated susceptibility to mycobacteria and a defect of the respiratory burst in macrophages only; (b) an autosomal dominant form of IRF8-deficiency, resulting from a lack of CD1c⁺ myeloid dendritic cells that would normally secrete IL-12. The clinical phenotype of MSMD may vary, depending on the nature of the genetic defect. Finally, GATA2 deficiency was recently identified as the cause of the Mono MAC syndrome, with multilineage cytopenias (of monocytes, peripheral dendritic cells, NK- and B-lymphocytes) resulting in opportunistic infections (including mycobacteria), alveolar proteinosis, and malignancy.

Table 6 | Defects in innate immunity.

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Affected cell	Functional defect	Associated features	OMIM number
1. Anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID)						
(a) EDA-ID, X-linked (NEMO deficiency)	Mutations of <i>NEMO</i> (<i>IKBKG</i>), a modulator of NF-κB activation	XL	Lymphocytes + monocytes	NF-κB signaling pathway	Various infections (bacteria, <i>Mycobacteria</i> , viruses, and fungi) Colitis EDA (not in all patients) Hypogammaglobulinemia to specific antibody polysaccharides deficiency	300248
(b) EDA-ID, autosomal-dominant ^a	Gain-of-function mutations of <i>IKBA</i> , resulting in impaired activation of NF-κB	AD	Lymphocytes + monocytes	NF-κB signaling pathway	Various infections (bacteria, viruses, and fungi) EDA T cell defect	612132
2. TIR signaling pathway deficiency						
(a) IRAK-4 deficiency	Mutations of <i>IRAK-4</i> , a component of TLR- and IL-1R-signaling pathway	AR	Lymphocytes + granulocytes + monocytes	TIR-IRAK signaling pathway	Bacterial infections (pyogenes)	607676
(b) MyD88 deficiency	Mutations of <i>MYD88</i> , a component of the TLR and IL-1R signaling pathway	AR	Lymphocytes + granulocytes + monocytes	TIR-MyD88 signaling pathway	Bacterial infections (pyogenes)	612260
3. HOIL1 deficiency ^a	Mutation of <i>HOIL1</i> , a component of LUBAC	AR	Lymphocytes + granulocytes + monocytes	NF-κB signaling pathway	Bacterial infections (pyogenes) Autoinflammation Amylopectinosis	Not assigned
4. WHIM (Warts, hypogammaglobulinemia, infections, myelokathexis) syndrome	Gain-of-function mutations of <i>CXCR4</i> , the receptor for CXCL12	AD	Granulocytes + lymphocytes	Increased response of the CXCR4 chemokine receptor to its ligand CXCL12 (SDF-1)	Warts/human papilloma virus (HPV) infection Neutropenia Reduced B cell number Hypogammaglobulinemia	193670
5. Epidermodysplasia verruciformis EVER1 deficiency	Mutations of <i>EVER1</i>	AR	Keratinocytes and leukocytes	EVER proteins may be involved in the regulation of cellular zinc homeostasis in lymphocytes	HPV (group B1) infections and cancer of the skin (typical EV)	226400
EVER2 deficiency	Mutations of <i>EVER2</i>	AR	Keratinocytes and leukocytes	EVER proteins may be involved in the regulation of cellular zinc homeostasis in lymphocytes	HPV (group B1) infections and cancer of the skin (typical EV)	226400
6. Predisposition to severe viral infection						
(a) STAT2 deficiency ^a	Mutations of <i>STAT2</i>	AR	T and NK cells	STAT2-dependent IFN-α and -β response	Severe viral infections (disseminated vaccine-strain measles)	Not assigned

(Continued)

Table 6 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Affected cell	Functional defect	Associated features	OMIM number
(b) MCM4 deficiency ^a	Mutations in <i>MCM4</i>	AR	NK cells	DNA repair disorder	Viral infections (EBV, HSV, VZV) Adrenal failure Short stature	609981
7. Herpes simplex encephalitis (HSE)						
(a) TLR3 deficiency ^a	(b) Mutations of <i>TLR3</i>	AD AR	Central nervous system (CNS) resident cells and fibroblasts	TLR3-dependent IFN- α , - β , and - λ induction	Herpes simplex virus 1 encephalitis (incomplete clinical penetrance for all etiologies listed here)	613002
(b) UNC93B1 deficiency ^a	(a) Mutations of <i>UNC93B1</i>	AR	CNS resident cells and fibroblasts	UNC-93B-dependent IFN- α , - β , and - λ induction	Herpes simplex virus 1 encephalitis	610551
(c) TRAF3 deficiency ^a	(c) Mutations of <i>TRAF3</i>	AD	CNS resident cells and fibroblasts	TRAF3-dependent IFN- α , - β , and - λ induction	Herpes simplex virus 1 encephalitis	614849
(d) TRIF deficiency ^a	(c) Mutations of <i>TRIF</i>	AD AR	CNS resident cells and fibroblasts	TRIF-dependent IFN- α , - β , and - λ induction	Herpes simplex virus 1 encephalitis	614850
(e) TBK1 deficiency ^a	(c) Mutations of <i>TBK1</i>	AD	CNS resident cells and fibroblasts	TBK1-dependent IFN- α , - β , and - λ induction	Herpes simplex virus 1 encephalitis	Not assigned
8. Predisposition to invasive fungal diseases ^a						
CARD9 deficiency	Mutations of <i>CARD9</i>	AR	Mononuclear phagocytes	CARD9 signaling pathway	Invasive candidiasis infection Deep dermatophytoses	212050
9. Chronic mucocutaneous candidiasis (CMC)						
(a) IL-17RA deficiency ^a	(a) Mutations in <i>IL-17RA</i>	AR	Epithelial cells, fibroblasts, mononuclear phagocytes	IL-17RA signaling pathway	CMC Folliculitis	605461
(b) IL-17F deficiency ^a	(b) Mutations in <i>IL-17F</i>	AD	T cells	IL-17F-containing dimers	CMC Folliculitis	606496
(c) STAT1 gain-of-function	(c) Gain-of-function mutations in <i>STAT1</i>	AD	T cells	Gain-of-function STAT1 mutations that impair the development of IL-17-producing T cells	CMC Various fungal, bacterial, and viral (HSV) infections Autoimmunity (thyroiditis, diabetes, cytopenia) Enteropathy	614162
(d) ACT1 deficiency ^a	(c) Mutations in <i>ACT1</i>	AR	T cells, fibroblasts	Fibroblasts fail to respond to IL-17A and IL-17F, and their T cells to IL-17E	CMC Blepharitis, folliculitis, and macroglossia	615527
10. Trypanosomiasis ^a	Mutations in <i>APOL1</i>	AD		APOL1	Trypanosomiasis	603743

(Continued)

Table 6 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Affected cell	Functional defect	Associated features	OMIM number
11. Isolated congenital asplenia (ICA)	Mutations in <i>RPSA</i>	AD	Spleen	<i>RPSA</i> encodes ribosomal protein SA, a component of the small subunit of the ribosome	Bacteremia (encapsulated bacteria) No spleen	271400

XL, X-linked inheritance; AR, autosomal recessive inheritance; AD, autosomal dominant inheritance; NF- κ B, nuclear factor kappa B; TIR, Toll and interleukin 1 receptor; IFN, interferon; HVP, human papilloma virus; TLR, Toll-like receptor; IL, interleukin.

^aTen or fewer unrelated cases reported in the literature.

Eight new disorders have been added to **Table 6**. Three new entries have been added in the table. One is a new PID with the association of recurrent bacterial infections, autoinflammation, and amylopectinosis caused by AR HOIL1 mutations found in two kindreds. The second is severe viral infection, for which three genetic etiologies have been discovered. AR-STAT2 deficiency and AR-CD16 deficiency have been found in one kindred each. AR MCM4 deficiency has been found in several Irish kindreds. The third is isolated congenital asplenia identified in 18 patients from 8 kindreds.

XR-EDA-ID is highly heterogeneous clinically, both in terms of developmental features (some patients display osteopetrosis and lymphedema, in addition to EDA, while others do not display any developmental features) and infectious diseases (some display multiple infections, viral, fungal, and bacterial, while others display a single type of infection). The various OMIM entries correspond to these distinct clinical diseases.

Table 7 | Autoinflammatory disorders.

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Affected cells	Functional defects	Associated features	OMIM number
1. Defects effecting the inflammasome						
(a) Familial Mediterranean fever	Mutations of <i>MEVF</i> (<i>lead to gain of pyrin function, resulting in inappropriate IL-1β release</i>)	AR	Mature granulocytes, cytokine-activated monocytes	Decreased production of pyrin permits ASC-induced IL-1 processing and inflammation following subclinical serosal injury; macrophage apoptosis decreased	Recurrent fever, serositis, and inflammation responsive to colchicine. Predisposes to vasculitis and inflammatory bowel disease	249100
(b) Mevalonate kinase deficiency (hyper IgD syndrome)	Mutations of <i>MVK</i> (<i>lead to a block in the mevalonate pathway. Interleukin-1β mediates the inflammatory phenotype</i>)	AR		Affecting cholesterol synthesis; pathogenesis of disease is unclear	Periodic fever and leukocytosis with high IgD levels	260920
(c) Muckle–Wells syndrome	Mutations of <i>CIAS1</i> (<i>also called PYPAF1 or NALP3 lead to constitutive activation of the NLRP3 inflammasome</i>)	AD	PMNs monocytes	Defect in cryopyrin, involved in leukocyte apoptosis and NF- κ B signaling and IL-1 processing	Urticaria, SNHL, amyloidosis	191900
(d) Familial cold autoinflammatory syndrome	Mutations of <i>CIAS1</i> (see above) Mutations of <i>NLRP12</i>	AD	PMNs, monocytes	Same as above	Non-pruritic urticaria, arthritis, chills, fever, and leukocytosis after cold exposure	120100

(Continued)

Table 7 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Affected cells	Functional defects	Associated features	OMIM number
5. Neonatal onset multisystem inflammatory disease (NOMID) or chronic infantile neurologic cutaneous and articular syndrome (CINCA)	Mutations of <i>CIAS1</i> (see above)	AD	PMNs, chondrocytes	Same as above	Neonatal onset rash, chronic meningitis, and arthropathy with fever and inflammation	607115
2. Non inflammasome-related conditions						
(a) TNF receptor-associated periodic syndrome (TRAPS)	Mutations of <i>TNFRSF1</i> (resulting in increased TNF inflammatory signaling)	AD	PMNs, monocytes	Mutations of 55-kDa TNF receptor leading to intracellular receptor retention or diminished soluble cytokine receptor available to bind TNF	Recurrent fever, serositis, rash, and ocular or joint inflammation	142680
(b) Early-onset inflammatory bowel disease	Mutations in <i>IL-10</i> (results in increase many proinflammatory cytokines)	AR	Monocyte/macrophage, activated T cells	IL-10 deficiency leads to increase of TNF γ and other proinflammatory cytokines	Early-onset enterocolitis enteric fistulas, perianal abscesses, chronic folliculitis	124092
(b) Early-onset inflammatory bowel disease	Mutations in <i>IL-10RA</i> (see above)	AR	Monocyte/macrophage, activated T cells	Mutation in IL-10 receptor alpha leads to increase of TNF γ and other proinflammatory cytokines	Early-onset enterocolitis enteric fistulas, perianal abscesses, chronic folliculitis	146933
(b) Early-onset inflammatory bowel disease	Mutations in <i>IL-10RB</i> (see above)	AR	Monocyte/macrophage, activated T cells	Mutation in IL-10 receptor beta leads to increase of TNF γ and other proinflammatory cytokines	Early-onset enterocolitis enteric fistulas, perianal abscesses, chronic folliculitis	123889
(c) Pyogenic sterile arthritis, pyoderma gangrenosum, acne (PAPA) syndrome	Mutations of <i>PSTPIP1</i> (also called C2BP1) (affects both pyrin and protein tyrosine phosphatase to regulate innate and adaptive immune responses)	AD	Hematopoietic tissues, upregulated in activated T cells	Disordered actin reorganization leading to compromised physiologic signaling during inflammatory response	Destructive arthritis, inflammatory skin rash, myositis	604416
(d) Blau syndrome	Mutations of <i>NOD2</i> (also called CARD15) (involved in various inflammatory processes)	AD	Monocytes	Mutations in nucleotide binding site of CARD15, possibly disrupting interactions with lipopolysaccharides and NF- κ B signaling	Uveitis, granulomatous synovitis, camptodactyly, rash, and cranial neuropathies, 30% develop Crohn's disease	186580
10. Chronic recurrent multifocal osteomyelitis and congenital dyserythropoietic anemia (Majeed syndrome) ^a	Mutations of <i>LPIN2</i> (increased expression of the proinflammatory genes)	AR	Neutrophils, bone marrow cells	Undefined	Chronic recurrent multifocal osteomyelitis, transfusion-dependent anemia, cutaneous inflammatory disorders	609628

(Continued)

Table 7 | Continued

Disease	Genetic defect/ presumed pathogenesis	Inheritance	Affected cells	Functional defects	Associated features	OMIM number
11. DIRA (deficiency of the interleukin 1 receptor antagonist) ^a	Mutations of <i>IL1RN</i> (see functional defect)	AR	PMNs, monocytes	Mutations in the IL-1 receptor antagonist allow unopposed action of Interleukin 1	Neonatal onset of sterile multifocal osteomyelitis, periostitis, and pustulosis	612852
12. DITRA – deficiency of IL-36 receptor antagonist	Mutation in <i>IL36RN</i> (see functional defect)	AR	Keratinocyte leukocytes	Mutations in IL-36RN leads to increase IL-8 production	Pustular psoriasis	614204
13. SLC29A3 mutation	Mutation in <i>SLC29A3</i> (?)	AR	Leukocyte, bone cells	Macrophage activation?	Hyperpigmentation hypertrichosis	602782
14. CAMPS (CARD14 mediated psoriasis)	Mutation in <i>CARD14</i> (see functional defect)	AD	Mainly in keratinocyte	Mutations in CARD14 activate the NF-κB pathway and production of IL-8	Psoriasis	173200
15. Cherubism	Mutation in <i>SH3BP2</i> (see functional defect)	AD	Stroma cells, bone cells	Hyperactivated macrophage and increased NF-κB	Bone degeneration in jaws	11840
16. CANDLE (chronic atypical neutrophilic dermatitis with lipodystrophy)	Mutation in <i>PSMB8</i> (see functional defect)	AD	Keratinocyte, B cell adipose cells	Mutations cause increase IL-6 production	Dystrophy, panniculitis	256040
17. HOIL1 deficiency	Mutation in <i>HOIL1</i> (see functional defect)	AR	PMNs, fibroblast	Mutation in <i>HOIL1</i> leads to IL-1 β dysfunction	Immunodeficiency autoinflammation amylopectinosis	610924
18. PLAID ($PLC\gamma 2$ associated antibody deficiency and immune dysregulation)	Mutation in <i>PLCG2</i> (see functional defect)	AD	B cells, NK, mast cells	Mutations cause activation of IL-1 pathways	Cold urticaria hypogammaglobulinemia	614878

AR, autosomal recessive inheritance; AD, autosomal dominant inheritance; PMN, polymorphonuclear cells; ASC, apoptosis-associated speck-like protein with a caspase recruitment domain; CARD, caspase recruitment domain; CD2BP1, CD2 binding protein 1; PSTPIP1, proline-serine/threonine phosphatase-interacting protein 1; SNHL, sensorineural hearing loss; CIAS1, cold-induced autoinflammatory syndrome 1.

^aTen or fewer unrelated cases reported in the literature.

Autoinflammatory diseases are clinical disorders marked by abnormally increased inflammation, mediated predominantly by the cells and molecules of the innate immune system, with a significant host predisposition. While the genetic defect of one of the most common autoinflammatory conditions, PFAPA, is not known, recent studies suggest that it is associated with activation of IL-1 pathway and response to IL-1beta antagonists.

Muckle–Wells syndrome, familial cold autoinflammatory syndrome and neonatal onset multisystem inflammatory disease (NOMID), which is also called chronic infantile neurologic cutaneous and articular syndrome (CINCA) are caused by similar mutations in CIAS1 mutations. The disease phenotype in any individual appears to depend on modifying effects of other genes and environmental factors.

Table 8 | Complement deficiencies.

Disease	Genetic defect; presumed pathogenesis	Inheritance	Functional defect	Associated features	OMIM number
1. C1q deficiency	Mutation in <i>C1QA</i> , <i>C1QB</i> , <i>C1QC</i> : classical complement pathway components	AR	Absent CH50 hemolytic activity, defective activation of the classical pathway Diminished clearance of apoptotic cells	SLE, infections with encapsulated organisms	120550; 601269; 120575
2. C1r deficiency	Mutation in <i>C1R</i> : classical complement pathway component	AR	Absent CH50 hemolytic activity, defective activation of the classical pathway	SLE, infections with encapsulated organisms	216950
3. C1s deficiency	Mutation in <i>C1S</i> : classical complement pathway component	AR	Absent CH50 hemolytic activity, defective activation of the classical pathway	SLE, infections with encapsulated organisms	120580
4. C4 deficiency	Mutation in <i>C4A</i> , <i>C4B</i> : classical complement pathway components	AR	Absent CH50 hemolytic activity, defective activation of the classical pathway, defective humoral immune response to carbohydrate antigens in some patients	SLE, infections with encapsulated organisms	120810; 120820
5. C2 deficiency	Mutation in <i>C2</i> : classical complement pathway component	AR	Absent CH50 hemolytic activity, defective activation of the classical pathway	SLE, infections with encapsulated organisms, atherosclerosis	217000
6. C3 deficiency	Mutation in <i>C3</i> : central complement component	AR, gain-of-function AD	Absent CH50 and AH50 hemolytic activity; defective opsonization Defective humoral immune response	Infections; glomerulonephritis Atypical hemolytic–uremic syndrome with gain-of-function mutations	120700
7. C5 deficiency	Mutation in <i>C5</i> : terminal complement component	AR	Absent CH50 and AH50 hemolytic activity; defective bactericidal activity	Neisserial infections	120900
8. C6 deficiency	Mutation in <i>C6</i> : terminal complement component	AR	Absent CH50 and AH50 hemolytic activity; defective bactericidal activity	Neisserial infections	217050
9. C7 deficiency	Mutation in <i>C7</i> : terminal complement component	AR	Absent CH50 and AH50 hemolytic activity; defective bactericidal activity	Neisserial infections	217070
10. C8 α – γ deficiency	Mutation in <i>C8A</i> , <i>C8G</i> : terminal complement components	AR	Absent CH50 and AH50 hemolytic activity; defective bactericidal activity	Neisserial infections	120950
11. C8b deficiency	Mutation in <i>C8B</i> : Terminal complement component	AR	Absent CH50 and AH50 hemolytic activity; defective bactericidal activity	Neisserial infections	120960
12. C9 deficiency	Mutation in <i>C9</i> : Terminal complement component	AR	Reduced CH50 and AP50 hemolytic activity; deficient bactericidal activity	Mild susceptibility to Neisserial infections	613825

(Continued)

Table 8 | Continued

Disease	Genetic defect; presumed pathogenesis	Inheritance	Functional defect	Associated features	OMIM number
13. C1 inhibitor deficiency	Mutation in <i>SERPING1</i> : regulation of kinins and complement activation	AD	Spontaneous activation of the complement pathway with consumption of C4/C2 Spontaneous activation of the contact system with generation of bradykinin from high molecular weight kininogen	Hereditary angioedema	138470
14. Factor B ^a	Mutation in <i>CFB</i> : activation of the alternative pathway	AD	Gain-of-function mutation with increased spontaneous AH50	aHUS	138470
15. Factor D deficiency	Mutation in <i>CFD</i> : regulation of the alternative complement pathway	AR	Absent AH50 hemolytic activity	Neisserial infections	134350
16. Properdin deficiency	Mutation in <i>CFP</i> : regulation of the alternative complement pathway	XL	Absent AH50 hemolytic activity	Neisserial infections	312060
17. Factor I deficiency	Mutation in <i>CFI</i> : regulation of the alternative complement pathway	AR	Spontaneous activation of the alternative complement pathway with consumption of C3	Infections, Neisserial infections, aHUS, preeclampsia, membranoproliferative glomerulonephritis (MPGN)	610984
18. Factor H deficiency	Mutation in <i>CFH</i> : regulation of the alternative complement pathway	AR	Spontaneous activation of the alternative complement pathway with consumption of C3	Infections, Neisserial infections, aHUS, preeclampsia, membranoproliferative glomerulonephritis (MPGN)	609814
19. Factor H-related protein deficiencies	Mutation in <i>CFHR1-5</i> : bind C3b	AR	Normal CH50, AH50, autoantibodies to Factor H	aHUS	235400
20. Thrombomodulin ^a	Mutation in <i>THBD</i> : regulates complement and coagulant activation	AD	Normal CH50, AH50	aHUS	188040
21. MASP1 deficiency	Mutation in <i>MASP1</i> : cleaves C2 and activates MASP2	AR	Deficient activation of the lectin activation pathway, cell migration	Infections, 3MC syndrome	600521
22. MASP2 deficiency ^a	<i>MASP2</i> : cleavage of C2 and C4	AR	Deficient activation of the lectin activation pathway	Pyogenic infections; inflammatory lung disease, autoimmunity	605102
23. 3MC syndrome COLEC11 deficiency ^a	Mutation in <i>COLEC11</i> : binds MASP1, MASP3	AR	Loss of neural crest cell migration signals	A developmental syndrome of facial dysmorphism, cleft lip and/or palate, craniosynostosis, learning disability, and genital, limb, and vesicorectal anomalies (3MC syndrome)	612502

(Continued)

Table 8 | Continued

Disease	Genetic defect; presumed pathogenesis	Inheritance	Functional defect	Associated features	OMIM number
24. Complement receptor 2 (CR2) deficiency ^a	Mutation in <i>CD21</i>	AR	See CD21 deficiency in Table 3		120650
25. Complement receptor 3 (CR3) deficiency	Mutation in <i>ITGB2</i>	AR	See LAD1 in Table 5		116920
Membrane cofactor protein (CD46) deficiency	Mutation in <i>CD46</i> : dissociates C3b and C4b	AD	Inhibitor of complement alternate pathway, decreased C3b binding	aHUS, infections, preeclampsia	120920
Membrane Attack Complex inhibitor (CD59) deficiency ^a	Mutation in <i>CD59</i> : regulates the membrane attack complex formation	AR	Erythrocytes highly susceptible to complement-mediated lysis	Hemolytic anemia, polyneuropathy	107271
Ficolin 3 deficiency ^a	Mutation in <i>FCN3</i> : activates the classical complement pathway	AR	Absence of complement activation by the Ficolin 3 pathway	Respiratory infections, abscesses	604973

XL, X-linked inheritance; AR, autosomal recessive inheritance; AD, autosomal dominant inheritance; MAC, membrane attack complex; SLE, systemic lupus erythematosus; MBP, mannose-binding protein; MASP2, MBP-associated serine protease 2.

^aTen or fewer unrelated cases reported in the literature.

New entities added to **Table 8** demonstrate the important role of complement regulators in a group of well-described inflammatory disorders. In particular, we have added mutations in membrane bound as well as surface attached soluble complement regulatory proteins recognized in hemolytic–uremic syndrome, age-related macular degeneration, and preeclampsia. The connecting theme of these otherwise unrelated clinical events is excessive activation or insufficient regulation of C3; these events lead to recruitment of leukocytes and permit secretion of inflammatory and anti-angiogenic mediators that disrupt the vascular bed of the target organ. Alterations in the genes for Factor B (*CFB*), Factor I (*CFI*), Factor H (*CFH*), and *CD46* act as susceptibility genes rather than disease causing mutations. Population studies reveal no detectable increase in infections in MBP (also known at mannose-binding lectin – MBL) deficient adults. The 3MC syndrome, a developmental syndrome, has been variously called Carnevale, Mingarelli, Malpuech, and Michels syndrome.

Table 9 | Phenocopies of PID.

Disease	Genetic defect/ presumed pathogenesis	Circulating T cells	Circulating B cells	Serum Ig	Associated features/ similar PID
Associated with somatic mutations					
(a) Autoimmune lymphoproliferative syndrome (ALPS–SFAS)	Somatic mutation in <i>TNFRSF6</i>	Increased CD4 [−] CD8 [−] double negative (DN) T alpha/beta cells	Normal, but increased number of CD5 ⁺ B cells	Normal or increased	Splenomegaly, lymphadenopathy, autoimmune cytopenias Defective lymphocyte apoptosis/ALPS–FAS (=ALPS type I ^m)
(b) RAS-associated autoimmune leukoproliferative disease (RALD)	Somatic mutation in <i>KRAS</i> (gain-of-function)	Normal	B cell lymphocytosis	Normal or increased	Splenomegaly, lymphadenopathy, autoimmune cytopenias, granulocytosis, monocytosis/ALPS-like

(Continued)

Table 9 | Continued

Disease	Genetic defect/ presumed pathogenesis	Circulating T cells	Circulating B cells	Serum Ig	Associated features/ similar PID
(c) RAS-associated autoimmune leukoproliferative disease (RALD)	Somatic mutation in <i>NRAS</i> (gain-of-function)	Increased CD4 ⁻ CD8 ⁻ double negative (DN) T alpha/beta cells	Lymphocytosis		Splenomegaly, lymphadenopathy, autoantibodies/ <i>ALPS-like</i>
Associated with autoantibodies					
(a) Chronic mucocutaneous candidiasis (isolated or with APECED syndrome)	Germline mutation in <i>AIRE</i> AutoAb to IL-17 and/or IL-22	Normal	Normal	Normal	Endocrinopathy, chronic mucocutaneous candidiasis/ <i>CMC</i>
(b) Adult-onset immunodeficiency	AutoAb to IFN gamma	Decreased naive T cells	Normal	Normal	Mycobacterial, fungal, <i>Salmonella</i> VZV infections/ <i>MSMD</i> , or <i>CID</i>
(c) Recurrent skin infection	AutoAb to IL-6	Normal	Normal	Normal	Staphylococcal infections/ <i>STAT3</i> deficiency
(d) Pulmonary alveolar proteinosis	AutoAb to GM-CSF	Normal	Normal	Normal	Pulmonary alveolar proteinosis, cryptococcal meningitis/ <i>CSF2RA</i> deficiency
(e) Acquired angioedema	AutoAb to C1 inhibitor	Normal	Normal	Normal	Angioedema/ <i>C1 INH</i> deficiency (hereditary angioedema)

The rapid advances in gene identification technology, including the widespread use of whole exome and whole genome sequencing, has meant that the ability to identify gene defects in affected families and even single individuals with inherited diseases has grown enormously. In this report, over 30 new gene defects have been added that were identified since the previous classification in November, 2011. These defects can be found in all major groups of PIDs included in this report. In many cases, the mutations are not necessarily in genes formally implicated in immune cell function but are genes involved in essential cell processes. The more detailed analysis and functional consequences of such defects as illustrated by these PIDs will increase our understanding of the interplay between different cellular processes in the development and function of the immune system.

Among the newly identified, gene defects are many that are to date particular to a single pedigree or individual; such defects may prove exceedingly rare, or indeed may not necessarily be found to recur in other individuals. We have marked conditions for which there are 10 or fewer reported individuals with an asterisk, although historically, following the description of the first few cases, additional individuals with a similar PID phenotype and genotype have often been recognized. It is likely that we will uncover many more “personal” or very rare gene defects over time

and that the spectrum of PIDs will become increasingly diverse and complex, due to contributions of both environmental exposures and genetic modifiers to each affected individual. The value of this report therefore to capture and catalog the full spectrum at any one time point becomes increasingly important.

The goal of the IUIS Expert Committee on PIDs is to increase awareness, facilitate recognition, and promote optimal treatment for patients with PIDs. In addition to the current report and previous “classification table” publications, the committee has also produced a “Phenotypic Approach for IUIS PID Classification and Diagnosis: Guidelines for Clinicians at the Bedside,” which aims to lead physicians to particular groups of PIDs starting from clinical features and combining routine immunological investigations. Together, these contributions will hopefully allow a practical clinical framework for PID diagnosis. The committee also aims to establish a classification of PIDs based on other aspects and will work on publishing further guidelines in due course.

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