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Adaptive immunity in local tissues

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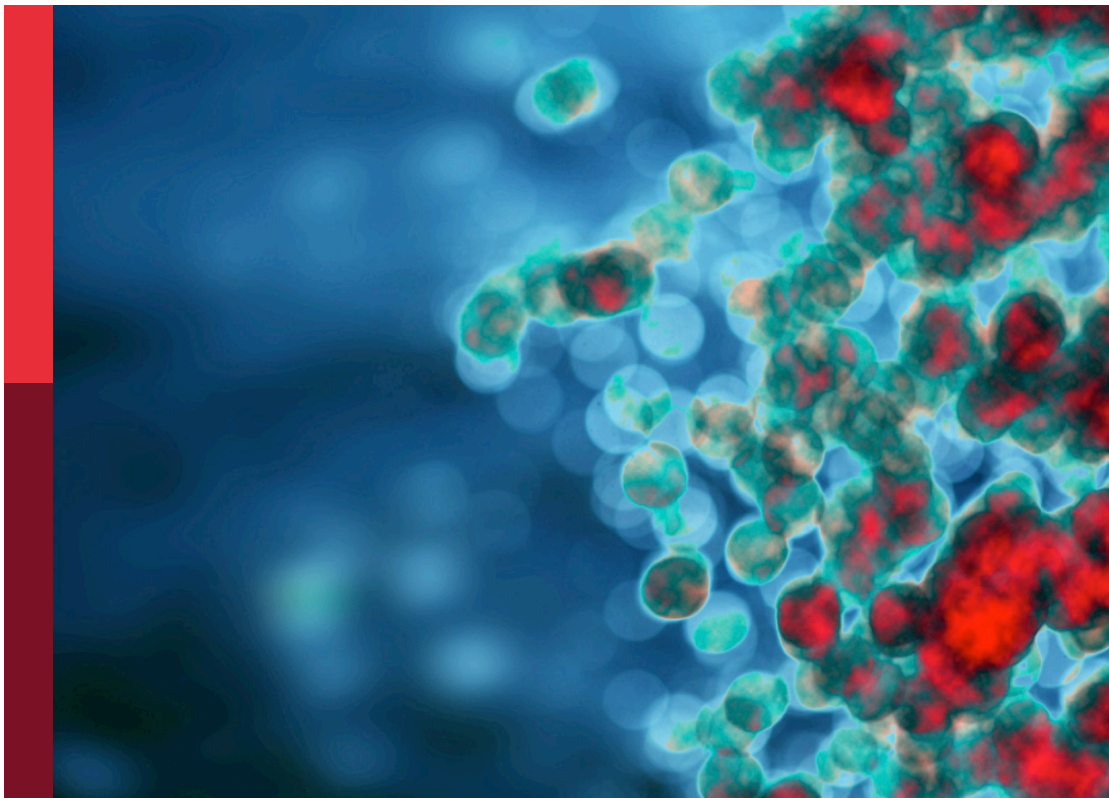

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Adaptive immunity in local tissues

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Editorial: Adaptive immunity in local tissues

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Editorial on the Research Topic
Adaptive immunity in local tissues

Traditionally, it has been thought that adaptive immune cells are activated in secondary lymphoid organs and migrate to peripheral tissues to perform their effect functions. However, more and more evidence has demonstrated that there are also naïve or resident adaptive immune cells in peripheral tissues (1, 2), and tissue immunology is beginning to reveal how these resident adaptive immune cells are integrated with organ physiology during both tissue development and disease evolution. Due to their unique microenvironment, with its own composite of cytokines and metabolites, immune cells in these peripheral tissues might have developed special immune characteristics that are different from those of immune cells in secondary immune organs, and participate directly in the pathophysiological activities of the local tissues (Figure 1). Since peripheral tissues may well be the direct site of infection or inflammation, the behavior of the adaptive immune cells in the local tissues is more closely related to the development of diseases than that in systematic lymphoid organs, especially at early stages of the pathogenesis (3). Therefore, understanding the characteristics of adaptive immunity in different peripheral tissues may be useful to identify novel therapeutic targets to develop immediate and effective remedies for regional diseases.

Several reviews and original research articles on adaptive immunity in various peripheral tissues are presented in this Research Topic. These cover the phenotype and function of T or B cells in peripheral tissues, as well as their roles in disease and possible therapeutic approaches.

T cells play an important role in maintaining homeostasis and inflammatory responses as helper or effector cells in adaptive immunity, which are activated and regulated by antigen-presenting cells (APCs) via cognate antigens presented and cytokines secreted respectively. Depending on the different microenvironment in the peripherial tissues, T cells can differentiate into different subtypes with different functions such as promoting inflammation, mediating immune tolerance, supporting cellular immunity, or favoring humoral immunity (4; Sun et al.; Sun et al.).

Barrier tissues are high-risk sites for infection and inflammation as the first line of defense against pathogens, Neuwirth et al. reviewed the commonalities and differences of the interactions between APCs and T cells in a variety of barrier tissues, including the skin, intestine, and female reproductive tract, under both homeostatic and infectious conditions. They pointed out that there are significant differences between T cells and the subsets of

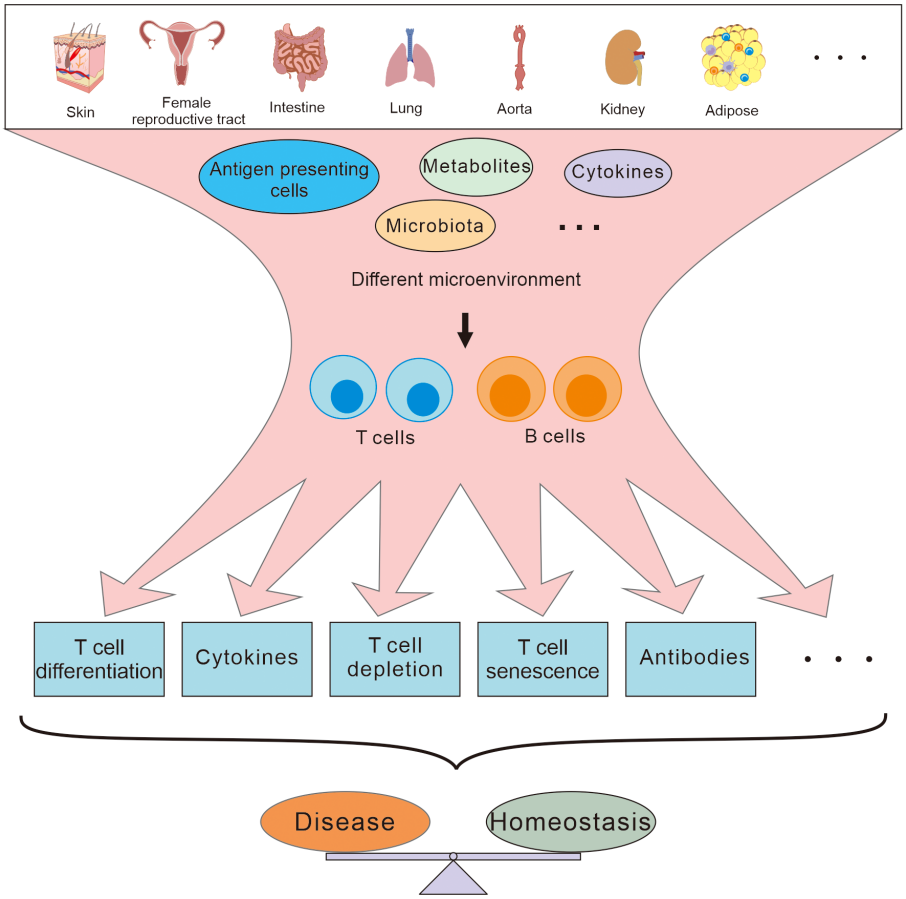

FIGURE 1
T and B cells residing in different peripheral tissues are involved in maintaining the homeostasis and disease development in different ways depending on the different microenvironment.

dendritic cells in different barrier tissues, controlling the balance between immune tolerance and immune responses mediated by regulatory T cells and other effector T cells respectively. Shirakawa and Sano summarized the transformation of $CD4^+$T cells in visceral adipose tissue as a result of obesity. It was shown that the functionalities of $CD4^+$T cells were closely associated with endocrine and metabolic homeostasis or dysfunction in visceral adipose tissue as well as obesity-associated chronic inflammation. Obesity-associated microenvironmental conditions could result in $CD4^+$ T cell depletion and accelerate $CD4^+$T cell senescence. Understanding these unique changes of $CD4^+$T cells in specific adipose tissues will assist in the development of drugs for obesity-related diseases.

Murayama et al. found that $CD4^+CD8^+$ Tfh cells, as a heterogeneous subpopulation of Tfh cells, were enriched in IgG4-related disease lesions of palatine tonsils. These $CD4^+CD8^+$ Tfh cells might regulate IgG4 production by memory B cells through cytotoxic effects and are potential targets for regulating regional humoral immunity.

Ma´rquez-Sa´nchez et al. summed up the functions of various adaptive immune cells, together with their innate counterparts, in abdominal aortic aneurysms, and concluded that there are special roles for angiotensin II and microbiota in the activation of adaptive immune responses in the vasculature and perivascular adipose, as well as in the regulation of this disease.

The results of Wang et al. led to the proposal that CARDS toxin activates a positive feedback loop of type I immune responses in the lung during M. pneumonia infection. This putative mechanism could be useful in future approaches to investigate immune interventions for M. pneumoniae pneumonia.

Liu et al. summarized recent advances in the insights on the role of T cells and their products in type 2 diabetic kidney disease, pointing out that T cells played protective or pathogenic roles through various means such as inducing insulin resistance, mediating podocyte injury, participating in fibrosis and regulating proteinuria, and T cell- directed therapies in type 2 diabetic kidney disease were established with preliminary results.

In addition to αβ T cells, which are restricted by MHC-II, T cells that play innate immune roles, such as γδ T cells and natural killer T cells, are prevalent in peripheral tissues and have been found to have important roles in regulating adaptive immunity (2, 5, 6). Chen et al. summarized the role of γδ T cells present in the mucosa and skin in a wide variety of vector-borne diseases. The paper pointed out that γδ T cells could secrete multiple cytokines for immune regulation, formed immune memory and responded rapidly by proliferation in secondary infections.

Understanding the characteristics of T cell responses in different tissues may be useful in identifying new therapeutic targets for translational gains. In this regard, studies and the development of drugs targeting T cells in periphery tissues may be of great interest. In a systematic review of the TCR-like antibodies and their application in identifying autoantigen-presenting APCs, Li et al. suggested that TCR-like antibodies could play an important role in the study and treatment of autoimmune diseases. Along the same line, Su et al. found that the regulation of Th2/Th22 differentiation by the Galectin-9/T cell immunoglobulin mucin-3 pathway in skin was closely associated with the development of atopic dermatitis. Furthermore, Yan et al. summarized the effects of platelets on various immune cells and suggest that platelets could regulate the production of leukocyte cytokines, depending on the severity of the disease.

In addition to T cells, tissue-resident B cells, the other arm of adaptive immunity, are also mentioned in this Research Topic. Lee and Oh reviewed the history, localization, origin, and markers of tissue-resident memory B cells, and summarized the unique characteristics of humoral immunity in peripheral tissues like skin, intestine, and female reproductive tract.

Although the important roles of regional adaptive immunity in disease have been recognized, there is still much to learn about the regulation of diseases by the regional adaptive immunity. This Research Topic makes timely selection of articles highlighting the current understanding of adaptive immunity in such disease-affected tissues as barrier tissue, visceral adipose tissue, and the vascular walls, and discusses possible research and therapeutic tools in these areas so that the better understanding of regional immunopathophysiology in the diseased tissues can be achieved for effective therapeutic intervention.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

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REVIEW
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Resident Memory B Cells in Barrier Tissues

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Epithelial barriers, which include the gastrointestinal, respiratory, and genitourinary mucosa, compose the body's front line of defense. Since barrier tissues are persistently exposed to microbial challenges, a rapid response that can deal with diverse invading pathogens is crucial. Because B cells have been perceived as indirectly contributing to immune responses through antibody production, B cells functioning in the peripheral organs have been outside the scope of researchers. However, recent evidence supports the existence of tissue-resident memory B cells (BRMs) in the lungs. This population's defensive response was stronger and faster than that of their circulating counterparts and could resist heterogeneous strains. With such traits, BRMs could be a promising target for vaccine design, but much about them remains to be revealed, including their locations, origin, specific markers, and the mechanisms of their establishment and maintenance. There is evidence for resident B cells in organs other than the lungs, suggesting that B cells are directly involved in the immune reactions of multiple non-lymphoid organs. This review summarizes the history of the discovery of BRMs and discusses important unresolved questions. Unique characteristics of humoral immunity that play an important role in the peripheral organs will be described briefly. Future research on B cells residing in non-lymphoid organs will provide new insights to help solve major problems regarding human health.

Keywords: resident memory B cells, respiratory infection, vaccine, humoral immunity, barrier tissues, mucosal immunity

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INTRODUCTION

Immune memory is an important component of our body's immune system. It enables rapid and strong responses to a pathogen by pathogen-specific memory cells. Another important protective component is the barrier tissues of the body. Mucosal barrier tissues, including the lung, intestine, skin, and female reproductive tract (FRT), etc., block pathogens from invading our body at its front line. Secretory IgAs, broadly neutralizing antibodies and neutralizing antibodies are secreted to the mucosa and bind to invading pathogens, thereby isolating these harmful organisms in the mucosa and excluding them from infecting host cells. This effector mechanism is not only efficient but is also safe because it causes less inflammatory response at the site of infection, while T cell-mediated responses usually cause collateral damage to the host (1).

During infection, mature naïve B cells specific to the pathogen can enter one of four differentiation fates (2, 3). In the earlier stages of immune response, the extrafollicular response generates short-lived antibody-secreting cells (ASCs) and germinal center (GC)–independent memory B cells (MBCs) that have undergone class-switch recombination but have minimal somatic hypermutation. The extrafollicular response is in charge of the early response against influenza virus, but it also the major mechanism protecting against several pathogens, including malaria and *Salmonella* (4). In the GC, a repetitive course of affinity maturation produces plasmablasts (PBs) and MBCs that synthesize high-affinity immunoglobulins. PBs migrate to the bone marrow (BM) where they differentiate into long-lived plasma cells (PCs), but MBCs circulate through the body until they re-encounter the specific antigen. These MBCs have the capacity to re-enter the GCs or generate ASCs, providing a rapid and stronger immunity for defense upon secondary infection (5).

Since the major basis of B-cell immunity is circulating antibodies, it was speculated that there is no need for resident MBCs in the peripheral tissues. Moreover, for proper antibody production, support from GC reactions should be provided. As GCs is a complex system built on the collaborative interactions of special types of stromal cells and immune cells, it is reasonable to question whether B cells in the mucosal tissues have access to this support. Therefore, in contrast to the research on T cells, there are few studies considering the concept of tissue-resident B cells.

A recent study provided direct evidence for the existence of resident memory B cells (BRMs) in the lungs, but no direct evidence supporting BRMs in other organs has been reported (6). In this review, we will skim through the history of the discovery of BRMs and the humoral immunity of non-lymphoid barrier tissues. The probability of the existence of BRMs in non-lymphoid organs other than the lungs will be examined. Last, in anticipation of identifying BRM-specific markers, markers for MBCs and their tissue residency will be reviewed in comparison with those of resident memory T cells (TRM).

TISSUE-RESIDENT MEMORY B CELLS

Evidence for Tissue-Resident Cells

It is now evident that some lymphocyte subsets are present *in situ* in non-lymphoid tissues and do not recirculate. Multiple experimental models have been used to demonstrate the residency of various cell types, including subsets of innate lymphoid cells, T cells, and recently B cells. Intravenous antibody labeling (iv-labeling) is a method that captures cells in circulation (7). Antibodies are injected intravenously into a mouse a few minutes before euthanasia. Circulating cells are captured by the antibodies but cells situated in the tissue are protected from them, allowing cells in each compartment to be distinguished from one another. The parabiosis model directly demonstrates the residency of the sessile cells. It is created by surgically joining two mice expressing distinct alleles. Circulating cells reach an equilibrium in both parabionts through the conjoined circulatory system, but the tissue-resident cells stay in the tissue, demonstrating that they reside in the tissue and do not recirculate (8–10). When infected tissues containing primed resident cells that express congenic markers are transplanted to naïve organisms, the primed resident cells do not recirculate to the recipient. Upon reactivation, local cells undergo secondary restimulation exclusively in the grafts, without the involvement of the recipient (11, 12). Models in which circulating lymphocytes have been ablated with cell type-specific antibodies have also been used (8, 13).

Based on studies using these experimental methods, the paradigm of TRM was established and intensively investigated over a decade. In contrast to TRMs, the history of BRMs is short and began with direct experimental evidence for resident non-circulating MBCs in the respiratory system (6). Since this discovery, subsequent studies have elucidated the unique characteristics of BRMs, and B cells in the human peripheral organs with resident phenotypes have been reported as well.

Brief History of BRMs

Only recently was a subset of B cells acknowledged to be resident in the lungs, but the notion of MBCs at the periphery that are distinct from the circulating MBCs and that do not recirculate is not new (**Table 1**). In 2008, an analysis of the dispersion of MBCs after local influenza infection was reported (14). In the analysis, after intranasal influenza virus infection, the distribution of MBCs in the blood, lung, and lymphoid tissues including mediastinal lymph nodes (mLNs), Peyer's patches, and spleen was examined. Among multiple organs, MBCs were found in the lymphoid tissues of the respiratory system, namely the mLNs and nasal-associated lymphoid tissue. Interestingly, a number of MBCs comparable to that in the mLNs were found in lungs 9 days post-infection. These lung MBCs persisted for at least 84 days, suggesting the establishment of peripheral MBCs in response to local antigen encounters. The authors anticipated the existence of mechanisms for tissue homing and delayed egress resembling those of TRMs. A few years later, more focused examinations of lung MBCs were performed. Cells situated in the lungs were separated from circulating cells by perfusing the right ventricle (RV) with PBS to clear the lungs of blood (15). Lungs harvested from influenza-infected mice after 160 days of infection still possessed MBC cells. When isolated MBCs were transferred into *scid* mice, lung MBCs outperformed MBCs derived from the mLN and the spleen in clearing the virus. These cells expressed higher levels of CD69, CXCR3, and IgA compared with MBCs in the mLNs and the spleen. These data suggest that lung MBCs are imprinted to migrate to the lungs and stay there. Next, the cross-reactive nature of lung MBCs was revealed, and it was shown that local lung GCs were responsible for supplying these cells (16). As antigenic drift is the main problem in confronting influenza virus infection, this result shows the importance of local lung mucosal immunity in defending against the infection. These early studies demonstrated interesting characteristics of a novel B-cell subset in the periphery but did not provide direct evidence for MBCs that are sessile in the lungs.

In 2019, through a parabiosis model and iv-labeling, lung MBCs were identified as a resident subset of cells in the lungs (6). In this study, resident lung MBCs were established upon local

TABLE 1 | Brief history of BRM research.

2008	2012	2015	2019	2020	2021	2022
MBCs are distributed in the lungs	**Lung MBCs discovered**	**Cross-reactive nature of lung MBCs**	**Lung BRMs that do not recirculate**	**Gut MBCs with resident phenotype**	**BRMs, a common feature of infected lungs**	**BRMs, a transcriptionally & functionally distinct B cell subset**
MBCs remained in the lungs beyond 12 weeks post infection.	Lung MBCs were isolated by RV perfusion.	Many cross-reactive lung MBCs originate from lung GCs, which show distinct selection features.	Non-circulating BRMs were discovered through a parabiosis model and iv-labeling.	Majority of human gut $CD27^+$ MBCs were CD45RB and CD69 double positive.	BRMs form in *S. pneumoniae* infection.	Human and murine BRMs have a transcriptional profile distinct from that of MBCs in PBMC and SLOs.
	When transferred into *scid* mice, the subset migrated to the lungs and was superior in resisting secondary viral challenge.		BRMs are independent from their circulating counterparts.	Gene sets of lung CD4 and CD8 TRMs were enriched in gut MBCs.	BRMs are also found in the pneumococcal pneumonia patients.	BRMs are the main source of respiratory IgAs.
			BRMs are established upon encountering local antigen.			BRMs migrate to sites of inflammation and differentiate into PCs.
Joo et al., 2008	Onodera et al., 2012	Adachi et al., 2015	Allie et al., 2019	Weisel et al., 2020	Barker al., 2021	Mathew et al., 2021 Oh et al., 2021 Tan et al., 2022 Maclean et al., 2022

antigen infection, but not through systemic immunization, and did not access the circulation of the parabiont. Also, this cell population was preserved *in situ* when the provision of B cells from the circulation was blocked by fingolimod (FTY720), implying the independence of the subset from the circulation. Functionally, these resident B cells differentiated rapidly into ASCs during secondary infection, providing a rapid antibody response against the pathogen. These results suggest that BRMs are a key component in mucosal humoral immunity. Local resident MBCs have also been shown to contribute to the secretion of local IgA (17). Importantly, mice with these cells showed superior protection against both the homologous and heterologous strains of influenza virus, supporting the cross-reactivity of local humoral immunity.

Following BRMs' identification as resident lymphocytes, diverse aspects of their biology have been investigated. First, BRMs are not a pathogen-specific cell population: establishment of BRMs in the lungs is detected in the *S. pneumococcus*–infected model as well as influenza virus infection (18). Second, MBCs in human lungs and gut with resident phenotypes have also been described (18–20). IgD^-CD27^+ MBCs from the lungs and draining LNs expressed higher levels of CD69, a representative marker for tissue-resident lymphocytes, compared with the spleen (20). When the phenotypes of $CD27^+$ MBCs derived from multiple human organs including the spleen, blood, BM, LN, tonsil, and the gut were investigated, $CD27^+$ MBCs in the gut included a higher percentage of CD45RB and CD69 double-positive cells (19). Also, an analysis of transcriptional profiles showed that lung MBCs cluster discretely from MBCs in lung-draining LNs or PBMCs, implying that lung BRMs have distinctive features other than the expression of CD69 (20). Markers and phenotypes of BRMs will be further discussed below.

Recently, the fate of BRMs upon secondary challenge was reported (21). In a live-imaging analysis, alveolar BRMs of influenza-infected mice were attracted by CXCL9 and CXCL10 induced by alveolar macrophages and migrated to inflammation foci to differentiate into PCs upon secondary challenge. The study not only demonstrates how BRMs react upon secondary challenge but also suggests that they interact with surrounding cells.

QUESTIONS ABOUT RESIDENT MEMORY B CELLS

Location of Resident Memory B Cells

Besides their presence, many aspects of BRMs are not discovered yet, including their location, markers, origin, the underlying mechanism that triggers their generation, and the environment that supports their maintenance (**Figure 1**). Regarding location, two studies using different infection models, one influenza virus and the other *S. pneumoniae*, have reported seemingly contradictory results (18, 20). The study using the influenza virus model showed that BRMs reside in the inducible bronchus-associated lymphoid tissues (iBALTs) by demonstrating the presence of antigen-specific B cells in the iBALTs beyond 110 days after infection. But BRMs were also present in an *S. pneumococcus*-infected model, in which iBALTs do not form. These are possibly complementary results, showing that BRMs not only reside in the tertiary lymphoid organs where survival

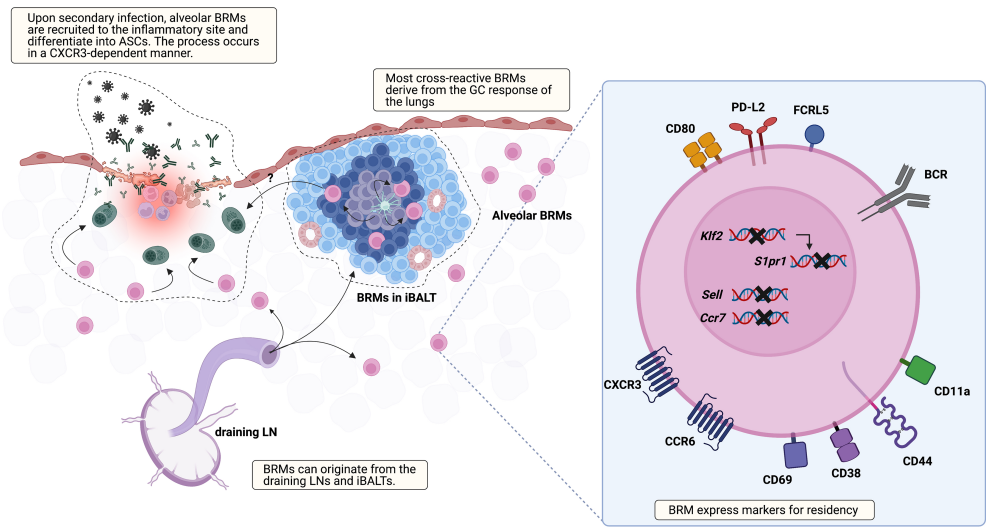

FIGURE 1 | Overview of lung BRM. BRMs are established in sites exposed to local antigens. It is likely that early lung BRMs derive from the draining LNs and late cross-reactive BRMs originate from lung GC reactions, which occur in iBALTs. Lung BRMs can be located within the iBALTs or reside throughout the lung parenchyma in close contact with alveoli, independent of iBALTs. Upon secondary infection, alveolar BRMs migrate to the inflammation foci, which is mediated by alveolar macrophages, and differentiate into PCs. The corresponding functional role of BRMs in iBALT has not been reported. The illustration of the BRM shows most of the surface molecules described in published studies so far. The upregulation of CD69, which reduces surface expression of S1PR1, is consistently reported. *Klf2* is a transcription factor that mediates S1PR1 expression. The expression of genes encoding the LN homing molecules *Sell* and *Ccr7* is downregulated. CXCR3 and CCR6 facilitate recruitment and/or retention of BRM in the lung. In addition, CCR6 is related to BRM differentiation into PCs in recall response. The upregulation of CD44 and CD11a in BRM is also reported. These may serve as an adhesion molecule for BRMs, but their functional role needs to be validated. Compared with circulating MBCs, lung BRMs have been shown to express higher levels of FCRL5, CD80, and PD-L2.

niches are provided but also can persist in the bare lung parenchyma with minimum support.

A recent study reported that BRMs not situated in the iBALTs, namely alveolar BRMs, relocate themselves to the inflammatory foci upon secondary challenge in an influenza virus infection model (21). In this study, aggregates of previously activated B cells within iBALT that express tdTomato in *Aicda* $(AID)^{Cre/+}$ $Rosa26^{tdTomato}$ reporter mice expressed the GC B-cell marker GL7 as well. The authors also showed that the cells within iBALT-like structures display typical extensive but confined motility behavior associated with GC B-cell characteristics, suggesting that the cells in the iBALT are GC B cells, not BRMs. As previous studies suggested BRMs residing within iBALT, the question of the differences of BRMs located in each locus remains. The mechanism that supports long-standing BRMs in the lung requires further investigation, especially alveolar BRMs minimally supported by adjacent cells. This could resemble that of PCs in the gut lamina propria. A subset of lamina propria PCs live for decades and their survival is supported by surrounding cells (22). Cytokine profiling of biopsy cultures has revealed the presence of IL-6 and APRIL, which are B-cell survival factors (23). Production of these cytokines by gut epithelium, eosinophils, macrophages, and DCs may provide the survival niche for PCs (24, 25). The possibility that innate immune cells and the induced stromal cells support the survival of alveolar BRMs needs to be examined.

Origin of Resident Memory B Cells

Given that the fate of B cells can be determined by signals provided by the surrounding tissues, the environment where BRMs are generated would affect the characteristics of BRMs (3). The GCs in the mLNs or GC-like structures of the iBALTs have been suggested as the sites for BRM generation in nasal influenza virus infection (6, 16). BRM cells are proposed to be generated in a T-cell-dependent manner at an early time point after infection (6). IgM^+ BRMs can be identified in the lungs at day 10, but isotype-switched ones require a longer time, not exceeding 30 days. Given that GC B cells emerge earlier in the mLNs than in the lungs, it is reasonable to think that early BRMs originate from the mLNs (16). Still, specific labeling of the lungs with EdU revealed that BRMs can also arise from the lymphoid structures in the lungs (16). These results led to the proposal that early BRMs originate from B cells that migrate from the draining lymph nodes and late BRMs originate from the iBALTs (16). Questions remain as to whether iBALT-originated MBCs can access the circulation and whether B cells are determined to become BRMs during the GC reaction or if any MBCs have the capacity to become resident cells when proper conditions are provided.

Cells of different origins would have different features. Lung-specific EdU labeling has also revealed that a larger proportion of BRMs originating from the lungs are cross-reactive, in comparison with BRMs derived from the draining LN (16). This suggests that lung GC reactions are distinct from

those of mLNs and that this may endow BRMs with different features (26). Fate-mapping techniques that can distinguish MBCs originating from different organs would be valuable in elucidating the heterogeneous characteristics of BRMs in the lungs.

Markers for Resident Memory B Cells

Elucidating the nature of BRMs urgently requires specific markers. Although BRM-specific markers have not yet been discovered, they can be identified by using gating schemes combining MBC with resident markers. Classically, it has been thought that MBCs are generated from the GCs in a T-cell-dependent manner by which they lose the expression of IgM and IgD and gain somatic hypermutation (27, 28). Therefore, in both humans and mice, isotype-switched B cells have been widely accepted as a surrogate population for MBCs. But this approach can be misleading since studies have revealed that a large compartment of MBCs is generated through a route independent of GC reaction. It is evident that IgM-expressing MBCs exist (29–34). Also, though in rare cases, the presence of IgD-only and IgD/IgM double-positive MBCs has been suggested in humans (35, 36).

In humans, CD27 is expressed by most MBCs and is commonly accepted as a defining marker of this population, but this is not true in mice (35, 37). In the absence of a definitive surface marker that encompasses murine MBCs, B cells that express CD38, a surface molecule downregulated in the PC and GC B cells and have an isotype-switched phenotype are considered to be MBCs (38–40).

Systematic analysis of MBC gene expression has suggested CD80, PD-L2, and CD73 as MBC markers, and the combination of these markers divides MBCs into three major subsets (41–43). These subsets are CD80 PD-L2 double negative, PD-L2 single positive, and CD80 PD-L2 double positive. These three subsets differ in their general properties of B cells, such as isotype switching and somatic hypermutation. Regarding antibody isotypes, 95% of the $CD80^-PD\text{-}L2^-$ subset express IgM, about 40% of $CD80^+PD\text{-}L2^+$ cells have IgM, and 90% of $CD80^-PD\text{-}L2^+$ cells express IgM (43). When the BCR mutation burden was evaluated, $CD80^-PD\text{-}L2^-$ cells were less mutated, whereas 80% of $CD80^+PD\text{-}L2^+$cells had a mutated Vλ1 gene segment, and $CD80^-PD\text{-}L2^+$ cells were in between (43, 44). In line with these findings, $CD80^-PD\text{-}L2^-$ cells were found to be produced earlier in the GC reaction, around day 5 post-infection, while the production of $CD80^+$ $PD\text{-}L2^+$ cells dominated after 12 days, and this subset required a stronger signal from CD40-CD40L interaction with T cells. $CD80^-PD\text{-}L2^+$ MBCs peaked between these time points (34). Functional studies have revealed that three MBC subsets enter distinct routes of differentiation upon reactivation. $CD80^-$ $PD\text{-}L2^-$ cells predominantly reenter the GC reaction and generate most of the ASCs that appear later. $CD80^+PD\text{-}L2^+$ MBCs generate IgG ASCs at an earlier time point. Again, $CD80^-$ $PD\text{-}L2^+$ subsets are intermediate in that they can choose either route (5). Analysis of RNA expression patterns also supports this feature (5). Microarray data suggest that $CD80^-PD\text{-}L2^-$ MBCs display higher expression levels of genes encoding cell cycle–promoting molecules, and $CD80^+PD\text{-}L2^+$ MBCs express higher levels of *Zbtb32*, which is related to PC differentiation (45).

MBCs of peripheral organs express these markers as well. MBCs from the Peyer's patches were isolated by gating $CD138^-$ $CD9^-CD80^+CD73^+$ B cells (46). These markers have also been detected in BRMs in the lungs. Compared with MBCs in the mLN and spleen, lung BRMs were found to express fewer CD73 but more PD-L2 (6). These markers are evidence of the heterogeneous nature of MBCs.

Currently, iv-labeling is used to identify resident subsets of MBCs. To find specific markers for BRMs, such as CD69 and CD103 for $CD8^+$ TRMs, transcriptional profiles of both murine and human BRMs have been analyzed (17, 20, 47). From their first appearance, lung MBCs showed higher expression of CXCR3 and CD69 compared with their counterparts in the mLN and spleen. Higher expression of these two molecules has been consistently reported in subsequent studies on BRMs. This expression pattern suggests the tendency of BRMs to head toward peripheral tissues and the operation of a mechanism delaying their egress, which is also observed in TRMs. Analysis of TRM transcriptional profiles has revealed the downregulation of S1PR1, the key receptor that recognizes the egression element S1P (48–50). In the TRM the transcription factor KLF2, which mediates the expression of S1PR1, is downregulated (49), and CD69, which internalizes and degrades S1PR1, is expressed (51, 52). Similarly, in the mouse model, lung BRMs, which were iv-labeling negative, were clustered discretely from iv-labeling-positive lung MBCs and MBCs from the blood, spleen, and mLN (20). The marked expression pattern of lung BRMs was the downregulation of *Ccr7, Sell, S1pr1*, and *Klf2*, and upregulation of *Cxcr3, Ccr6, Ccr1*, and *Cd69*. In addition, BRMs in a pneumococcal pneumonia model upregulated CD11a and CD44 but downregulated CD62L, a phenotype similar to that of lung CD4 TRM cells (53, 54). A similar pattern is also observed in human organs. Upregulation of CD69 and the two chemokine receptors CXCR3 and CCR6 has been detected in $CD27^+$ B cells from human lungs (18, 20). At the transcript level, downregulation of *S1PR1*, *SELL*, and *CCR7* was observed. Also, as mentioned above, MBCs in the gut are mostly CD69 positive (19). These results imply that BRMs share underlying mechanisms that are in common among lymphocytes resident in non-lymphoid organs.

Other surface markers or transcriptional regulators specific to BRMs need to be identified. CD103, a marker for $CD8^+$ TRMs, is not expressed in lung BRMs (6). In the case of TRMs, several transcription factors (TFs) that regulate the development and maintenance of resident cells are known (10). Blimp-1, Hobit (a homolog of Blimp-1), and AhR promote the generation and maintenance of resident cells, while the expression of Klf2 and the T-box TFs Eomes and T-bet oppose it. Some of these TFs have an effect on B cells but in a cell-type-specific manner (3, 55, 56), and studies testing these TFs on BRM formation have yet to be reported. The fact that the transcriptional program that decides the differentiation fate of MBCs is still not fully discovered is an obstacle to identifying regulatory factors in BRM formation. However, since rapid responsiveness and cross-reactivity make

BRMs a promising cell type that can aid resistance to fatal infection, the underlying transcriptional program should be thoroughly revealed in order to utilize this cell population.

HUMORAL IMMUNE CELLS SITUATED IN NON-LYMPHOID BARRIER ORGANS

Resident Memory B Cells and Antibody-Secreting Cells in the Intestine

The intestine is a unique organ in the sense that it harbors numerous lymphoid organs, the gut-associated lymphoid tissues (GALT), from birth and is the site where active interaction with the environment shapes the humoral immunity of the region. The humoral immunity of the intestine is well described in other reviews (57, 58). After pointing out several aspects of the gut humoral immune system, this review will focus on the resident memory B cells in the gut.

Factors that promote the dominance of IgA in the mucosa-associated lymphoid tissues (MALTs) are fairly well investigated in the gut. In T-cell-dependent class-switch recombination, CD40 signaling and TGF-β play an important role. It appears that NO produced by inducible nitric-oxide synthase-expressing DCs induces the expression of TGFβRII (59). Also, DCs are major players in the T cell-independent response. These cells provide proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF) that promote IgA-specific class switching. These cells are activated by commensal microbiota through toll-like receptor signaling. Dietary factors also have an effect on the production of IgA. Retinoic acid signaling is suggested to be important in generating IgA, and short-chain fatty acids produced by gut microbiota support antibody production by controlling the metabolism of B cells (60, 61). Collectively, these results show the tight relationship between the microbiota and the humoral immunity of the gut.

Another interesting example of the interaction between the environment and the immune system is the imprinting of GALT-derived ASCs by gut-homing molecules (**Figure 2A**). Retinoic acid secreted by DCs in the GALTs induces the expression of these molecules, which are integrin α4β7 that binds to mucosal addressin cell adhesion molecule 1 (MAdCAM-1) expressed on endothelial cells in the lamina propria, and the chemokine receptors CCR9 and CCR10, which respond to CCL25 and CCL28 produced by the intestinal epithelium (62–64). ASCs expressing these molecules home back to the gut lamina propria, where they secrete antibodies. Human IgM^{hi} transitional B cells expressing α4β7 tend to migrate to the intestine to enrich the GALTs (65). MBCs are not an exception. These molecules have been suggested to be essential for the recruitment of IgA^{+} MBCs to the intestine (66, 67).

Returned MBCs are likely to recirculate between different Peyer's patches and re-enter the GC response, resulting in the persistence and evolution of the IgA repertoire (46). BCR repertoire analysis has revealed that PCs are more clonally related to MBCs, suggesting that MBCs recirculate. New clones

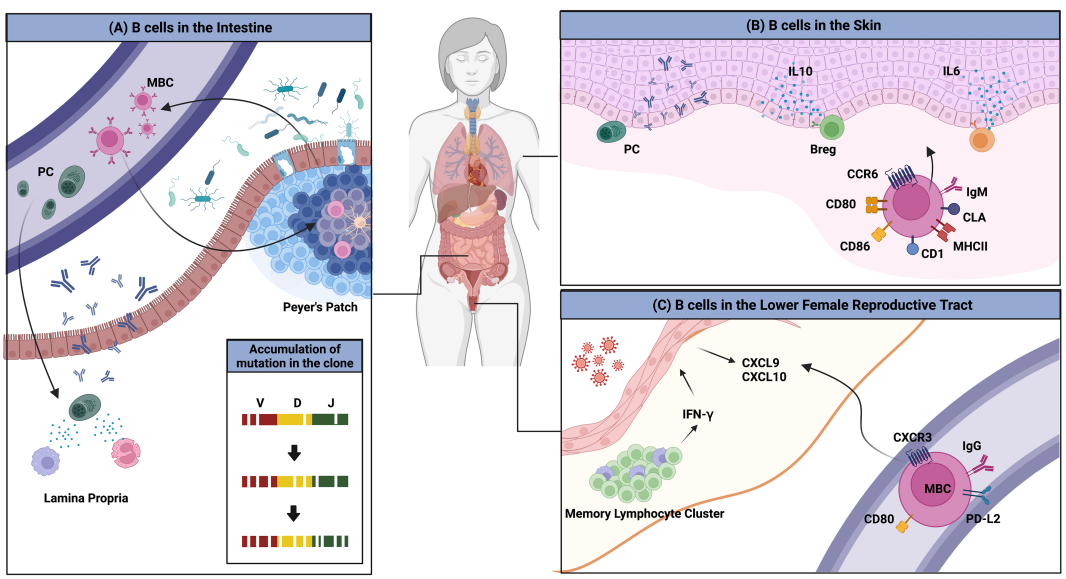

FIGURE 2 | Overview of B cells in multiple peripheral organs. **(A)** The gut microbiota is a consistent stimulus to immune cells in the gut. MBCs and PCs that are formed access the circulation and return to the gut; this migration is mediated by the chemokine receptors CCR9 and CCR10, and integrin α4β7. PCs reside in the lamina propria and MBCs enter the GC reaction, which results in the evolution of BCR repertoires. **(B)** Several clues for the existence of skin-associated B cells are provided. B cells that migrate to the skin have higher expression of MHCII, CD1, CD86, CD80, and IgM. The migration to the organ is mediated by CCR6 and CLA. B cells in the skin produce antibodies locally and regulate the immune reaction directly by producing the pro-inflammatory cytokines such as IL-6 or the anti-inflammatory cytokine, IL-10. **(C)** BRMs are not established in the lower FRT. Upon secondary infection, circulating MBCs rapidly migrate to the tissues in a CXCR3-dependent manner. These cells show higher expression of MBC markers including PD-L2 and CD80. These cells could not stay long in the lower FRT tissues.

were introduced upon new infection. The authors discussed this observation and proposed that this mechanism is necessary for stable interaction between the host and microbiota (46). In humans, a clonal relationship between IgM^+ MBCs and IgA^+ MBCs and PCs has been observed, suggesting that gut IgM^+ MBCs can switch to expressing IgA (68). Recently, a study reported that the majority of $CD19^+CD27^+$ B cells in the human gut are CD45RB CD69 double-positive, a distinguishing feature of gut MBCs (19). $CD4^+$ and $CD8^+$ lung TRM gene sets were found to be enriched in this double-positive subset. These data imply the possibility that some unidentified portion of recruited MBCs may reside in the gut for a long period and operate in a unique manner.

Skin-Associated B Cells and Their Function

The skin is the largest barrier tissue that faces a variety of daily threats, including UV, injuries, pathogens, and commensals. Traditionally, the skin was thought to lack B cells and contain mostly T cells (69, 70). However, recent studies have reported the presence of B cells with interesting functions, including antibody production and antibody-independent function, in both healthy and diseased skin (**Figure 2B**) (71).

Clonally restricted B cells have been observed in normal skin, implying the existence of B cells specific for local skin antigens (72). In normal skin of an ovine model, skin-associated B cells were found to be a heterogeneous population that comprised a B-1 B-cell–like phenotype, IgM^{hi} and $CD11b^{hi}$, and an activated phenotype, expressing higher levels of MHC II and CD80/86 (73). IgM ASCs that reside in healthy mouse and human skin have also been observed (74). The survival of ASCs was dependent on APRIL and BAFF produced at the site. It was suggested that these B cells migrate to the skin through a CCR6-CCL20 axis (73). Cutaneous lymphocyte antigen is the molecule that guides T cells into the skin. As parenteral immunization induced its expression in ASCs, it appears that B cells are recruited to the skin in a similar manner (75). Though direct evidence of skin BRMs was not provided, these results imply their possible existence. In addition, skin-associated B cells appear to be directly involved in immune reactions in the skin.

The functions of B cells in the skin in pathologic conditions are relatively well studied. One is local antibody production. For example, pemphigus is characterized by circulating anti-desmoglein 1/3 (Dsg1/3) autoantibodies that target the desmosomal adhesion molecules anchoring epidermal keratinocytes (76). It has been suggested that Dsg1/3-specific B cells infiltrate the lesion and that autoantibodies can be produced locally (77). B cells can secrete cytokines to promote inflammation. A study using a bleomycin-induced scleroderma model reported an accumulation of IL-6–producing B cells in the inflamed skin, and the skin and lung fibrosis were attenuated in IL-6 deficient mice (78). The result demonstrates the antibody-independent function of B cells in the skin.

Regulatory B cells (Bregs) are capable of suppressing the inflammatory response by producing the anti-inflammatory cytokine IL-10. A subset of both mice and human skin–associated B cells with innate-like phenotypes, which are $CD1d^{hi}$ $CD5^+$ in mice and $CD11b^+$ in humans, is reported to produce IL-10 upon stimulation (79). Bregs have been found to limit inflammation in several disease models. IL-10–deficient mice show more severe fibrosis in the scleroderma model mentioned above (78). Peritoneal B-1a cells display a regulatory function in a contact hypersensitivity model, and IL-10–producing $CD1d^{hi}CD5^+$ B cells can negatively regulate inflammation in an imiquimod-induced psoriasis model (80, 81).

Given the diverse role of B-cell inflammatory skin disorders, depleting pro-inflammatory B cell subsets while retaining the regulatory subset would be a promising means for treating these diseases, but the identity of Bregs is not fully elucidated. The question of whether these cells are a specific lineage or if any B cell subsets can become Bregs under certain conditions should be answered first. If the latter is the case, the conditions should be specified (82).

Memory B Cells in the Lower Female Reproductive Tract

In terms of BRM, the lower FRT is the lungs' opposite. Circulating antibodies are unable to enter the tissue or reach the lower FRT lumen (83, 84). Local immunization, however, can increase the titer of antibodies in the vaginal lumen, with the activity of these antibodies being higher than that of their circulating counterparts (85, 86). Also, antibodies that are passively transferred to the lumen are capable of controlling infection (87). These findings imply that antibodies in the lower FRT lumen are produced locally. This hypothesis is supported by the presence of PCs in the lower FRT of mice locally immunized with attenuated HSV type 2. These antibody-producing cells appeared under the epithelium after secondary challenge with wild-type viruses and lasted for 10 months. The increment of IgG-producing cells was more than 10 times higher than the increment of IgA-producing plasma cells, which explains why IgG is the dominant antibody isotype in the lower FRT (86). Similar results were found in a study of HIV-1: the level of vaginal secretion of anti–HIV-1 antibodies was higher than that in the serum (85). A study on SIVmac239Δnef vaccination also showed that PCs appeared after vaccination, supporting the presence of local antibody production (88).

Notably, tissues that compose the lower FRT do not permit access by circulating B cells. Immunization with attenuated HSV-2 is insufficient to establish PCs and MBCs in the tissue. In the mouse genital herpes model, only after a secondary challenge with wild-type virus were IgG^+ circulating MBCs recruited, and they contributed to the proper antibody production (**Figure 2C**) (87). These cells express high levels of CD80, PD-L2, and CXCR3. Their migration is mediated by CXCR3-ligand chemokines induced by IFN-γ produced from CD4 TRM maintained in memory lymphoid clusters, which are immune clusters composed mainly of CD4 TRMs and macrophages (89). However, in contrast to the lung, in which BRMs are embedded for at least 120 days, BRMs do not form in the lower FRT (87). This discrepancy may result from the different microenvironments the two organs provide to B cells.

CONCLUDING REMARKS

In this review, we have briefly described the discovery of BRMs in the lung. The timeline is short but several studies highlighting its distinguishing features have been published recently. The rapid response of BRMs upon secondary infection and their cross-reactive potential make them a valuable target for vaccine design. To control this cell population, several questions including their location, origin, specific markers, and transcriptional regulators must be answered. The different features of BRMs and their survival niches in different locations should be identified. Identifying the origin of BRMs and the cross-talk between BRMs and the microenvironment will help to determine the factors that regulate the generation and establishment of BRMs. Although there is no direct evidence of BRM existence in other barrier tissues rather than the lung, B cells and ASCs have diverse properties and play important roles in multiple barrier tissues. Further investigation is required to elucidate the characteristics and the residency features of these cells. Understanding the molecular pathways that regulate the interaction of these cells and their microenvironment could reveal the key factors that determine tissue-specific immune properties.

AUTHOR CONTRIBUTIONS

CL and JO wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Galectin-9 contributes to the pathogenesis of atopic dermatitis *via* T cell immunoglobulin mucin-3

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Background: Atopic dermatitis (AD), a common type 2 inflammatory disease, is driven by T helper (T_H) 2/T_H22polarization and cytokines.Galectin-9 (Gal-9), *via* its receptor T cell immunoglobulin- and mucin-domain-containing molecule-3 (TIM-3), can promote T_H2/T_H22 immunity. The relevance of this in AD is largely unclear.

Objectives: To characterize the role of TIM-3 and Gal-9 in the pathogenesis of AD and underlying mechanisms.

Methods: We assessed the expression of Gal-9 and TIM-3 in 30 AD patients, to compare them with those of 30 healthy controls (HC) and to explore possible links with disease features including AD activity (SCORAD), IgE levels, and circulating eosinophils and B cells. We also determined the effects of Gal-9 on T cells from the AD patients.

Results: Our AD patients had markedly higher levels of serum Gal-9 and circulating TIM-3-expressing T_H1 and T_H17 cells than HC. Gal-9 and TIM-3 were linked to high disease activity, IgE levels, and circulating eosinophils and/or B cells. The rates of circulating TIM-3-positive $CD4^+$ cells were positively correlated with rates of T_H2/T_H22 cells and negatively correlated with rates of T_H1/T_H17 cells. Gal-9 inhibited the proliferation and induced the apoptosis of T cells in patients with AD, especially in those with severe AD.

Conclusion: Our findings suggest thatGal-9, *via* TIM-3, contributes to the pathogenesis of AD by augmenting T_H2/T_H22 polarization through the downregulation of T_H1/T_H17immunity. This makes Gal-9 and TIM-3 interesting to explore further, as possible drivers of disease and targets of novel AD treatment.

KEYWORDS

T cell immunoglobulin- and mucin-domain-containing molecules-3 (TIM-3), Galectin-9 (Gal-9), T_H1 cells, T_H2 cells, T_H17 cells, T_H22 cells, atopic dermatitis

Introduction

Atopic dermatitis (AD) is a common T-cell mediated skin inflammatory disease, with abnormal activation of several subpopulations of T helper (T_H)cells (1–3). We and others have shown that AD, in many patients, is characterized by excess T_H2/T_H22 cell activity (4–8). The increased production of T_H2 cytokines such as interleukin (IL)-4 and IL-13 initiates a complex immune cascade that includes the generation of allergen-specific IgE-producing B cells and eosinophil migration to AD skin lesions (9–14), two hallmark features of AD. Furthermore, T_H2 and T_H22 cytokines inhibit skin barrier protein-encoding genes such as filaggrin, loricrin, and involucrin (15) and the production of antimicrobial peptides, both of which are held to contribute to the increased susceptibility to skin infections in patients with AD (16). The key role of T_H2/T_H22 cytokines in the pathogenesis of AD is supported by the efficacy of treatment with the anti-IL-4 receptor antibody dupilumab and an anti-IL-22 antibody (ILV-094) (17–19). As of now, it is largely unclear what drives T_H2/T_H22 skewing in AD.

Galectin-9 (Gal-9) is a tandem-repeat type galectin with two carbohydrate-recognition domains, and it was first identified as an eosinophil chemoattractant and activation factor (20, 21). It is universally expressed in a wide range of immune and non-immune cells and is known to regulate different biological functions, such as cell adhesion, differentiation, aggregation, and cell death (22). Galectin-9 is a versatile immunomodulator that has recently been shown to be associated with the pathogenesis of AD. For example, the skin of AD patients exhibits increased levels of Gal-9, especially in the epidermis, and increased numbers of Gal-9 positive eosinophils and mast cells (23). Blood levels of Gal-9, in patients with AD, were reported to be significantly higher than in healthy controls (HC) and correlated with disease activity (24).

Gal-9 exerts its biological functions *via* multiple receptors, including CD44 and T-cell immunoglobulin and mucin containing-protein 3 (TIM-3). TIM-3 is expressed by several populations of immune cells including terminally differentiated T_H1, T_H17, and Tc1 lymphocytes as well as NK, monocytes, and myeloid cells, whereas T_H2/T_H22 cells do not express TIM-3 (25). Gal-9 signaling *via* TIM-3 is held to modulate immune responses and diseases. For example, we have previously shown upregulation of Gal-9 and TIM-3 in the serum and peripheral blood mononuclear cells of patients with systemic lupus erythematosus (SLE), and this was closely related to disease activity (26). Gal-9, *via* TIM-3, induces apoptosis in T_H1 and T_H17 cells (27, 28), is involved in tolerance induction and T cell exhaustion (25, 27, 29, 30), and downregulates T_H1/T_H17-biased immune responses resulting in T_H2 polarization. Whether or not TIM-3 plays a role in AD is currently unknown.

To address this question, we investigated patients with AD and HC for their Gal-9 serum levels and rates of circulating TIM-3-positive cells, we characterized the clinical relevance of Gal-9 and TIM-3 in AD, and we explored potential mechanisms that underlie their role in the pathogenesis of AD.

Materials and methods

Study conduct, patients, and control subjects

Ethical approval from the Ethics Committee of The First Affiliated Hospital of Soochow University (Suzhou, China, No. 2014809026) was obtained prior to the study. All patients provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association. AD was diagnosed in accordance with the criteria of Hanifin and Rajka and disease severity was evaluated using the SCORing Atopic Dermatitis index (SCORAD), with 0–24, 25-50, and >50 points reflecting mild, moderate, and severe AD,

Abbreviations: AD, Atopic dermatitis; T_H2, T helper T_H 2; TIM-3, T cell immunoglobulin- and mucin-domain-containing molecules-3; Gal-9, Galectin-9; HC, Healthy controls; TIM, T cell immunoglobulin mucin; PBMCs, Peripheral Blood Mononuclear Cells; AV/PI, Annexin V/propidiumiodide.

respectively (31). At the time of the study and for one month prior, none of the patients were treated with systemic steroids or other immunosuppressant treatments, or potent topical steroids, or topical corticosteroids as well as other medications (e.g. antibiotics, light therapy ect.). Patients with other allergic conditions, e.g., pollen allergy, food allergy, or allergic asthma, et al. were excluded. Age-matched healthy blood donors were recruited as controls, all of whom were without any allergic conditions (n =30, female: 19; mean age: 10.4 ± 4.7 years).Pediatric allergy specialists and trained field technicians performed the physical examinations, SCORAD score assessments, and collected blood samples.

Laboratory investigation including blood routine examination and IgE levels. Total and specific IgE levels measured at the central laboratory (Central Labor, The Second Affiliated Hospital of Soochow University) using Immuno CAP System (Phadia Laboratory Systems, Thermo Fisher Scientific Inc, Uppsala, Sweden).

Peripheral blood mononuclear cell purification

PBMCs were immediately isolated and purified from drawn blood as previously published (32). Briefly, PBMCs were isolated from heparinized venous blood on Ficoll-Hypaque gradients (Pharmacia, Uppsala, Sweden) and re-suspended in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with gentamicin (40μg/mL) and 10% pooled type AB normal human serum (Sigma-Aldrich).

Flow cytometric analyses

T cells were stained with fluorescent-labeled monoclonal antibodies against CD-4-FITC (300538, Clone: RPA-T4, Biolegend), TIM-3-PE (345006, Clone: F38-2E2, Biolegend), IFN-γ-APC (502512, Clone: 4S.B3, Biolegend), IL-17A-APC (512334, Clone: BL168, Biolegend), IL-4-APC (500812, Clone: MP4-25D2, Biolegend), and IL-22-APC (366706, Clone: 2G12A41, Biolegend). Intracellular staining was performed as follows: Surface staining was performed for 20 minutes with CD-4-FITC and/or TIM-3-PE antibodies on ice. Cells were washed and resuspended in fixation/permeabilization solution (420801/421002, Biolegend) and stained with IFN-γ-APC, IL-17A-APC, IL-4-APC, and IL-22-APC. B cells were stained with CD19-PerCP (392510, Clone: 4G7, Biolegend). Armenian hamster IgG (400908, Clone: HTK88, Biolegend), mouse IgG1 (400108, Clone: RTK2071, Biolegend), mouse IgG2a (400246, Clone: MOPC-173, Biolegend), and mouse IgG 2b (400314, Clone: MPC-11, Biolegend) were used as isotype controls. Cells were analyzed with a Coulter Epics XL Flow cytometer (Beckman) and a Coulter FC 500 ANALYZER (Beckman Coulter); the relevant data were obtained and analyzed using FlowJo software, version 7.6.

Gal-9, PBMC proliferation, apoptosis, and cytokine production analysis

For analysis of the Gal-9 level, serum was obtained by centrifuging peripheral blood samples (PBs) from patients with AD; the level of expression of Gal-9 in serum was detected using ELISA kits (AMS Biotechnology, UK), and the PBMC were separated by density gradient centrifugation. Cells from the interphase were collected and washed twice with Dulbecco's PBS. For analysis of proliferation, apoptosis, cytokine production, freshly isolated PBMCs (1×10^5 cells/well) were cultured in RPMI 1640 medium (Gibco, USA) containing 10% human AB serum (Gibco) with Recombinant Gal-9 (0.5μg/mL, 1μg/mL, 2μg/mL, and 4μg/mL, ICA309Bo01, LMAI Bio) and LEAFTM Purified Anti-Human CD3 Antibody (100 ng/mL, BioLegend) in 96-well plates for 72 hours, respectively. For analysis of cell proliferation, cell viability was determined using a Cell Counting Kit-8 (CCK-8) assay kit (Beyotime Institute of Biotechnology, Beijing, China). Cells were stained with annexin V-FITC and PI to detect early apoptotic cells (annexin V positive, PI negative) and late apoptotic cells (annexin V positive, PI positive) by flow cytometry (BD PharMingen).

Statistical analysis

Statistical analysis and Figures were performed or made using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA), respectively. The distribution of numerical variables were analyzed with the Kolmogorov-Smirnov test. Nonparametric tests were used for not normally distributed data. The relation between TIM-3 or Gal-9 expression level and clinical and laboratory characteristics was examined by Spearman's or Pearson's correlation coefficient rank test. Comparison analyses between the groups were carried out using the χ^2 test, the Mann-Whitney U test, and the Friedman test. A P-value ≤ 0.05 was considered statistically significant.

Results

Blood levels of Gal-9 and TIM-3-positive T cells are markedly increased in patients with AD

A total of 30 AD patients (female: 20; mean age: 11.1 ± 6.0 years; aged 1-3 years, n=2; aged 3-5 years, n=5; aged 5-12 years, n = 10; aged 12-18 years, n=10; aged 18-20, n=3) were included after informed consent. As was previously reported, patients with AD had markedly higher serum levels of Gal-9, as

compared to HC (3,030 ± 208 *vs* 1,330 ± 90 pg/ml, P<0.0001, Figure 1A). In addition, AD patients showed significantly higher rates of TIM-3-positive (TIM-3$^+$) circulating CD4$^+$ T cells (27.2 ± 2.9%*vs* 11± 1.1%, P<0.0001, Figure 1B and S1).In CD4$^+$IFN-γ^+ cells(T_H1 cells), rates of TIM-3 expression were more than 3-fold higher in AD patients (17.7 ± 3.6% *vs* 4.9± 0.9%, P=0.003, Figure 1C, and S2), and rates in CD4$^+$ IL-17A$^+$ cells(T_H17 cells) were twice as high, as compared to HC (12.5± 2.7%*vs* 6.1± 1.2%, P=0.033, Figure 1D and S3) (Table 1).

Furthermore, serum Gal-9 levels were strongly and positively correlated with rates of circulating TIM-3$^+$CD4$^+$ T cells (r=0.6364, P=0.0002, Figure 2A). AD patients with high serum levels of Gal-9 had markedly higher rates of TIM-3$^+$CD4$^+$ T cells as compared toAD patients with low serum levels ofGal-9 (32.5 ± 4%*vs* 19.3± 3.9%, P=0.029, Figure 2B).Vice versa, patients with high rates of TIM-3$^+$CD4$^+$ T cells had markedly higher serum levels of Gal-9 than patients with low rates (3,532 ± 253 *vs* 2,456 ± 273 pg/ml, P=0.0074, Figure 2C).

In AD, high rates of circulating TIM-3$^+$T cells are linked to high disease activity, IgE levels, and circulating eosinophils and B cells

When we assessed these findings for their clinical relevance, increased circulating TIM-3$^+$CD4$^+$T cell populations in our AD patients were linked to higher disease activity, i.e. SCORAD values (r=0.6060, P=0.0004, Figure 3D), higher serum levels of total IgE (r=0.3633, P=0.048, Figure 3A), as well as higher number of circulating blood eosinophils (r=0.6126, P=0.0003, Figure 3B) and CD19$^+$B cells (r=0.5120, P=0.0038, Figure 3C). Gal-9 serum levels showed similar links, albeit less pronounced (Figure 4), suggesting that TIM-3 and Gal-9 contribute to the course and pathogenesis of AD.

In AD, rates of circulating TIM-3-positive CD4$^+$ cells are positively correlated with rates of T_H2/T_H22 cells and negatively correlated with rates of T_H1/T_H17 cells

Next, we explored the role of TIM-3 and possible underlying mechanisms in AD. High rates of TIM-3$^+$CD4$^+$T cells in the blood of AD patients were strongly linked with high rates of T_H22 cells (r=0.7633, P<0.0001, Figure 5A), and, in addition, with those of T_H2 cells (r=0.5481, P<0.01, Figure 5B). In contrast, the rates of TIM-3$^+$CD4$^+$ T cells in the blood of our AD patients were negatively correlated, albeit weakly, with those of T_H17 cells (r=-0.4372, P<0.05, Figure 5C), and, additionally, with those of T_H1 cells (r=-0.4652, P<0.01, Figure 5D). Serum levels of Gal-9, in our AD patients, were also positively and negatively correlated with circulating T_H22 cells (r=0.5904, P<0.001, Figure 6A) and T_H17 cells (r

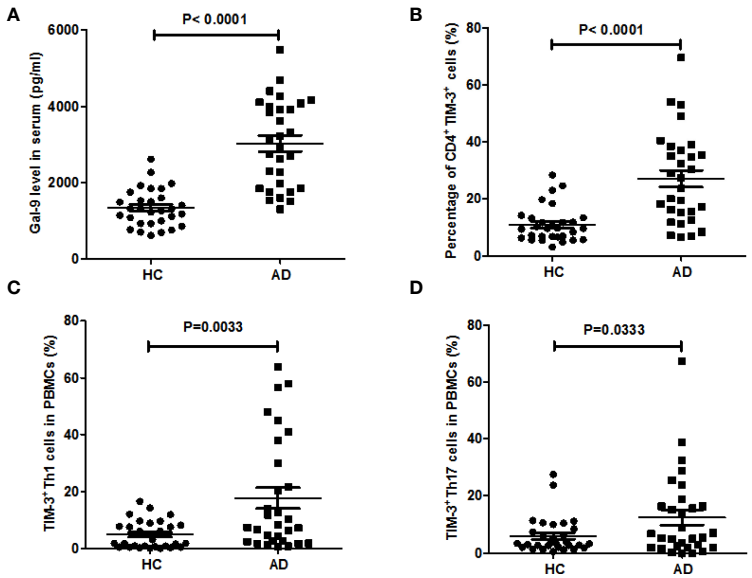

FIGURE 1
Blood levels of Gal-9 and TIM-3-positive T cells are markedly increased in patients with AD. **(A)** Serum Gal-9 levels of patients with atopic dermatitis involved in this study (AD, n = 30), as compared to healthy controls (HC, n = 30), **(B)** the percentage of CD4$^+$TIM-3$^+$ T cells in whole blood, the percentage of **(C)** TIM-3$^+$$T_H$1 cells and **(D)** TIM-3$^+$$T_H$17 cells in PBMCs of the above patients with AD compared to HC. These results are presented as means ± SEM.

TABLE 1 Characteristics of patients with atopic dermatitis in the present study.

Patient		Age at onset		Total serum	Absolute eosinophil count	Blood $CD19^+$ B cells	
no.	**Sex**	**(years)**	**SCORAD**	**IgE (U/ml)**	**(/nl) (0,02-0,52)**	**(%)**	**Allergens**
1	F	6	17.20	878	1.22	8.49	*D.pteronyssinus*
2	F	5	19.50	86.12	0.29	6.17	/
3	F	6	20.10	2979	3.76	9.25	/
4	F	5	23.70	199.40	1.87	15.60	Cat and Dog dander
5	F	11	16.20	2026	0.28	14.90	unknown
6	F	5	15.10	617	0.67	6.71	unknown
7	M	19	6.80	1586	1.73	13.80	Dog dander and Birch pollen
8	F	18	11.80	2773	0.02	12.10	*C. albicans*
9	F	20	12.60	3300	1.50	13.50	/
10	M	10	27.50	1084	3.84	11.80	/
11	F	2	29.20	710	1.90	13.20	/
12	F	13	30.50	2783	4.70	11.90	Birch pollen
13	M	6	32.50	2173	5.01	16.00	/
14	M	8	34.80	580	1.80	15.00	/
15	M	14	35.40	440	5.10	11.40	Peanut and Shrimp
16	M	16	37.20	2069	3.60	7.28	Cat dander
17	F	18	38.50	1099	1.80	13.40	Mugwort pollen
18	M	15	69.50	1945	0.10	9.67	Timothy pollen
19	F	16	70.20	2118	1.19	11.20	/
20	F	4	75.10	738	3.73	6.52	/
21	F	13	78.20	3769	2.03	17.20	*D.pteronyssinus*
22	M	18	53.00	1737	1.01	7.60	/
23	M	20	54.00	1165	3.04	9.88	Egg white and Cow's milk
24	M	19	56.50	1198	4.07	15.40	unknown
25	F	6	61.30	2945	0.12	6.39	unknown
26	F	8	68.40	3227	0.10	10.60	*D.pteronyssinus*
27	F	17	39.20	1175	0.26	7.48	/
28	F	1	40.50	2284	0.13	11.60	/
29	F	8	45.60	2889	0.30	13.60	/
30	F	7	49.00	2076	0.04	12.90	unknown

F, female; M, male; /, no testing for sensitization to allergens was performed. SCORAD, SCORing Atopic Dermatitis. Determination of suspected allergenswas performed by fluorescence enzyme immunoassay usingImmunoCAP System® (Sigma-Aldrich, Deisenhofen, Germany).

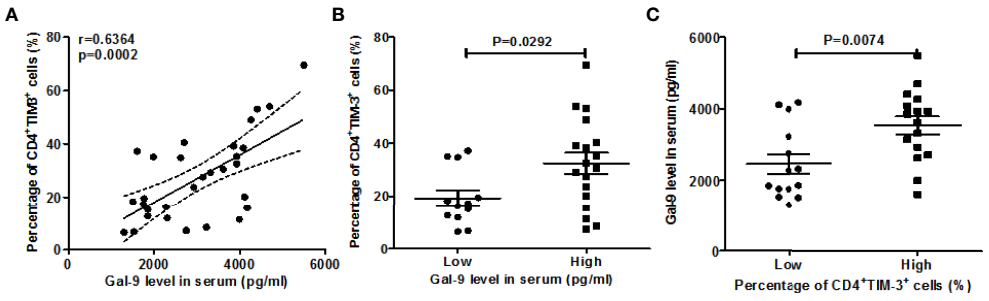

FIGURE 2
Correlation between Gal-9 levels and rates of TIM-3+CD4+ T cells. **(A)** Association between the percentage of TIM-3+CD4+ T cells and serum Gal-9 levels. For comparisons between groups, we divided the data based on Gal-9 level as low (<2659.13pg/ml), high (≥2659.13pg/m) and the frequency of TIM-3+ cells on CD4+ T cells as low (<21.9%), high (≥21.9%), respectively. The above cut-off values were 2 times the mean of HC test results. **(B, C)** The association of the serum Gal-9 level and the percentage of TIM-3+CD4+ T cells.

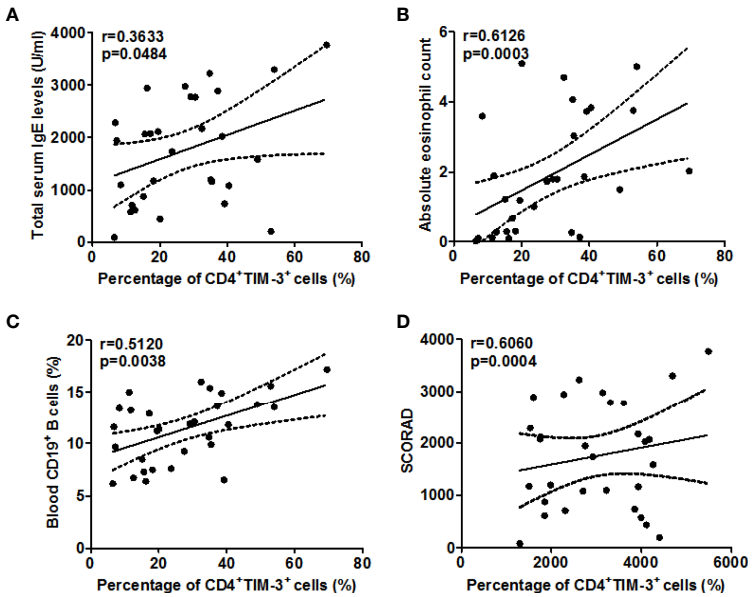

FIGURE 3
Association between the percentage of TIM-3$^+$CD4$^+$ T cells and total serum IgE **(A)**, circulating eosinophils **(B)**, blood CD19$^+$ B cells **(C)**, and disease activity as assessed by SCORAD **(D)** in patients with AD.

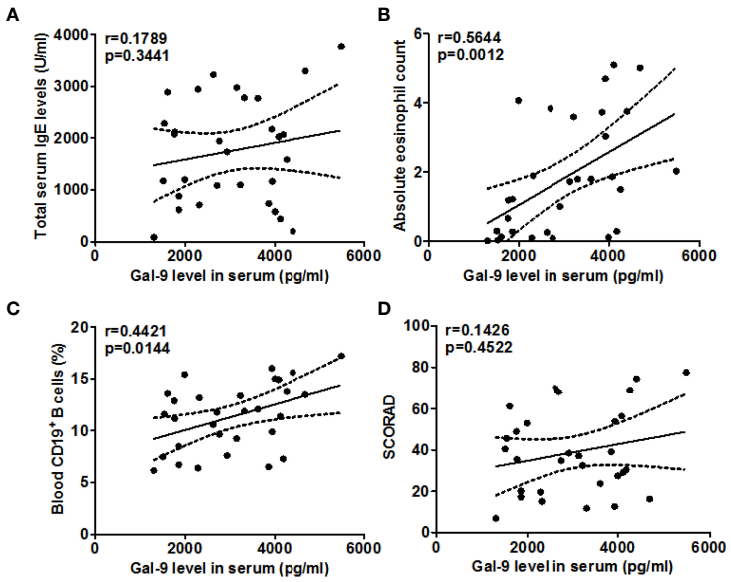

FIGURE 4
Association between the serum Gal-9 levels and total IgE **(A)**, eosinophils **(B)**, CD19$^+$ B cells **(C)**, and SCORAD **(D)** in patients with AD.

=-0.4647, P <0.01, Figure 6C), respectively, suggesting that Gal-9, *via* TIM-3, downregulates T_H1/T_H17-immunity and drives T_H2/T_H22 polarization. However, there were no significant correlations between Gal-9 serum levels and circulating TH2 cells (Figure 6B) and TH1 cells (Figure 6D).

Gal-9 inhibition of T cell proliferation and induction of T cell apoptosis are linked to AD severity

Finally, we characterized the effects of TIM-3 activation of T cells by Gal-9 in AD and their clinical relevance. To this end, we

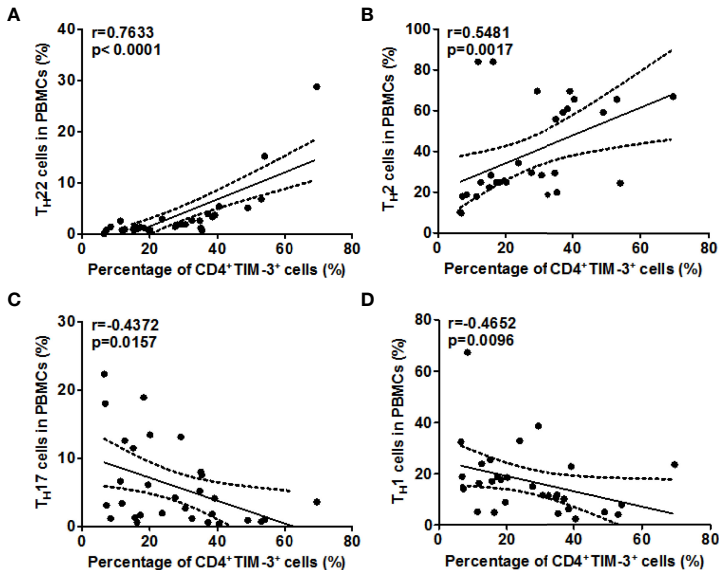

FIGURE 5
The correlation between TIM-3 levels and T_H2/T_H22 as well as T_H1/T_H17 cell ratios in AD. Association between the percentage of TIM-3^+CD4^+ T cells and the frequency of T_H1 cells **(A)**, T_H2 cells **(B)**, T_H17 cells **(C)**, and T_H1 cells **(D)** in patients with AD.

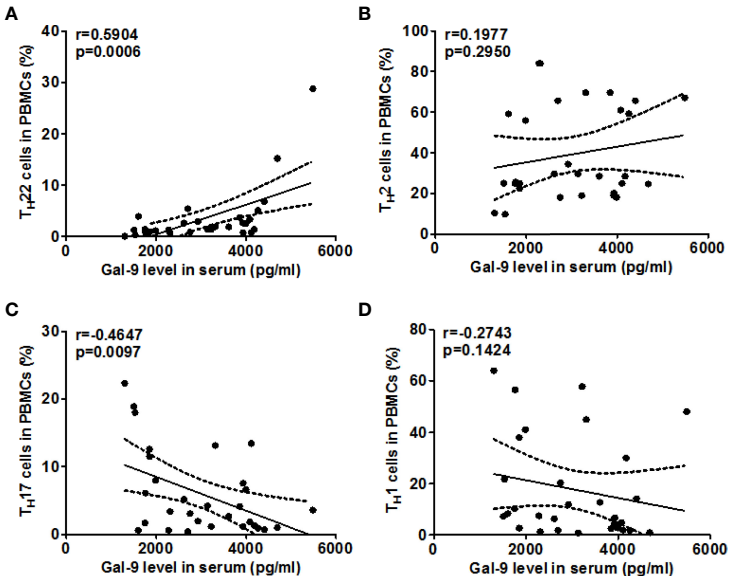

FIGURE 6
The correlation between Gal-9 levels and T_H2/T_H22 as well as T_H1/T_H17 cell ratios in AD. Association of Gal-9 levels and the percentage of TIM-3^+CD4^+ T cells and frequency of T_H1 cells **(A)**, T_H2 cells **(B)**, T_H17 cells **(C)**, and T_H22 cells **(D)** in patients with AD.

stimulated circulating T cells in PBMC samples of patients with mild, moderate, or severe AD with Gal-9 and anti-CD3 and then assessed their proliferation and apoptosis. Gal-9 dose-dependently inhibited proliferation (Figure 7A) and induced apoptosis (Figure 7B) in PBMCs of AD patients. The inhibition of proliferation and induction of apoptosis by Gal-9 were highest in PBMCs of patients with severe AD and lowest in patients with mild AD (Figures 7A, B), linking Gal-9 effects on T cells to AD disease severity.

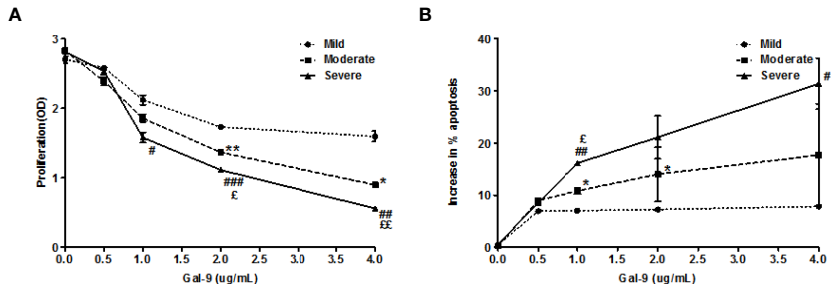

FIGURE 7
Gal-9 inhibits the proliferation and induces apoptosis in PBMCs from AD patients. **(A)** CCK-8 assay and **(B)** Apoptosis assay of PBMCs isolated from AD patients (Mild AD, n=11; Moderate AD, n=9; Severe AD, n=10) under graded doses of Gal-9 (0, 1.5, 5, 15, and 50 nM) and anti-CD3 (100ng/ml) for 72 h, respectively. These results are presented as means ± SEM. *P <0.05, **P <0.01, ***P <0.001 Mild AD *vs*. Moderate AD; #P < 0.05, ##P < 0.01, ###P < 0.001 Mild AD *vs*. Severe AD. ζP < 0.05, ζζP < 0.01, ζζζP < 0.001 Moderate AD *vs*. Severe AD.

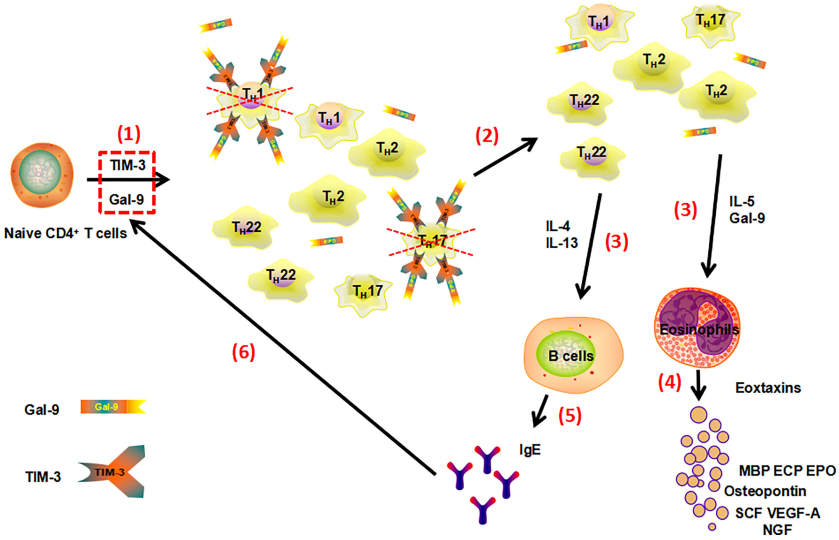

FIGURE 8
Proposed model of the role of Gal-9 and TIM-3 in the pathogenesis of AD. Gal-9, *via* TIM-3 expressed by T_H1/T_H17 cells, downregulates their numbers, by inhibiting proliferation and the induction of apoptosis (1). The reduction of T_H1/T_H17 immunity leads to T_H2/T_H22 polarization (2). Increased T_H2/T_H22 immunity and cytokines drive type 2 inflammation and disease activity (3) with higher numbers of eosinophils(4) and B cell class switching to IgE and elevated IgE levels (5). This, in turn, may drive further upregulation of Gal-9 and TIM-3 expression (6). MBP (Major basic protein), ECP (Eosinophil cationic protein), EPO (Eosinophilperoxidase), SCF (Stem cell factor), VEGF-A (Vascular endothelial growth factor-A), NGF (Nerve growth factor).

Discussion

This study tiesGal-9 and its receptor, TIM-3, to the pathogenesis of AD. Both are upregulated in patients in AD and linked to disease features and activity. Our findings support the notion that Gal-9, *via* TIM-3, augments T_H2/T_H22 polarization and down regulates T_H1/T_H17immunityvia effects on $CD4^+$ T_H1 and T_H17 cells.

That Gal-9 levels are elevated in AD is not a new finding (23, 33). In contrast, what our study shows for the first time, is that levels of $CD4^+$ T cells that express the Gal-9 receptor TIM-3 are also markedly increased in patients with AD. TIM-3 is specifically expressed in T_H1 and T_H17 cells, but not in T_H2 (33), and our AD patients showed triple and double the rate of TIM-3-expressing T_H1 and T_H17 cells, respectively, as compared to HC. These findings go against those reported by Kanai and coworkers, who reported numbers of TIM-3-expressing $CD4^+$T cells to be similar in 9 AD patients as compared to HC (34). Possible explanations for this discrepancy include differences in patient populations, i.e. young Han Chinese patients in our study *vs* middle-aged Japanese patients,

and the small number of patients studied. In addition to age, factors such as gender, genetics, and environmental factors also will influence the immunological profile of patients with AD (35).

Why are Gal-9 levels and rates of TIM-3$^+$CD4$^+$ T cells both upregulated in AD? Our study does and cannot answer this question and was not meant to. Further studies are needed to identify the underlying mechanisms. At least four scenarios could be relevant. First, elevated Gal-9 could increase the rate of TIM-3$^+$CD4$^+$ T cells. Second, TIM-3$^+$CD4$^+$ T cells could drive Gal-9 levels. Third, Gal-9 and TIM-3 expression may be upregulated by independent mechanisms. Fourth, increased Gal-9 and TIM-3 expression may be driven by the same signals. The first scenario is unlikely since Gal-9 inhibits the proliferation and induces apoptosis of TIM-3$^+$ cells, as previously reported (36)and demonstrated by our findings in AD. That TIM-3$^+$cells produce or induce the production of Gal-9, i.e. scenario two, is also unlikely. CD4$^+$T cells have been reported to produce Gal-9 (37, 38), but other cells such as keratinocytes and mast cells are probably much more relevant sources of Gal-9 in AD (24). As for the third and fourth options, the fact that Gal-9 levels and rates of TIM-3$^+$CD4$^+$T cells are strongly correlated suggests that the mechanisms that drive the elevation of both are shared, at least in part, rather than independent. Since both are not only correlated with each other, but also linked to disease activity and, to a lesser extent, AD features such as IgE and blood eosinophils and B cells, it appears likely that what drives the increase in Gal-9 levels and rates of TIM-3$^+$CD4$^+$T cells in AD is AD itself. Thus, Gal-9 and TIM-3 may act as amplifiers of AD pathogenesis. This notion is supported by the observation that effective treatment of AD can result in the decline of Gal-9 levels.

Our results clearly show that, regardless of the cause, high rates of circulating TIM-3$^+$T cells are linked to high AD disease activity, IgE levels, numbers of circulating eosinophils and B cells, as well as high rates of T_H2/T_H22 cells and low rates of T_H1/T_H17 cells. This was also so for Gal-9, albeit less pronounced. What explains this, at least in part, is that Gal-9, in our AD patients, inhibits T cell proliferation and induces T cell apoptosis and that both effects are linked to AD severity. The vicious feedback loop suggested by our results looks like this: High levels of Gal-9 and high levels of TIM-3 expressing T_H1/T_H17 cells make for strong inhibition of T_H1/T_H17 immunity and for T_H2/T_H22 polarization, which in turn comes with high levels of disease activity and inflammatory signals that may drive further Gal-9 and TIM-3 expressions.

As two target glycoproteins of Gal-9 have been identified, TIM-3 and CD44. Whether Gal-9 downregulates T_H1/T_H17 immunity *via* TIM-3 in AD? First, we observed that both Gal-9 level and the rate of TIM-3$^+$CD4$^+$ T cells are elevated in AD patients. Second, Gal-9 levels and rates of TIM-3$^+$CD4$^+$T cells are strongly correlated in our patients with AD. Third, in our AD patients, Gal-9 significantly inhibited T cell proliferation and induced T cell apoptosis. These results indicate that Gal-9 might *via* TIM-3 contributes to the inhibition of T_H1/T_H17 activation in AD. However, further experimental evidence is still needed, such as TIM-3 block experiment. And additional experiments with galectin inhibitors also need to be performed to clarify the specific mechanism of Gal-9-mediated suppression in AD.

Our study has several strengths and a few limitations. As for the former, for example, we assessed Gal-9 and TIM-3 in a sizeable and well-characterized patient population, together with clinical and other molecular markers. A major limitation of our study is its monocentric approach, which calls for confirmation of our results in a broader and more heterogeneous group of patients. A minor limitation of our study is that we only investigates the expression of Gal-9 and TIM-3 in blood samples. This is mainly due to blood samples are relatively easy to obtain, and blood source indicators have the potential to be developed into biomarkers in the later stage for AD. As skin biopsy is not a routine test for patients with AD. Besides, two studies have been reported that, increased Gal-9 expression in the skin lesions of AD patients (23, 24). Whereas, comparative studies on Gal-9 and TIM-3 expression in peripheral blood and lesions of AD are still needed.

Taken together, as summarized in **Figure 8**, upregulation of TIM-3/Gal-9 interaction, in AD, comes with downregulation of T_H1/T_H17 responses and more pronounced T_H2/T_H22 immunity. Our data suggest that the TIM-3/Gal-9 pathway may play an important role in the pathogenesis of AD, given that levels of TIM-3/Gal-9 are closely associated with disease activity, total serum IgE levels as well as blood eosinophil and B cell count. Further research is needed to clarify the molecular mechanisms that drive increased TIM-3/Gal-9 expression of T_H1/T_H17 cells in AD. In addition, future studies should aim to characterize TIM-3/Gal-9 expression on Tc1, NK, and myeloid cells as well as their levels in skin lesions of patients in AD.

What is already known about this topic?

- Atopic dermatitis (AD) is driven by T_H2/T_H22 polarization and cytokines.
- Galectin-9 (Gal-9) can promote T_H2/T_H22 immunity, *via* its receptor T cell immunoglobulin- and mucin-domain-containing molecule-3 (TIM-3).

What does this study add?

- Gal-9 and TIM-3 are markedly upregulated in AD and linked to disease features.
- Gal-9 and TIM-3 levels are positively correlated with rates of T_H2/T_H22 cells and negatively correlated with rates of T_H1/T_H17 cells.

- TIM-3/Gal-9 inhibits the proliferation and induces apoptosis in AD T cells, and both effects are linked to disease severity.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary material**. Further inquiries can be directed to the corresponding author/s.

Ethics statement

This study was reviewed and approved by The Ethics Committee of The First Affiliated Hospital of Soochow University (Suzhou, China, No. 2014809026). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

WS, JZ, SY, YS, and CL performed experiments and analyzed the data. MT and JJ recruited patients. QJ, JJ, and MM analyzed data and wrote the manuscript. MM contributed to the critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author WS was employed by China National Nuclear Corporation 416 Hospital.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.952338/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Representative flow cytometry analysis of TIM-3 expression on $CD4^+$T cells in whole blood of patients with AD and HC. Cells were initially gated on $CD4^+$ T cells. Subsequently, the frequency of $TIM\text{-}3^+$ cells $CD4^+$ T cells was analyzed by flow cytometry in the whole blood of AD patients and HC, respectively.

SUPPLEMENTARY FIGURE 2
Representative flow cytometry analysis of TIM-3 expression on $CD4^+$IFN-γ^+ T cells in PBMCs from AD patients and HC. Cells were initially gated on $CD4^+$ T cells. Then IFN-γ^+cells were selected out of the gated $CD4^+$ T cells. Subsequently, the frequency of $TIM\text{-}3^+$ cells on $CD4^+$IFN-γ^+ T cells was analyzed by flow cytometry in PBMCs from AD patients and HC, respectively.

SUPPLEMENTARY FIGURE 3
Representative flow cytometry analysis of TIM-3 expression on $CD4^+$IL-$17A^+$ T cells in PBMCs from AD patients and HC. The cells were initially gated on $CD4^+$ T cells. Then IL-$17A^+$ cells were selected out of the gated $CD4^+$ T cells. Subsequently, the frequency of $TIM\text{-}3^+$ cells on $CD4^+$IL-$17A^+$ T cells was analyzed by flow cytometry in PBMCs from AD patients and HC, respectively.

SUPPLEMENTARY FIGURE 4
Representative flow cytometry analysis of T_H22 and T_H22 in PBMCs from AD patients. **(A)** T_H22 cells: Cells were initially gated on $CD4^+$ T cells. Then IL-22^+cells were selected out of the gated $CD4^+$ T cells. **(B)** T_H2: Cells were initially gated on $CD4^+$ T cells. Then IL-4^+cells were selected out of the gated $CD4^+$ T cells.

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TCR-like antibodies targeting autoantigen-mhc complexes: a mini-review

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T cell receptors (TCRs) recognize peptide antigens bound to major histocompatibility complex (MHC) molecules (p/MHC) that are expressed on cell surfaces; while B cell-derived antibodies (Abs) recognize soluble or cell surface native antigens of various types (proteins, carbohydrates, etc.). Immune surveillance by T and B cells thus inspects almost all formats of antigens to mount adaptive immune responses against cancer cells, infectious organisms and other foreign insults, while maintaining tolerance to self-tissues. With contributions from environmental triggers, the development of autoimmune disease is thought to be due to the expression of MHC risk alleles by antigen-presenting cells (APCs) presenting self-antigen (autoantigen), breaking through self-tolerance and activating autoreactive T cells, which orchestrate downstream pathologic events. Investigating and treating autoimmune diseases have been challenging, both because of the intrinsic complexity of these diseases and the need for tools targeting T cell epitopes (autoantigen-MHC). Naturally occurring TCRs with relatively low (micromolar) affinities to p/MHC are suboptimal for autoantigen-MHC targeting, whereas the use of engineered TCRs and their derivatives (e.g., TCR multimers and TCR-engineered T cells) are limited by unpredictable cross-reactivity. As Abs generally have nanomolar affinity, recent advances in engineering TCR-like (TCRL) Abs promise advantages over their TCR counterparts for autoantigen-MHC targeting. Here, we compare the p/MHC binding by TCRs and TCRL Abs, review the strategies for generation of TCRL Abs, highlight their application for identification of autoantigen-presenting APCs, and discuss future directions and limitations of TCRL Abs as immunotherapy for autoimmune diseases.

KEYWORDS

TCR-like antibodies, autoimmune diseases, autoantigen presentation, immunotherapy, antigen-specific therapy

Introduction

To date, over 80 autoimmune diseases have been described (1), ranging from organ-specific (e.g., pancreas-specific Type 1 diabetes (T1D) and thyroid gland-specific Grave's disease) to systemic conditions (e.g., rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)). Curative approaches for autoimmunity are lacking. Despite diverse manifestations and autoantigen sources, these autoimmune reactions typically share stages of initiation, propagation, and for some, periods of clinical remission (2).

Although environmental factors are thought to be required as triggers for disease, predisposition to autoimmunity most often reflects inherited factors, with MHC (human leucocyte antigen (HLA) in humans) alleles conferring the highest risk (3, 4). Typically, class I (MHC-I or HLA-I in humans) genes encode proteins that present peptides from intracellular antigens to CD8 + T cells, and class II (MHC-II or HLA-II in humans) genes encode proteins that present extracellular/endosomal antigens to CD4+ T lymphocytes (5). A number of particular HLA-II (i.e., HLA-DR, -DQ, and -DP) alleles have been identified as critical risk factors for particular autoimmune diseases. For example, >90% celiac patients carry HLA-DQA1*05:01/HLA-DQB1*02:01 (6, 7), and >95% narcoleptic patients carry HLA-DQB1*06:02 (8, 9). In addition, the HLA-DRB1*04:01/*04:04 genotypes are risk alleles (odds ratios are ~4.14 and ~3.17, respectively) for RA (10) and the HLA-DRB1*15:01-DRB5*01:01 haplotype (up to 60% among Caucasians) is linked to multiple sclerosis (MS) (11). How these polymorphic MHC proteins interact with autoantigens and how autoantigen-MHC presenting APCs interact with autoreactive T cells are central questions in the field.

HLA-II+ APCs generate peptide/HLA-II (p/HLA-II) complexes (12, 13) that interact with cognate TCRs on CD4+ T cells, which orchestrate downstream autoimmune reactions (9, 14–16). Therefore, targeting autoantigen-HLA-II complexes on the APC surface with soluble TCR or TCRL reagents enables a specific way to investigate the initiation and propagation of autoimmunity. Here, we review current approaches and future directions for generating and using TCRL (also known as TCR mimic) Abs as research tools and potential therapeutics for autoimmune diseases.

Comparisons of TCRL Abs with TCRs

Abs share many similarities with TCRs in terms of diversity of the receptor repertoire and specificity for antigen recognition (17, 18). Abs, especially monoclonal Abs (mAbs) are widely used in research, diagnoses and therapies as specific immune-targeting agents (19), whereas TCRs have not been widely used (20). This is in large part due to the intrinsic difference in their antigen binding affinities. TCRs have micromolar affinities for cognate p/MHCs (21), whereas Abs have nanomolar affinities and interact with their specific antigens with >100x higher binding energies (22).

Each TCR contains two polypeptide chains: α and β, whereas each Ab consists of two heavy (H) and two light (L) chains. An Ab has two identical antigen-binding fragments (Fab, an H/L dimer) and a crystallizable fragment (Fc, from the H chain) that links the two Fab arms (22), yielding increased avidity for antigen. The Fab H/L heterodimer, like the TCR α/β heterodimer, uses two sets of complementarity-determining regions (CDRs) to directly contact the cognate antigen. The CDR regions are also referred to as the fragment variable (Fv) region. CDR3 of both Fabs and TCRs are hypervariable, with key amino acid residues governing antigen binding specificity. Residues within the germline-encoded CDR1 and CDR2 are less variable (17). Ab engineering usually focuses on CDRs of Fab or Fv heavy and/or light chains. To modify both chains using one gene cassette, a covalent link between the heavy and light chain fragments can be used, yielding single-chain Fv (scFv) for example.

As natural p/MHC receptors, TCRs have scientific, diagnostic and therapeutic potential, particularly if used as tetramers or higher order multimers to increase avidity (23), or if engineered to improve target affinity or avidity (23–25). Affinity improved and/or multimeric TCRs and TCR-engineered T cells have been used to target and clear tumor cells presenting cancer-related p/MHC-I (26). However, these reagents have seldom been used for autoantigen-MHC-II targeting, likely for several reasons. First, compared to TCRs recognizing foreign or neoantigens, MHC-II/autoantigen-reactive TCRs tend to have lower affinity, which typically allows their escape from thymic negative selection but activity for autoimmune responses (27); this affinity window is a poor starting point for affinity improvement by TCR engineering. Second, improved TCR affinity is often compromised by unpredictable cross-reactivity (28, 29), causing off-target staining during auto-APC characterization.

To resolve these issues stemming from natural TCRs, investigators developed TCRL mAbs by combining the high affinity of a mAb with the capacity to recognize p/MHC complexes (20). Some TCRL mAbs target intracellular antigens presented by MHC-I on tumor cells and have been applied as immunotherapeutics for cancers (30, 31). Crystallization studies have determined the structures of five p/MHC-I-specific TCRL mAbs in Fab formats binding to their p/MHC-I targets (32–35). Although CDR regions of all five TCRL Fab molecules interact with the peptide region of p/MHC-I complexes, only two (34) show the canonical docking geometry of TCRs with p/MHC (20). Thus, the TCR docking geometry that elicits TCR signaling (36) is not an absolute requirement for TCRL mAb development. Recently, the co-crystal structure of an MHC-II-restricted TCRL Fab bound by a gliadin peptide/HLA-

DQ2.5 (DQA1*05:01/DQB1*02:01) complex has been determined (37). This Fab has picomolar affinity, adopts the canonical TCR docking geometry (38), and demonstrates desirable properties for p/MHC-II staining and specific T cell inhibition relevant to celiac disease (37).

Generation of TCRL mAbs targeting p/MHC-II complexes

Naturally occurring Abs rarely mimic TCR specificity for p/MHC antigen(s); therefore, the TCRL feature of an Ab is typically obtained through target-driven *in vitro* selection and/or Ab engineering. Advances in hybridoma technology (39), recombinant p/MHC synthesis (40), and binder selection *via* phage or yeast display (41, 42) have enabled protein engineering of TCRL mAb. As other reviews have summarized TCRL mAb generation (20, 30, 31), we focus on the available approaches relevant to TCRL mAbs specific for p/MHC-II.

Initially, mice or rats immunized with p/MHC-II complexes expressed by cells or as soluble, recombinant proteins were used to produce a candidate B cell pool from which B cell hybridomas (immortal B cell lines producing candidate mAbs) were generated. Although TCRL specificity was possible (43, 44), most often, p/MHC-II-specific enrichment and screening were required to identify hybridomas producing TCRL mAbs. To date, >20 p/MHC-II-specific TCRL mAbs have been generated using this approach (20, 45, 46) and about half are relevant to autoimmune diseases (Table 1). However, challenges persist: 1) limited B cell clonal candidates with peptide specificities and more clones with monomorphic MHC specificity due to the framework differences of MHC-II alleles or MHC-II from different species (immunization of HLA-transgenic mice (49) may help enrich for peptide-specific responses, see discussion below); 2) low throughput of hybridoma production and labor-intensive screening for p/MHC-II binding; 3) non-human origin of the Ab itself, limiting their therapeutic use. Notably, a human B cell hybridoma expressing a TCRL mAb recognizing an HLA-A2-derived self-peptide bound to HLA-DR1 was generated using peripheral blood mononuclear cells (PBMC) (59).

To avoid the limitations of hybridoma approaches, phage display has been applied by several groups to screen Ab libraries for p/HLA-II binders (49, 52, 56) (Table 1). A typical library contains 10^8-10^{11} phage particles, each displaying an Ab variant on the surface. Phage display is achieved by covalently fusing Ab fragments, such as Fab and scFv, with a phage coat protein through molecular cloning (41, 70). Screening the library for binders to p/HLA-II relies on a process called "panning" or more recently "biopanning" (70). This process includes multiple rounds of negative selection (e.g., against irrelevant p/HLA-II) and positive selection (e.g., against target p/HLA-II). Designing Ab libraries in phage allows selection from mouse (49) or human (37, 52, 53, 56) antibody sources. To enrich for peptide-specific Abs in the mouse endogenous repertoire prior to construction of

TABLE 1 TCRL mAbs targeting autoimmunity-related p/MHC-II complexes.

mAb Clone	Species	Format	Method	Disease/Model	T cell antigen/MHC	References
B-7-1, B-18-7, C-34-72	Mouse	Full-length Ab	Hybridoma	MS/EAE model	$MBP_{87\text{-}99}$/I-A^s	(47)
S.1.6	Mouse	Full-length Ab	Hybridoma	MS	MBP/DR7	(48)
R.1.D12	Mouse	Full-length Ab	Hybridoma	MS	MBP/DRw11	(48)
MK16	Mouse	Fab	Phage display	MS	$MBP_{218\text{-}231}$/DR15	(49)
12A	Mouse	Full-length Ab	Hybridoma	RA	HC gp-$39_{263\text{-}275}$/DR4	(50, 51)
2E4, 1F11, 2C3, 3A3, 3H5	Human	Fab	Phage display	MS	$MOG_{35\text{-}55}$/DR15	(52)
G3H8	Human	Fab; reconstructed full-length Ab	Phage display	T1D	$GAD65_{555\text{-}567}$/DR4	(52, 53)
mAb287	Mouse	Full-length Ab	Hybridoma	T1D/NOD mice	Insulin $B_{9\text{-}23}$/I-A^{g7}	(54, 55)
FS1	Mouse	Full-length Ab	Hybridoma	Diabetes/NOD mice	p63/I-A^{g7}	(46)
106, 107	Human	scFv; reconstructed full-length Ab	Phage display	Celiac Disease	glia-α1a/DQ2.5	(56)
mAb757	Mouse	Full-length Ab	Hybridoma	T1D/NOD mice	Insulin $B_{9\text{-}23}$/I-A^{g7}	(57)
3-5	Mouse	Full-length Ab	Hybridoma	T1D/NOD mice	2.5HIP/I-A^{g7}	(58)
206, 3.C11	Human	scFv; reconstructed full-length Ab	Phage Display	Celiac Disease	glia-α2/DQ2.5	(37)
Selected other TCRL mAbs mentioned in this mini review						
Y-Ae	Mouse	Full-length Ab	Hybridoma	Self-antigen	Eα/I-A^b	(43, 44)
UL-5A1	Human	Full-length Ab	Hybridoma*	Self-antigen	HLA-$A2_{105\text{-}117}$/DR1	(59)
I-5	Mouse	Full-length Ab	Hybridoma	Self-antigen	CLIP/DR3	(60)
D-4, G-32, and G-35	Mouse	Full-length Ab	Hybridoma	Model antigen	MCC/I-E^k	(61, 62)
3M4E5 and 3M4F4	Human	Fab	Phage Display	Tumor antigen	NY-ESO-1/A*0201	(34)
13.4.1	Mouse	Fab	Phage Display	Viral antigen	$HA_{255\text{-}262}$/H-$2K^k$	(63)

*Human hybridoma. Note: See (20, 30, 31, 45, 46, 64–69) for a more comprehensive list of other TCRL mAbs, including anti-p/MHC-I reagents.

a phage-Fab library, the Fugger group immunized HLA-DR15 (DRA*01:01/DRB1*15:01) transgenic mice using DR15 molecules in complex with a myelin basic protein (MBP) peptide, leveraging the inherent DR15 tolerance of the model to skew the Ab response towards specificity for the MBP peptide (49). HLA-transgenic animal immunization followed by screening yielded a series of TCRL reagent findings, including the MBP/DR15-restricted TCRL mAb MK16 as mentioned (49), invariant chain peptide/HLA-DR mAb in another study (60), and an MHC-I-restricted TCRL mAb in additional work (63). Human Fab or scFv libraries built and expressed in phage have been mostly from large naïve repertoires (37, 52, 53, 56), which likely harbor TCRL candidates, albeit rare. Using stringent phage panning strategies, the Reiter and the Løset groups isolated DR-restricted (52, 53) and DQ-restricted (37, 56) human TCRL mAbs, respectively (Table 1). As these human Fabs or scFvs were not raised or matured against the target p/HLA-II, their affinities were suboptimal. Reconstructing a full-size Ab using the TCRL Fab or scFv increased the binding strength (37, 53). However, further affinity maturation may be useful. Recently, Frick et al. suggested a strategy to improve binder affinity *via* multiple rounds of phage-Ab library optimization and selection (37).

Combining phage display with yeast display is particularly useful for developing high affinity TCRL mAbs (71). Since first developed (42), yeast display technology has evolved, allowing surface display of monomeric or dimeric protein scaffolds (72, 73). Thus, either scFv or Fab identified from a phage-Ab library can be affinity matured using the yeast platform. Advantages of yeast display include 1) eukaryotic gene transcription and protein expression machinery for appropriate Ab folding; and 2) quantitative flow cytometry-based screening, ensuring high throughput selection for high-affinity Abs (74, 75).

TCRL mAbs as research tools and therapeutics for autoimmune diseases

Characterization of autoantigen-presenting APCs using TCRL mAbs

Presentation of autoantigen by APCs, especially professional MHC-II+ APCs, such as dendritic cells (DCs), macrophages (MΦs), and B cells, is critical for CD4+ T cell activation and differentiation into helper T effector (T_{eff}) or suppressive T regulatory (T_{reg}) cells during autoimmune responses. An imbalance of T_{eff} and T_{reg} functions upon autoantigen recognition is believed to drive the loss of tolerance, with subsequent autoreactive T cell responses and production of autoantibodies (2). Therefore, the study of MHC-II+ autoantigen presenting cells (auto-APCs) is fundamental for understanding disease pathogenesis and may lead to novel immunotherapies. Murine models allow direct evaluation of tissue-resident and circulating APC subsets and enable genetic modifications of these APCs to assess their autoreactive functions. For example, using an experimental autoimmune uveitis (EAU) mouse model, Lipski et al. analyzed disease-related infiltrating MΦs and resident retinal microglia by tissue immunostaining and cytometry-based immunophenotyping of isolated cells (76). In another model, single-cell sequencing was used to characterize tissue-infiltrating APCs in autoimmune diabetes (77). However, discoveries in murine models are not easily transferable to human diseases (78, 79).

Auto-APC identification using human samples is a preferred approach for clinical relevance. Early studies with human samples focused on APC enumeration in PBMC and biopsies. Increased frequency of circulating DCs was implicated in regulation of antigen presentation by islet cells and activation of autoreactive CD4+ T cells (80, 81). Recently, a novel approach was developed using PBMC to identify autoantigen-specific memory B cells, which are potent MHC-II+ APC (82). Therapeutic strategies focusing broadly on APC function have been developed, such as B cell depletion in SLE (83) and tolerogenic DC adoptive therapy (84). However, the key to the optimization of APC-directed immunotherapy is identification of autoantigen specificity.

Due to their high affinity and the ease with which they can be further engineered, TCRL mAbs have gradually replaced TCR-derived reagents in research and therapeutic development for autoimmunity. TCRL MK16, described above, identified microglia/MΦs rather than astrocytes as the predominant auto-APCs in MS lesions (49). Human cartilage glycoprotein (HC gp-39, residues 263-275) represents a candidate T cell autoantigen in RA and can be presented by the RA susceptibility allele, HLA-DR4 (DRA*01:01/DRB1*04:01) (50, 85). TCRL mAb 12A specific for gp-39 (263-275)/DR4 identified autoantigen-presenting DCs in synovial tissue of DR4+ patients, indicating local presentation of gp-39 in inflamed joints (50, 51). Recently reported are several TCRL mAbs, specific for different gluten-derived peptide epitopes in complex with the celiac disease risk allele, HLA-DQ2.5. These complexes are known to be recognized by CD4+ T cells that drive disease (16, 38). The TCRL mAbs identified plasma cells, an unexpected APC, as the most abundant cell type presenting gluten peptides in gut biopsies from celiac patients (37, 56).

Although murine models cannot directly identify auto-APCs that function in human diseases, applying TCRL mAbs in these models may shed mechanistic light on disease pathology. For example, with specificity for a model antigen, moth cytochrome c-derived peptide (MCC, residues 95-103) bound by mouse MHC-II I-E^k, TCRL mAb determined that a minimum of 200–400 p/MHC-II complexes per APC was necessary for T-cell stimulation (61). This number is at least an order of

magnitude higher than the minimum requirement of p/MHC-I complexes for licensing cytolytic activity of human CD8+ T cells (86, 87).

Therapeutic potential of TCRL mAbs in autoimmune diseases

TCRL mAbs have not been intensively investigated as therapeutics for autoimmune diseases, although their pre-clinical examination in cancers (30, 31) suggests therapeutic potential. In cancer, TCRL mAbs can target intracellular tumor antigens presented by cell surface MHC-I molecules, broadening the original oncoantigen spectrum targeted by Ab-based therapy. However, a limitation of TCRL mAb in this setting is low TCRL Ab coverage per cell due to MHC-I downregulation on tumors (30). In contrast, MHC-II is typically up-regulated on auto-APCs in autoimmunity. Further, the tight linkage of particular autoimmune diseases with particular MHC-II alleles (3) provides defined allelic targets for TCRL mAbs. Although depletion of pathology-driving cells, as in cancer therapy, is a therapeutic option in autoimmunity, TCRL mAb therapy typically aims to reestablish healthy immune balance among cells like CD4+ T_{eff} and T_{reg} cells by non-depleting mechanisms (Figure 1). Here, we propose a few options for future TCRL autoimmune therapeutics, based on advances in TCRL mAb cancer therapies (30, 31) and Ab therapies for autoimmune diseases (19, 88).

Antibody treatment can induce target cell apoptosis (89) or (via Ab Fc region) lead to antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), or complement-dependent cytotoxicity (CDC) (88, 90) (Figures 1A, B). For example, anti-CD20 mAbs, FDA-approved for RA and primary progressive MS (91, 92), appear to work by depletion of CD20+ B cells, including those that present autoantigen to T cells and give rise to autoantibody-producing plasma cells. However, unclear long-term benefits and side effects (e.g., lack of vaccine Ab response) of broad B cell elimination are concerns (88). Alternatively, one may consider engineering a bispecific Ab (BsAb), coupling specificity of anti-

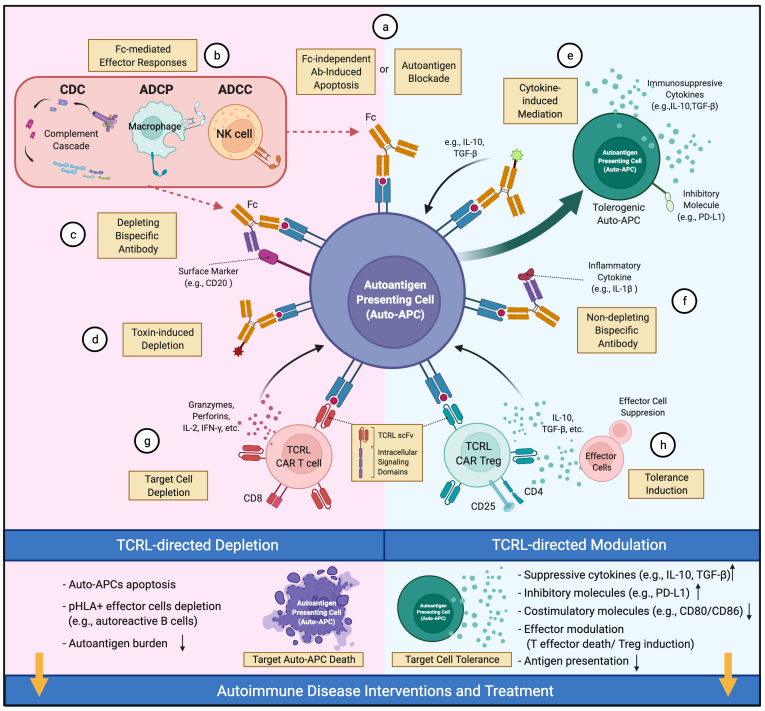

FIGURE 1
Therapeutic potential of TCRL mAbs in autoimmune diseases. TCRL mAbs specific for autoantigen/HLA complexes can elicit therapeutic effects *via* depleting (pink) or non-depleting (cyan) mechanisms. **(A)** TCRL mAbs either block autoantigen presentation or induce apoptosis of target cells. **(B)** TCRL mAbs induce Fc-mediated cytotoxicity through various effector mechanisms. **(C, F)** Bispecific antibodies targeting autoantigen/HLA complexes and either a surface marker of target cells or a pathogenic-related cytokine; **(D)** TCRL mAb–toxin conjugates induce auto-APC depletion by payload effector molecules, including cytokines, toxins or radioactive substances. **(E)** TCLR mAb-cytokine conjugates guide the delivery of immunomodulatory cytokines (e.g., IL-10, TGF-β) to auto-APCs for tolerance induction. **(G)** TCRL scFv fragments are reformatted into CARs for auto-APC targeting and depletion. **(H)** CD4+CD25+ TCRL CAR T_{reg} cells suppress T_{eff} function and induce tolerance. (This figure was created with BioRender.com).

CD20 and anti-autoantigen p/MHC for targeted depletion of pathology-related B cells (Figure 1C). In NOD mice, an autoimmune diabetes model, TCRL mAb alone were reported to delay diabetes onset, likely due to selective deletion of auto-APCs (54, 55); detailed mechanism and systemic immune impact await further investigation.

Non-depleting TCRL mAbs, for example those with low FcR binding (93, 94), provide additional avenues for therapeutic interventions. TCRL mAbs can limit autoantigen-MHC accessibility and reduce activation of cognate T cells (Figure 1A). This has long been the rationale for evaluating TCRL mAb specificity and functionality *in vitro* or in mouse models (37, 49, 50, 53, 54). Additionally, autoimmune modulators conjugated to or coupled with TCRL mAbs could facilitate modulator delivery to autoantigen-MHC-II-enriched sites of disease. Such modulators include toxins (Figure 1D), immunoregulatory cytokines, and antibodies that neutralize effector molecules or regulate effector cell activity (88). Cytokines like IL-10 and TGF-β that induce tolerogenic DC (95) with therapeutic efficacy (84) might reestablish tolerance at sites harboring auto-APCs (Figure 1E). Coupling TCRL mAb to FDA-approved antibodies that target inflammatory cytokines, as available for TNF, IL-6 and IL-1β, could localize their immunosuppressive effect to the sites of pathology (Figure 1F). TCRL mAbs could also be used in a chimeric antigen receptor (CAR) format for constructing CAR T cells (Figure 1G). In diabetic NOD mice, CAR T cells expressing an insulin peptide/MHC-II TCRL mAb modulated autoimmunity (54, 55). In addition, re-directing T_{reg} cells to the autoimmune milieu was shown to suppress autoreactive T_{eff} cells in several models (96). Thus, it may be fruitful to introduce TCRL CARs into T_{reg} cells for autoantigen-MHC directed T_{reg} cell activity (Figure 1H).

Potential side effects of TCRL mAb therapy targeting autoantigen-MHC complexes

For TCRL mAbs that are on-target (specific for autoantigen-MHC) and on-tissue (targeting autoimmune lesion), their primary actions will be to deplete auto-APCs and/or to modulate the CD4+ T cell-mediated immune responses (**Figure 1**). However, adverse effects may arise after target auto-APC depletion or following immunomodulation. A potential concern with cell-depleting TCRL mAbs is autoantigen release from apoptotic auto-APCs, which may propagate autoimmunity (2). On-target but off-tissue or off-target binding by TCRL mAbs raises other risks, such as unpredictable cross-reactive interaction between these Abs and highly homologous HLA-II allelic proteins or mimetic self-peptides. For example, unexpected cross-reactivity of affinity-enhanced TCR reagents targeting cancer-related MAGE A3/HLA-A*01 complex was reported to result in fetal cardiotoxicity (29). Regardless of target specificity, immune activation or suppression subsequent to TCRL mAb administration may lead to unpredictable toxicities, such as new autoimmune reactions or reduced host defense. In general, most safety and side effect concerns associated with traditional Ab therapies (97, 98) are worthy of attention during TCRL Ab development and preclinical evaluation. To minimize the chance of causing adverse effects, efforts in 1) Fc engineering/modification to control Fc-mediated effector function, 2) advanced affinity maturation to avoid exaggerated/prolonged mAb binding to the target, and 3) rigorous immunopharmacology studies *in vitro* and in animal models (97), will be crucial at stages prior to clinical trials.

Future directions

Despite great promise, effectively leveraging modern TCRL technologies in autoimmune therapy still requires optimization: First, advanced tools and innovative strategies for autoantigen discovery are still needed, as highly accurate identification and characterization of HLA-restricted peptide antigens are a prerequisite for downstream development of TCRL agents. Secondly, directed evolution and affinity maturation for low affinity TCRL candidates are still challenging, although combinatorial libraries designed using phage and yeast display platforms offer potential solutions. As more and more TCR and TCRL mAb structures emerge, machine learning (99) may offer more guidance on TCRL engineering. Last, for use in physiologic conditions, protein scaffolds other than mAbs sometimes possess better properties including protein stability, reduced immunogenicity, and increased tissue penetration (90, 100). Lessons learned from TCRL mAb development can be applied to alternative protein scaffolds (90) to expand TCRL methodology. Ongoing TCRL projects are focusing on resolving these issues, in hopes of opening an era for next generation autoimmune research and therapies.

Author contributions

YL, WJ, and EM wrote the manuscript. All authors approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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A diversified role for γδT cells in vector-borne diseases

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Vector-borne diseases have high morbidity and mortality and are major health threats worldwide. γδT cells represent a small but essential subpopulation of T cells. They reside in most human tissues and exert important functions in both natural and adaptive immune responses. Emerging evidence have shown that the activation and expansion of γδT cells invoked by pathogens play a diversified role in the regulation of host-pathogen interactions and disease progression. A better understanding of such a role for γδT cells may contribute significantly to developing novel preventative and therapeutic strategies. Herein, we summarize recent exciting findings in the field, with a focus on the role of γδT cells in the infection of vector-borne pathogens.

KEYWORDS

γδT cells, vector-borne diseases (VBDs), host immune response, infection, pathogens

Introduction

Vector-borne emerging and re-emerging infectious diseases are major public health problems worldwide, accounting for more than one sixth of all infectious diseases (1, 2). They are caused by pathogens carried and transmitted by vectors, such as mosquitos, ticks, fleas, flies, lice, snails, and triatomine bugs (Figure 1).

WHO recently announced the spread of the vector-borne pathogens, primarily including parasites, viruses and bacteria (https://www.who.int/zh/news-room/fact-sheets/detail/vector-borne-diseases). Specifically, the parasites included were lymphatic filariasis (mosquito), schistosoma (aquatic snail), onchocodium filariasis (black fly) and trypanosoma (triatomine bug, tsetse fly) and etc; viruses include mosquito-borne chikungunya fever, dengue, lymphatic filariasis, Rift Valley fever, yellow fever, Zika, and tick-borne Crimean-Congo hemorrhagic fever virus, borrelia burgdorferi, tick-borne encephalitis virus, and etc.; and bacteria mainly include Typhoid, Coxella bainiensis, spot fever rickettsia, and etc. (Figure 1).

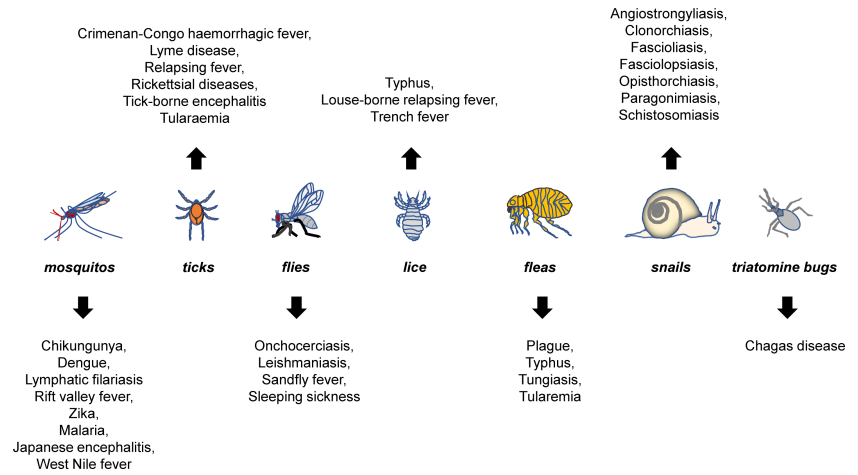

FIGURE 1
Major vector-borne diseases and their vectors. Mosquitos, ticks, fleas, flies, lice, snails, and triatomine bugs are best-characterized vectors that can carry pathogens for a variety of diseases. The listed are representative rather than a complete list of major vector-borne diseases that are known to be transmitted by each of the vectors.

Vector-borne diseases may not be directly disseminated between humans. Under certain circumstances, they can be transmitted to different hosts through the bite by pathogen-infected vectors (3, 4). As emerging infectious diseases (including vector-borne diseases) have certain relationships with social and economic development (5), a better understanding of the emerging and recurrent infectious diseases, especially how these diseases are transmitted, has profound significance for both human health and social development.

In response to pathogen invasion, human immune system acts as an advanced structural and functional architecture, in which all components (e.g. immune organs and cells, inflammatory factors, humoral factors, cytokines and chemokines) are highly orchestrated towards eliminating the invaded pathogen (6). During the invasion, both innate and adaptive immune responses can be triggered. Compared to innate immunity, adaptive immunity utilizes antigen and antibody specificity to eliminate the pathogen, thereby maintaining a steady state of the host and creating immunological memory for combating potential re-invasion of the same pathogen.

Different leukocytes, for example B and T cells, are known to play differential roles during these processes (7). T cells can be categorized to conventional T cells (αβT cells) and unconventional T cells (γδT cells) according to the types of their cell surface antigen receptors (8). Although αβT and γδT originate (differentiate) from the same thymic precursors, there are huge differences of biological functions and structures between the two types of T cells. The αβT cell receptors are expressed by ~95% of the T cells in the spleen, lymph nodes, and circulation system, and by ~60-70% of T cells in the peripheral blood. They have α and β chains and exhibit MHC restriction during the recognition of antigens (9).

On the contrary, the γδT cells express γ and δ chains of T cell receptors, account for ~5-15% T cells in the peripheral blood, and do not have MHC restriction during the recognition of antigens (10, 11). At the initial stage during the invasion of pathogens, γδT cells apparently exert innate immunity functions (6), so that they can rapidly respond by recognizing some common antigen components expressed by the invading pathogens, including glycolipids, glycoproteins, and mycobacterial derivatives (12). Although γδT cells have been known to act primarily in innate immunity, more and more findings have shown that these cells also exert fundamental functions in adaptive immune responses, for instance, by secreting cytokines and presenting antigens. Therefore, they have been considered as a bridge connecting innate immunity and adaptive immunity. However, the biological functions of γδT cells are not entirely dependent on HLA recognition mechanism.

γδT accounts for only a small part of the T cell population and is widely distributed in different parts of the human body, such as skin and intestinal tract (7). Human γδT cells are mainly categorized by the usage of δ chain, whereas mouse γδT cells are often categorized by the usage of γ chains. As such, human γδT cells can be divided into γδ1, γδ2, and γδ3 T cells (13), with their distribution and function varying from each other (14). γδ1 T cells are mainly distributed in the mucosal epithelium and play an important role in cell infection by listeria and cytomegalovirus. γδ2 T cells are relatively high in peripheral

blood γδT cells and show strong immune response to mycobacterium and influenza virus (15, 16). They destroy pathogens or infected cells by interacting rapidly with them (17, 18). γδ3 T cells, which account for a small proportion of γδT cell and are abundant in the liver, act during chronic viral infection (19).

The common and more harmful vector-borne diseases include dengue virus, Japanese encephalitis, Lyme disease and malaria. γδT cells play a key role in the host immune responses to the invasion of arbo-borne pathogens. More and more studies have shown that γδT cells are critical for antiviral and immunomodulatory activities in the first stage of arbo-borne pathogen infection. They are activated and participate in innate immune responses by producing cytokines associated with appropriate T-assisted responses during the early stages of microbial infection, either intracellular or extracellular (20). In addition to directly fighting against invading pathogens, γδT cells can also respond by recruiting other natural immune cells such as neutrophils and macrophages (21).

Infectious diseases are caused mainly by pathogenic microorganisms such as bacteria, viruses and parasites. γδT cells play important roles in responding to the invasion of common pathogens. Zhao and colleagues have summarized the role played by γδT cells in host responses to mycobacterium tuberculosis, Listeria monocytogenes, influenza viruses, HIV, EBV, and HBV (13). However, little is known about the effects of vectors on host γδT cells. Emerging vector-borne infectious diseases are an important part of emerging infectious diseases and have been in an intensified form globally. Many social and natural factors, including environmental pollution and modern transportation and logistics, make it more convenient for vectors to transmit arboreal pathogens.

Traditionally, many vector-borne diseases can be treated by antibiotics-based therapeutics. However, at least partly due to the antibiotics abuse in clinic, a variety of pathogens have developed resistance to common antibiotics, leading to poor clinical outcomes when using antibiotics to treat infected patients (22). To address this problem, it is critical for developing novel therapeutic approaches. In the past decades, scientists and clinicians have focused on the roles of conventional T cells-mediated immune responses during the pathogen infection. Notably, more and more evidence has uncovered previously-unrecognized key roles of unconventional T cells in this process. Therefore, we feel it is important to summarize recent progresses in the field investigating the functions of T cells (Table 1), especially the unconventional γδT cells, during the host immune responses to vector-borne pathogens, such as plasmodium, borrelia

TABLE 1 Potential roles for γδT cells in vector-borne diseases.

Disease	Pathogen	Involved γδT cells and their potential roles	References
Chikungunya (mosquito-borne)	chikungunya fever virus	γδT cells; likely involved in promoting protective immunity	(23–25)
Rift Valley Fever (mosquito-borne)	Rift Valley Fever virus	$CD11b^+$ γδT; may be critical for the host responses in sheep	(26–28)
Yellow fever (mosquito-borne)	Yellow fever virus	γδ2T cells; can respond quickly to virus infection and produce IFN-γ	(29)
Dengue fever (mosquito-borne)	Dengue fever virus	γδ2-T-cells; may serve as the early source of IFN-γ during dengue virus infection and promote the host immune responses by eliminating the virus-infected cells	(30–37)
Zika fever (mosquito-borne)	Zika virus	γδ2T; unclear	(38–40)
West Nile fever (mosquito-borne)	West Nile virus	γδT cells; may serve as the main source of IFN-γ and may also promote DC maturation and $CD4^+$ T cell infiltration	(41–45)
Malaria (mosquito-borne)	plasmodium parasite	γδT cells, Vg9Vd2 subpopulation, and $γδ2^+$ γδT cells; play both anti-pathogen and pathogenic roles	(46–60)
Lyme disease (tick-borne)	borrelia burgdorferi	γδT cells; may act indirectly through the actions of Toll-like receptors of DCs or monocytes, and may also act to activate the host acquired immunity during the infection of the pathogen	(61–66)
Tularaemia (tick-borne)	Francisella tularensis	γδT cells can be increased and maintained for up tyo a year in the peripheral blood from tularaemia patients	(67–69)
Leishmaniasis	leishmania	γδT cells; a potential role for γδT cells in eliminating the infected parasites, but long-term parasite infection may lead to γδT lymphoma	(70–73)
South American trypanosomiasis	Trypanosoma cruzi	γδT cells; may act by secreting IL-10 to facilitate host responses	(74)

burgdorferi, and dengue fever, with a hope to accelerate our efforts in developing novel and effective clinical therapeutic strategies.

Immune responses of the host γδT cells to mosquito-borne pathogens

Mosquito-borne viruses

Chikungunya

Chikungunya is caused by the infection of chikungunya fever virus. Its clinical manifestations include headache, fever, and serious joint pains (23–25). Vectors for chikungunya fever mainly include Aedes Aegypti and Aedes albopictus (also called Asian tiger mosquito) (75). Currently, there is no effective drugs or vaccines available for treating or preventing chikungunya (76). Different T cell family members play differential roles after the invasion of chikungunya fever. Rapidly accumulated $CD8^+$ T cells have been detected in the joints of mice that are acutely infected by the virus to promote protective immunity, but the lack of $CD8^+$ T seems to have no effect in preventing arthrophlogosis of the infected mice (77). On the contrary, the virus will not be able to induce joint diseases after the exhaustion of $CD4^+$ T cells (77, 78). Activated $CD4^+$ T cells have been shown to be implicated in the pathogenesis of arthrosis swell (77–79).

Unconventional γδT cells are also likely involved in promoting protective immunity in the host against chikungunya invasion. The numbers of γδT cells in the feet and lymph nodes are significantly increased after the mice are infected by chikungunya. Mice defective in γδT cells are more susceptible to chikungunya infection, exhibiting more severe foot swell and inflammation in the ankles, as well as increased oxidative damages, suggesting that γδT cells play critical roles in the protective immunity during the infection of chikungunya and subsequent inflammation and tissue damage (80).

Rift Valley Fever

Rift Valley Fever is a type of zoonosis caused by Rift Valley Fever virus, transmitted mainly by aedes and culex (26). Although most of infected patients only have minor fever, headache, and muscle pains, some patients may develop serious symptoms, including retinopathy, meningoencephalitis symptoms, and hemorrhagic fever (27). A possible role for γδT cells in Rift Valley Fever has been reported for infected sheep. Similar to other ruminants, the sheep's γδT cells account for a major population of its peripheral blood mononuclear cells. When recombinant Rift Valley Fever vaccine has been injected into the sheep, the percentage of $CD11b^+$ γδT in its peripheral blood mononuclear cells can be significantly increased, suggesting that these cells may be critical for the host responses in respond to the virus infection (28).

Yellow fever virus

Yellow fever virus belongs to the Flaviviridae, transmitted primarily by aedes and haemophilus mosquito. The symptoms for yellow fever commonly include fever, headache, jaundice, muscle pains, and emesia. Some patients may develop more serious symptoms and die quickly (29). So far, the YF-17D vaccine for yellow fever is probably one of the most effective vaccines. When it is inoculated into human hosts, γδ2T cells can respond quickly and produce IFN-γ within a week (28).

Dengue fever

Dengue virus is a mosquito-borne pathogen that is transmitted between hosts by mosquito bite (30). In clinic, most patients with slight infection will not have complications, and only a small population of patients will progress into severe disease states, exhibiting thrombocytopenia, end-organ damage, and other symptoms (31). critically ill patients commonly develop secondary infection, which is closely related with innate immunity (32, 33). In the host immunity, T cells are critical for eliminating pathogen invasion. In *in vitro* experiments, $CD8^+$ αβT cells can respond to dengue virus, and many evidence have shown that these cells play important roles in the host responses (34, 35).

We know relatively less about the roles for γδ T cells in the dengue virus infection. Eleonora Cimini and colleagues analyzed peripheral blood mononuclear cells from 15 dengue fever patients, the results show a significant decrease of γδ2-T-cell frequency and an increase of failure markers. In addition, the ability of γδ2-T-cells to produce IFN-γ in response to the phosphor-antigen was limited (36). Interestingly, primary human γδT has been shown to be able to kill dengue virus *in vitro*, suggesting a potential role for these cells in the anti-dengue virus process. Further investigations by Chen-Yu Tsai and colleagues have shown that primary γδT cells serve as the early source of IFN-γ during dengue virus infection and promote the host immune responses by eliminating the virus-infected cells. Monocytes can act as helper cells to participate in the virus infection and enhance the immune reponses in an IL-18-dependent manner (37).

Zika virus

Similar to dengue virus, Zica virus is primarily transmitted by infected aedes mosquitoes in tropical and subtropical regions. The infection of Zica virus can cause Guillain-Barre syndrome, neuropathy and myelitis. The infection during pregnancy may lead to the development of microcephaly and other congenital abnormalities in fetuses and newborns (38), and there has been a lack of clear treatment strategy. Previous reports have shown significantly increased Th1, Th2, Th9, and Th17 during acute Zica infection (39), suggesting that conventional T cells may dominate the host responses to Zica invasion. However, it should be noted that Eleonora Cimini and colleagues have also

specifically detected γδ2TCR in Zika virus-infected patients and a significantly increased expression level of $CD3^+CD4^-CD8^-$ T cell subsets (40), implying a possible role for unconventional γδT cells.

West Nile virus

West Nile virus is a type of flavivirus, primarily transmitted by culex pipiens. Most West Nile virus-infected patients exhibit no significant symptoms (asymptomatic). However, the incidence of severe cases increases significantly in immunocompromised populations (41), and there is no targeted vaccine for such cases. γδT cells are thought to play an essential role in the early control of infection. They respond rapidly by producing large amounts of IFN-γ (42). In addition to serving as the main source of IFN-γ, γδT cells may also promote DC maturation and $CD4^+$ T cell infiltration, as suggested by the observations that the expression of the dendric CD40, CD80, CD86 and MHC II molecules, as well as the expression of IL-12, are lower in γδT-deficient mice compared to those in wild-type mice. Furthermore, West Nile virus-induced activated γδT cells can promote the maturation of DC and the initiation and excitation of $CD4^+$ cells (43) to combat against virus invasion.

Besides of above-mentioned roles, West Nile virus-activated γδT cells are also critical for limiting the invasion of the virus into the brain central vervous system, which is essential for protecting most infected-hosts from developing fatal encephalitis. Thomas Welte and colleagues have shown that, compared with young mice, older/aged mice are more susceptible to virus infection and have slower $Vg1^+$ responses but more $Vg4^+$ cells, which in turn produce TNF-α, a factor implicated in the destruction of the blood-brain barrier. On the other hand, low $Vg4^+$ cells will allow the virus load in the brain, whereby reducing the mortality rate of virus-caused severe encephalitis (44). In the acquired immunity against West Nile virus infection, γδT cells also actively participate in the host defense process, and decreased memory responses of $CD81^+$ T cells likely easily lead to secondary infection of the virus in γδT-deficient mice (45).

Mosquito-borne parasite

Malaria

Malaria is estimated to affect more than 200 million people each year (46). It is an arbo-borne disease transmitted by the bite of mosquito-borne plasmodium parasite. Despite more and more significant progresses in the control and reduction of malaria cases in the past decade, it still remains a major threat to global health (47). After invading into the host, malaria parasites parasitize their spores in the liver of the host, then start to grow and eventually form merozoites to invade red blood cells, leading to significant clinical symptoms and death (48, 49).

The invasion of plasmodium parasites can cause complicated immune responses, including humoral and cellular immunity responses. We have very limited knowledge about the nature of these responses, especially the cellular immunity responses. Previously, it has been reported that γδT cells can be activated by phosphor-antigen of the parasites (50),

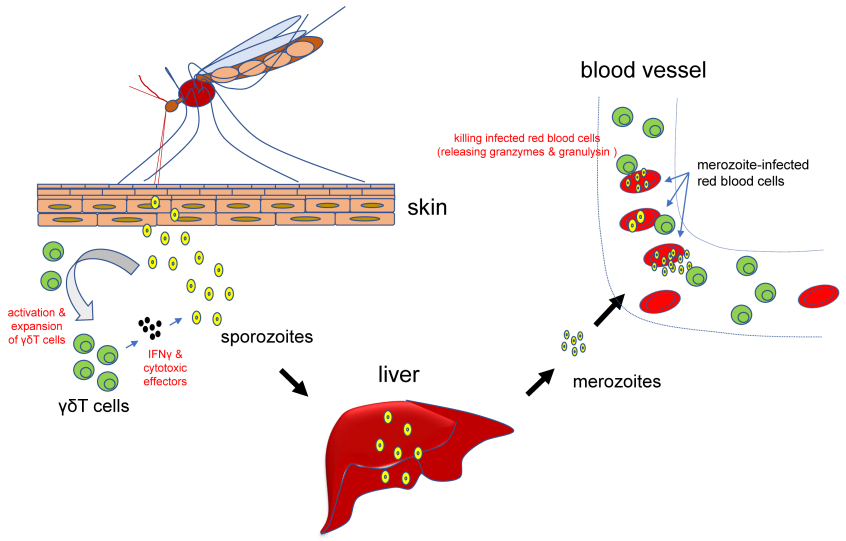

FIGURE 2
Roles for γδT cells in malaria parasites infection. At the early stage of infection, γδT cells can be activated and expanded and subsequently secret IFN-γ and other cytotoxic effectors to prevent or attenuate the infection. After the parasites have infected blood cells, activated γδT cells can also bind to the infected cells, release granzymes and granulysin, and kill the invaded plasmodium parasites and infected red blood cells.

leading to a quick increase of γδT cells, especially the Vg9Vd2 subpopulation (51, 52) (Figure 2). Such activation and/or expansion of γδT cells appear to be persistent after the invasion of the plasmodium parasites and can occur during secondary infections (53–55). It is already known that the main cause of the high morbidity and mortality of malaria patients is the successful survival and exponential proliferation of plasmodium parasites within the host blood. In the supernatants from the co-culture of plasmodium parasites, γδT cells can be specifically expanded and promoted to acquire the parasite-lysing potential through the up-regulated expression of IFN-γ and other cytotoxic effector proteins.

Subsequently, γδT cells can directly kill plasmodium parasite-infected red blood cells to prevent or attenuate further infections (56) (Figure 2). These killer cells can bind to the infected red blood cells, release granzymes and granulysin to kill the invaded plasmodium parasites (46). Therefore, a decrease of the numbers of γδT cells may reversely facilitate the tolerance of plasmodium parasites. Accordingly, repeated plasmodium parasites infection may contribute to the development of clinical immunity in children living in plasmodium parasites-infested regions, which is characterized by decreased numbers of patients with clear symptoms, accompanied by increased numbers of asymptomatic patients (57, 58).

During the repeated plasmodium parasites infections, the numbers of $γδ2^+$ γδT cells will be decreased in the peripheral blood, along with down-regulated production of cytokines and up-regulated immune-related factors. As such, repeated plasmodium parasites infections in the childhood will drive a progressive loss of the $γδ2^+$ γδT cells, leading to increased immune tolerance of the patients to plasmodium parasites (59).

Notably, besides above-mentioned anti-pathogen roles, γδT cells may also have a paradoxical role in driving or participating in the pathogenesis of cerebral malaria, as the incidences of cerebral malaria complications is lower in infants with lower γδT reactivity. Julie Ribot and colleagues have shown that the γδT-deficient mice are more resistant to the development of cerebral malaria when infected with the plasmodium berghei ANKA sporozoa. Conversely, the presence of γδT cells can enhance the production of the plasmodium immune factors at the stage of liver infection and subsequently promote the inflammation reactions at the blood infection stage (60). Together, these findings demonstrate that γδT cells can promote the pathogenesis of IFN-γ-dependent plasmodium infection.

Immune responses of the host γδT cells to tick-borne pathogens

Borrelia burgdorferi

Compared to mosquito-borne pathogens, there have been less reports regarding the roles of γδT cells in the infection of tick-borne pathogens. The Lyme disease is the most frequently seen natural epidemic disease in the United States of America (61) and is transmitted through bites from different hosts by borrelia burgdorferi-carrying ticks (62). Initial symptoms after the pathogen invasion in most Lyme disease patients are characterized by chronic erythema migrans. Several weeks after the disease onset, some patients may develop neurological and cardiac problems. After several months, most patients will have recurrent symptoms such as joint pain or arthritis (63). Under the stimulation by borbora burgdorferi, an accumulation can be detected within the inflated joints of the patients (64), suggesting a possible role for these cells in the host immune responses to the pathogen. Similarly, proliferated γδT cells have also been detected in leukocytes from micewith Lyme disease and in human peripheral blood after tick bites (65). However, the responses of γδT cells may be indirectly through the actions of Toll-like receptors of DCs or monocytes, rather than through a direct mechanism by themselves (64). In addition, γδT cells may also act to activate the host acquired immunity during the infection of the pathogen (66).

Tularaemia

The pathogen for tularaemia is Francisella tularensis, a type of gram-negative bacterium, which can be transmitted by tick bites from different hosts and cause acute febrile disease (67). Increased numbers of γδT cells can be detected in the blood from tularaemia patients (67), possibly attributable to non-specific phosphor-molecules (68). Further investigations by M. KROCA and colleagues have revealed that the frequency of γδT cells can be increased and maintained for up tyo a year in the peripheral blood from tularaemia patients (69).

Immune responses of the host γδT cells to other vector-borne diseases

Leishmaniasis

Leishmaniasis is disease caused by the infection of leishmania and primarily transmitted by the bites from different hosts by leishmania-infected female diptera insect phlebotomus fly. Leishmaniasis can be categorized into three major subtypes, including visceral leishmaniasis, cutaneous leishmaniasis, and mucocutaneous leishmaniasis. Visceral leishmaniasis is also called kala-azar (black sickness) and is the most severe subtype of leishmaniasis. Visceral leishmaniasis leads to symptoms including irregular fever, weight loss, hepatosplenomegaly, and anamenia, and may eventually cause patient death. Cutaneous leishmaniasis is the most popular subtype and mainly causes skin ulcer. Mucocutaneous leishmaniasis mainly causes mucous membrane injury within the oral and nasal cavity. Leishmania belongs to parasites, and cellular immunity plays a central role in the host responses to its infection. An accumulation of γδT cells has been detected in the skin and blood from human hosts infected with leishmania (70,

71), suggesting a potential role for γδT cells in eliminating the infected parasites. Consistently, it has been reported that natural killer cells and γδT cells act through secreting INF-γ and TNF-α, respectively, to exert their functions in the host innate immunity against the leishmania invasion (72). Compared with healthy individuals, double negative T cells from about 75% of the cutaneous leishmaniasis patients express aβT cell receptors, and the rest of the double negative T cells express γδT cell receptors (73). In addition, dogs severely infected with leishmania may develop γδT cell lyphoma (71), suggesting that long-term stimulation by leishmania may lead to malignant transformation and lyphoma pathogenesis, but the underlying mechanism has been unclear.

South American trypanosomiasis

South American trypanosomiasis is also known as Chagas' disease and mainly caused by direct contact with the excrement or urinate of Trypanosoma cruzi-infected trypanosoma triatoroae (stink bug). Currently there is no vaccine available for this disease. During the acute stage, the infected patients mainly exhibit symptoms including cyanosis swelling on one side of the skin or eyelid, headache, difficulty in breath, and muscle pains. At the chronic stage, the parasites parasitize in the intestine tracts and the heart. Years later after the infection, some patients may develop heart and digestive tract diseases. By utilizing γδT cell-deficient mice as a model, a recent report has shown that the γδT cells may not play a critical role in the elimination of the parasites at the acute stage of the disease but may contribute to tissue damage and pathogenesis. In cutaneous leishmaniasis patients, αβT cells and γδT cells secret inflammatory factors and IL-10, respectively to protect the hosts against the parasites invasion (74). Moreover, the frequency of IL-10 expression by γδT cells have been linked to an improvement of cardiac functions of cutaneous leishmaniasis patients, suggesting a potentially important role for γδT cells in the host responses (74).

Conclusions

It is estimated that vector-borne diseases lead to more than half million of global deaths each year, and some types of vector-borne diseases, such as chikungunya, leishmaniasis, and lymphatic filariasis may cause life-long diseases. Vaccines or other clinically effective drugs for many vector-borne diseases are still lacking, further worsening the life quality of the infected patients. As such, understanding better the host-pathogen interactions is critical for future developing novel and curative therapeutics.

Compared to unconventional γδT cells, the role for conventional αβT cells in the host responses to vector-borne pathogens has been more extensively and comprehensively studied, for instance Chikungunya virus (81–83), Rift Valley Fever virus (28), Yellow fever virus (84, 85), Zika virus (86, 87), West Nile virus (88), Malaria (89, 90), Borrelia burgdorferi (91), and Leishmaniasis (73, 92). However, as above-reviewed, our understanding of the role for γδT cells in these processes has been preliminary and incomplete.

It should be noted that most types of pathogens for vector-borne diseases are carried and disseminated by mosquitos and ticks, which transmitted the pathogens through the bites of host skin (93, 94). Given that γδT cells primarily reside in skin and mucosal tissues (95), these cells apparently are in the frontline to respond to pathogen invasion at the earliest stage. Therefore, it is important and urgent to gain a better understanding of the role for γδT cells during these precesses.

Previous studies have shown that αβT and γδT cells cooperate with each other and act synergistically towards eliminating pathogen invasion. As a bridge between innate and adaptive immunity, γδT cells have been known to play active roles during the first and secondary infections by pathogens and may serve as targets for clinical development. However, we still have limited knowledge about details of how they appropriately respond to vector-borne pathogen infection to facilitate the host immune responses.

During the early events of pathogen infection, activated γδT cells can secret multiple cytokines and inflammatory factors to induce the acquired immunity (96–98). Further studies in rodent models of infection of listeria, cytomegalovirus, and plasmodium parasites have revealed that γδT cells can strongly respond and quickly expand during secondary infections, suggesting that they have acquired certain levels of immune memory. These findings also suggest that the mode by which γδT cells respond to pathogen infections may be more complicated than previously appreciated.

Herein, we have reviewed recent findings related to the potential roles of γδT cells in response to several types of vector-borne pathogens, especially the mosquito- and tick-borne pathogens. We expect that these findings, together with those from more studies to analyze the interactions between γδT cells and vector-borne pathogens in the future, will provide useful information for developing clinically relevant targeted therapeutics.

Author contributions

CC and YY wrote the manuscript; AC and YY contributed to figures in this manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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$CD4^+CD8^+$ T follicular helper cells regulate humoral immunity in chronic inflammatory lesions

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T follicular helper (Tfh) cells drive humoral immunity by facilitating B cell responses at the initial and recall phases. Recent studies have indicated the possible involvement of Tfh cells in the process of chronic inflammation. However, the functional role of Tfh cells in persistent immune settings remains unclear. Here, we report that $CD4^+CD8^+$ (double-positive, DP; $CD3^+CD4^+CD8^+CXCR5^{hi}PD\text{-}1^{hi}$) Tfh cells, a subset of germinal-center-type Tfh cells, were abundantly present in the fibroinflammatory lesions of patients with immunoglobulin G4-related disease (IgG4-RD). Transcriptome analyses showed that these DP-Tfh cells in the lesions of IgG4-RD preferentially expressed signature genes characteristic of cytotoxic $CD8^+$ T cells, such as Eomes, CRTAM, GPR56, and granzymes, in addition to CD70. Scatter diagram analyses to examine the relationships between tissue-resident lymphocytes and various clinical parameters revealed that the levels of DP-Tfh cells were inversely correlated to the levels of serum IgG4 and local IgG4-expressing ($IgG4^+$) memory B cells ($CD19^+CD27^+IgD^-$) in patients with IgG4-RD. Cell culture experiments using autologous tonsillar lymphocytes further suggested that DP-Tfh cells possess a poor B-cell helper function and instead regulate memory B cells. Since $CD4^+$ (single positive, SP; $CD3^+CD4^+CD8^-CXCR5^{hi}PD\text{-}1^{hi}$) Tfh cells differentiated into DP-Tfh cells under stimulation with IL-2 and IL-7 as assessed by *in vitro* experiments, these data imply that SP-Tfh cells are a possible origin of DP-Tfh cells under persistent inflammation. These findings highlight the potential feedback loop mechanism of Tfh cells in immune tolerance under chronic inflammatory conditions. Further studies on DP-Tfh cells may facilitate control of unresolved humoral responses in IgG4-RD pathological inflammation.

KEYWORDS

DP-Tfh cells, memory B cells, chronic inflammation, IgG4-RD, tonsils

Introduction

$CD4^+$ T helper cells as well as their diverse distinct subsets drive humoral and cellular immune responses for host defense under pathological conditions (1). Humoral immunity plays a cardinal role in immune responses, therefore, the T follicular helper cells (Tfh cells) which are an effector subset of helper $CD4^+$ T cells, have received much interest (2, 3). Tfh cells play an essential role in facilitating germinal center (GC) formation by B cells to generate high-affinity antibodies as well as long-lived plasma cells and memory B cells. The antagonistic functions of the two transcription factors, B-cell lymphoma 6 (Bcl6), which is a master regulator of Tfh cells, and B lymphocyte-induced maturation protein 1 (Blimp1), toward each other during the development of Tfh cells from naïve $CD4^+$ T cells underlie a mechanism for bimodal cell fate decision-making to specify Tfh cell identity among effector helper $CD4^+$ T cells (4). The productive interactions of Tfh and B cells are controlled by the C-X-C motif chemokine receptor 5 (CXCR5) and sphingosine-1 phosphate receptor 2 (S1PR2) expressed on Tfh cells, which are responsible for the effective distribution of Tfh cells to tissues to enable their cognate interactions with B cells (5). After activation of Tfh cells in lymphoid tissues, a portion of tissue-resident Tfh cells enters the systemic circulation as memory-like Tfh cells (Tfh1, Tfh2, and Tfh17 cells) with the functional feature of a class switch recombination for B-cell activation (6). In these contexts, studies on Tfh cells and their related lymphocytes can renew the landscape of Tfh cell-mediated humoral immunity in immunocompetent settings as well as in the aberrant immune responses underlying pathological chronic inflammation (7, 8).

Immunoglobulin G4-related disease (IgG4-RD) belongs to a unique category of chronic immune-mediated diseases, that are primarily recognized as an archetypal Mikulicz's disease of IgG4-related dacryoadenitis and sialadenitis (9, 10). IgG4-RD occurs in multiple organs including salivary glands, lacrimal glands, pancreas, thyroid glands, lung, biliary tree, and retroperitoneum, and is characterized by the presence of high serum levels of IgG4. In tissue lesions of IgG4-RD, ectopic GCs are observed as a common histopathological feature in addition to marked infiltration of IgG4-positive B cells, storiform-pattern fibrosis, and obliterative phlebitis. While steroid therapy is effective in the treatment of IgG4-RD, relapse occasionally occurs after dose tapering or intermittent administration of glucocorticoids (11). Although the pathogenesis of IgG4-RD remains controversial, rituximab, an anti-CD20 antibody that decreases B-cell activity, is generally beneficial for IgG4-RD treatment, suggesting that B cells are the target cells of steroid therapy for IgG4-RD (12). In this regard, dysregulation of B-cell-mediated immunity is thought to be related to the pathogenesis of IgG4-RD. Accumulating evidence has shown that the tissue lesions of IgG4-RD preferentially harbor Tfh cells (13, 14); however, an understanding of the functional role of Tfh cells in B-cell regulation and its mechanism of action within the inflammatory environment of IgG4-RD has been a topic of debate in recent years.

To address the possible role of Tfh cells in IgG4-RD, we investigated Tfh cells and other lymphocyte subsets in tissue lesions of IgG4-RD in this study. Ectopic GCs are commonly formed in IgG4-RD lesions; therefore, we focused on GC-type Tfh cells ($CD3^+CD4^+CXCR5^{hi}PD\text{-}1^{hi}$) and comprehensively analyzed their transcriptome (15). In comparison with GC-type Tfh cells in tonsils, GC-type Tfh cells in the inflammatory lesions of IgG4-RD preferentially expressed CD8 ($CD3^+CD4^+CD8^+CXCR5^{hi}PD\text{-}1^{hi}$). These cells are thus referred to as $CD4^+CD8^+$ (double-positive; DP) Tfh cells. The CD8 expression in DP-Tfh cells was lower than that in canonical $CD8^+$ cytotoxic T cells (CTLs). However, DP-Tfh cells expressed CTL-related signature genes such as eomesodermin (Eomes), class I-restricted T cell-associated molecule (CRTAM), G protein-coupled receptor 56 (GPR56), perforin, and granzymes (16, 17). Interestingly, clinical studies of DP-Tfh cells in IgG4-RD and *in vitro* experiments using autologous lymphocytes of tonsils have suggested that memory B cells are a potential target of DP-Tfh cells. Since GC-type Tfh cells in tonsils upregulate CD8 upon stimulation with IL-2 and IL-7, which are usually associated with the maintenance of T cell activation and memory T cells, authentic $CD4^+$ GC-type Tfh cells may be able to generate DP-Tfh cells to regulate surrounding memory B cells under inflammatory conditions. By focusing on the functional and developmental processes of DP-Tfh cells, further studies could provide a novel modality to resolve undesirable immune responses in IgG4-RD and other pathological conditions underlying chronic inflammation.

Materials and methods

Study populations

The study populations are summarized in Supplementary Table S1. Diagnosis of IgG4-RD was performed in accordance with widely recognized diagnostic criteria (18). The type of organ involved in IgG4-RD patients is summarized in Supplementary Table S2. None of the recruited patients had received standard glucocorticoid therapy before surgical resection of submandibular glands (SMGs).

Tissues

Tissues from SMGs and palatine tonsils were obtained from patients with IgG4-RD and tonsillar hypertrophy for diagnosis or treatment at Sapporo Medical University Hospital, Japan. Cells in tissues were analyzed by flow cytometry and *in vitro*

studies, and a portion of the tissues was employed to prepare formalin-fixed paraffin-embedded (FFPE) sections.

Antibodies and other reagents

Antibodies for flow cytometry and immunohistochemistry as well as reagents used for *in vitro* studies are summarized in Supplementary Table S3.

Flow cytometry and cell sorting

Tissues were mechanically disrupted and lymphocytes in single-cell suspensions were prepared by density-gradient centrifugation with Lympholyte-H (Cedarlane, Burlington, Canada). Then, the cells were stained with antibodies to determine the expression of specific molecules using flow cytometry. The cells were analyzed or sorted using a FACS Canto II or FACS Aria II and III (BD Biosciences, New Jersey, USA) in combination with magnetic bead sorting (Miltenyi Biotec, North Rhine-Westphalia, Germany). In each experiment, samples were analyzed for singlet events with doublet discrimination. The purity of FACS-sorted cells reached 95% after validation by reanalysis using the FACS Canto II. The flow cytometry data were analyzed using FACS DiVA and FlowJo software (BD Biosciences).

Microarray analysis

Total RNA was extracted using TRIzol reagent (Life Technologies, California, USA) and validated with a 2100 Bioanalyzer (Agilent Technologies, California, USA) and NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Then, the RNA was amplified and labelled with Cy3-CTP to obtain cRNA using a Quick Amp Labelling kit (Agilent Technologies) and then hybridized to a microarray plate (SurePrint G3 Human GE 8×60K v3; Agilent Technologies). The obtained data were analyzed by bioinformatics software (Riken Genesis, Tokyo, Japan) and Heatmapper software (The University of Alberta, Alberta, Canada). Data were further investigated by gene set enrichment analysis (GSEA v2.0.13 software, UC San Diego, California, USA) and the iPathwayGuide platform (Advaita Bioinformatics, Ann Arbor, Michigan, USA).

RT-qPCR analysis

First-strand cDNA was synthesized from total RNA by using a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). Quantitative PCR analysis was conducted to detect gene-specific products using SYBR green and TaqMan probes with the Light Cycler 96 System (Roche, Basel, Switzerland). The PCR primer pairs and probes used are summarized in Supplementary Table S4.

Immunohistochemistry

FFPE tissue sections were immunestained using a standard protocol to detect IL-7. After staining, signals were visualized with 3,3′-diaminobenzidine and the sections were counterstained with hematoxylin. For histological differentiation, corresponding tissue sections were stained with hematoxylin and eosin.

Transmission electron microscopy

Transmission electron microscopy was performed using a standard protocol. Ultrathin sections were prepared using an ultramicrotome, mounted on a copper grid, and examined under a transmission electron microscope (JEM-1400; JEOL, Tokyo, Japan).

Cell culture experiments

FACS-sorted tonsillar lymphocytes were used for cell culture experiments in a humidified atmosphere with 5% CO_2 at 37°C. For co-culture of T and B cells, DP-Tfh or SP-Tfh cells were seeded in a 96-well round-bottom plate with autologous B cells at a 1:1 ratio (5×10^4 cells/well) in 200 µL of AIM-V medium containing 2 µg/mL anti-CD3 mAb, 2 µg/mL anti-CD28 mAb, and 1 µg/mL CD40L. After incubation for 7 days, the supernatants were analyzed using an ELISA kit to measure IgG (R&D Systems, Minneapolis, USA) and a cytotoxicity LDH assay kit-WST (Dojindo, Tokyo, Japan) to evaluate cytotoxicity. To examine the secretion of cytotoxic granules, cells (5×10^4 cells) were seeded in a 96-well plate in 200 µL of AIM-V medium containing 2 µg/mL anti-CD3 mAb and 2 µg/mL anti-CD28 mAb, and incubated for 7 days. Subsequently, the supernatants were analyzed by an ELISA kit for granzyme B (R&D Systems).

Statistical analyses

Results are expressed as mean ± SEM. Statistical analyses were performed using Mann–Whitney U test or Spearman's rank correlation test, where applicable. In the all analyses, $p < 0.05$ was considered significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$), while $p > 0.05$ was considered non-

significant (n.s.). The statistical tests were performed using GraphPad Prism 8 software (GraphPad, San Diego, CA).

Results

Tfh cells in IgG4-RD lesions have a cytotoxic phenotype

Following a previous study, which showed the abundance of tissue-resident Tfh cells ($CD3^+CD4^+CXCR5^+PD\text{-}1^+$) in SMG lesions in patients with IgG4-RD (14), we examined the gene expression profile of GC-type Tfh cells ($CD3^+CD4^+CXCR5^{hi}PD\text{-}1^{hi}$) in IgG4-RD lesions and compared it with that of GC-type Tfh cells in tonsils (Figure 1A). The results indicated that GC-type Tfh cells in IgG4-RD lesions significantly expressed signature genes of CTLs, including CD8, Eomes, CRTAM, and granzymes (Figure 1B and Supplementary Table S5). Further analysis of transcriptomes showed that other CTL-related genes, such as GPR56 and FAS ligand, were also upregulated in GC-type Tfh cells in the SMG lesions of IgG4-RD, whereas the expression levels of Tfh-related genes, such as IL-21, CD200, and Pou2af1 (also named as Bob1), in GC-type Tfh cells of IgG4-RD appeared to be relatively lower than those of GC-type Tfh cells in tonsils (Figure 1C). GC-type Tfh cells of IgG4-RD expressed low levels of genes related to Th2, Th17, and Treg cells, while genes related to Th1 cells and interactions of T cells with B cells such as CD70 were well expressed in these cells (19). Validation studies further showed that the GC-type Tfh cells in IgG4-RD lesions showed higher expression of CD8, Eomes, CRTAM, and granzymes in comparison with GC-type Tfh cells in tonsils (Figures 1D, E). Based on the expression profile of CD8, we here referred to CD8-expressing GC-type Tfh cells as $CD4^+CD8^+$ Tfh cells (double-positive Tfh cells, DP-Tfh cells; $CD3^+CD4^+CD8^+CXCR5^{hi}PD\text{-}1^{hi}$). The frequency of DP-Tfh cells in IgG4-RD lesions ranged from 1.1% to 62.3% (average, 14.5% of the total GC-type Tfh cells; n = 31; Figure 1E). DP-Tfh cells were also detected in tonsils and constituted about 0.1%–11.5% of the total GC-type Tfh cells (average, 2.3%; n = 71; Figure 1E). CD8 protein expression on DP-Tfh cells was lower than that on canonical $CD8^+$ CTLs in both IgG4-RD lesions and tonsils (Figure 1F). Additionally, CD8α and CD8β were almost equally expressed on DP-Tfh cells of IgG4-RD lesions and tonsils (data not shown). The ultrastructural analysis indicated the cytotoxic activity of GC-type Tfh cells, which possessed electron-dense granules similar to NK cells, but not of GC-type Tfh cells in tonsils (Figure 1G). Collectively, these data indicated that DP-Tfh cells with a possible CTL-like ability are frequently found in the GC-type Tfh cell population residing in IgG4-RD lesions. To a lesser extent, such DP-Tfh cells were detected among GC-type Tfh cells as a minor population in the tonsils. Most $CD3^+CD8^+CXCR5^{hi}PD\text{-}1^{hi}$ cells expressed CD4 in tonsillar lymphocytes as identified with DP-Tfh cells (Figure 1H).

Clinical significance of DP-Tfh cells in IgG4-RD

To address the functional role of DP-Tfh cells in the etiology of IgG4-RD, we analyzed scatter plot diagrams of the levels of DP-Tfh cells and various clinical parameters in patients with IgG4-RD. The results showed a marked inverse correlation of the levels of DP-Tfh cells with the serum IgG4 level (r = -0.4812, p = 0.0234), and the ratio of serum IgG4 to total IgG (r = -0.5234, p = 0.0124; Figure 2A). We also obtained similar results from scatter plot analysis of the level of DP-Tfh cells and the number of involved organs in IgG4-RD (r = -0.434, p = 0.0436; Figure 2A and Supplementary Table S2). Since the serum level of IgG4 is well associated with the disease severity of IgG4-RD (11), these findings imply a possible role of DP-Tfh cells in regulating a certain pathway(s) involved in the production of IgG4 in IgG4-RD. Next, we performed scatter chart analyses to determine the relationships between DP-Tfh cells and different B-cell subsets in IgG4-RD lesions (Figure 2B). Interestingly, the results indicated an inverse correlation between the levels of DP-Tfh cells and memory B cells (r = -0.5076, p = 0.0159; Figure 2C), which promptly and effectively induce a humoral recall response as antigen-experienced B cells (20). Conversely, the levels of other B-cell subsets, including naïve B cells, GC B cells, plasmablasts, plasma cells, and regulatory B cells, did not show any correlation to the level of DP-Tfh cells in the tissue lesions of IgG4-RD. Even though the SMG lesions of IgG4-RD with the formation of ectopic lymphoid structures could not fully harbor a memory B cell pool, IgG4-expressing ($IgG4^+$) memory B cells were fairly enriched in the lesions (Figures 2E–G). Thus, DP-Tfh cells could be considered to have a potential role in regulating IgG4 production by controlling $IgG4^+$ memory B cells in IgG4-RD lesions. While we did not find functional correlations between DP-Tfh cells and B-cell subsets residing in tonsils (Figure 2D), the clinical significance of DP-Tfh cells might be observed in ectopic lymphoid tissues under chronic inflammation rather than secondary lymphoid tissues.

DP-Tfh cells vary from SP-Tfh cells

To better understand the physiological characteristics of DP-Tfh cells, we conducted a comparative transcriptome analysis of tonsillar Tfh cell populations including DP-Tfh cells and single-positive (SP) GC-type Tfh cells ($CD3^+CD4^+CD8^-CXCR5^{hi}PD\text{-}1^{hi}$, SP-Tfh cells) used as a control. DP-Tfh cells were detected in the tonsils of young (e.g., adenoid) and adult patients (Figures 3A, 5A), whereas SP-Tfh cells were consistently found in the tonsils irrespective of age. Next, we investigated the

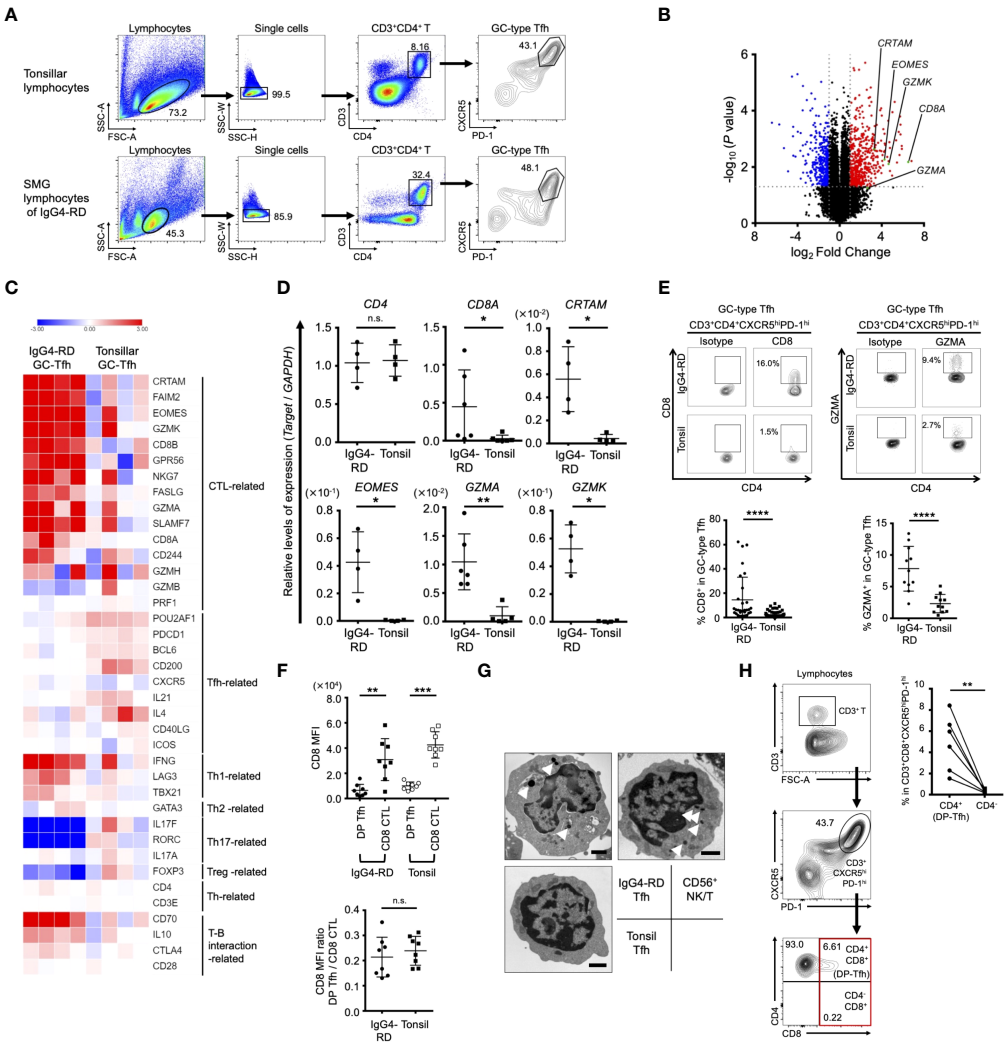

FIGURE 1
Phenotypic characteristics of GC-type Tfh cells in IgG4-RD lesions. **(A)** Flow cytometry profiles showing selected windows and gating strategy applied to identify GC-type Tfh cells ($CD3^+CD4^+CXCR5^{hi}PD\text{-}1^{hi}$) in lymphocytes of tonsils and SMG lesions of IgG4-RD. **(B)** Volcano plot identifying differentially expressed genes ($p < 0.05$) with more than two-fold expression in GC-type Tfh cells localized in SMG lesions of IgG4-RD versus GC-type Tfh cells in tonsils. The red and blue dots indicate upregulated and downregulated genes, respectively, in the Tfh cells of IgG4-RD. Data were obtained from microarray analysis of four specimens in each experiment group of IgG4-RD lesions or tonsils. **(C)** Heat map indicating relative abundances of transcripts identified in **(A)** for selected genes regulating helper $CD4^+$ T cells and CTLs. Relative values of gene expression are indicated by color. **(D)** Relative expression levels of genes in GC-type Tfh cells in the SMG lesions of IgG4-RD and tonsils as indicated in **(C)** assessed by RT-qPCR analysis. GAPDH was used as a control (IgG4-RD, n = 4-6; tonsil, n = 4-6). **(E)** Representative flow cytometry profiles of the expression of CD8 and granzyme A (GZMA) in GC-type Tfh cells in SMG lesions of IgG4-RD and tonsils (upper panels). Graphs indicating the expression of CD8 (IgG4-RD, n = 31; tonsil, n = 71) and GZMA (IgG4-RD, n = 11; tonsil, n = 11) in GC-type Tfh cells as assessed by flow cytometry (lower panels). **(F)** Expression levels of CD8 on DP-Tfh cells and $CD8^+$ CTLs in the lymphocytes of IgG4-RD lesions and tonsils (IgG4-RD, n = 8; tonsil, n = 8) assessed by flow cytometry. MFI, mean fluorescence intensity. **(G)** Transmission electron microscopy of FACS-sorted T cells, including GC-type Tfh cells from SMG lesions of IgG4-RD and tonsils and NKT cells ($CD3^+CD56^+$) from tonsils. Arrowheads indicate electron-dense granules in the cytosol. Scale bar: 1 µm. **(H)** DP-Tfh cells enriched in the $CD3^+CD8^+CXCR5^{hi}PD\text{-}1^{hi}$ T cell population in tonsils. Representative flow cytometry profiles to detect DP-Tfh cells and $CD3^+CD4^-CD8^+CXCR5^{hi}PD\text{-}1^{hi}$ T cells as indicated in the red square (left panels). A graph showing the $CD3^+CD8^+CXCR5^{hi}PD\text{-}1^{hi}$ T cell population preferentially containing DP-Tfh cells (right). Data from the same tonsils are paired (n = 6). Data in **(D–F, H)** were analyzed by the Mann–Whitney U test. Data in **(G)** were obtained from three independent experiments.

transcriptomes of pairs of DP-Tfh and SP-Tfh cells derived from each individual tonsil (Supplementary Table S6). The results showed that DP-Tfh cells preferentially expressed transcripts related to CTLs, such as CD8 (CD8A and CD8B), Eomes, CRTAM, FAS ligand (CD95L), granzymes, and SLAMF7, indicating a possible cytotoxic attribute of DP-Tfh cells (Figures 3B–E). Th1 cell-related signature genes were also expressed in DP-Tfh cells, and cytokines, such as interferon

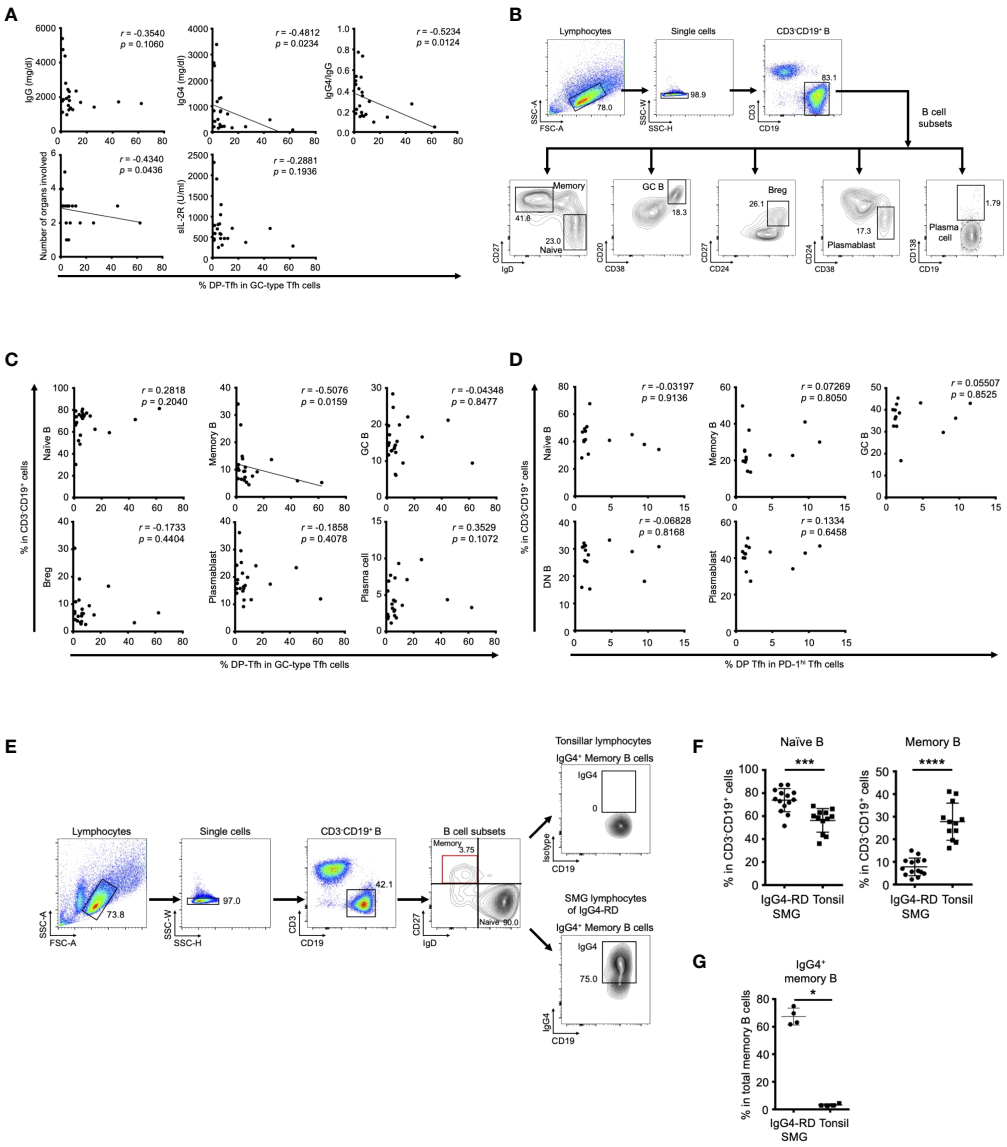

FIGURE 2
Clinical association of DP-Tfh cells in IgG4-RD lesions. **(A)** Scatter plots showing relationships between various clinical parameters associated with the IgG4-RD disease state and the ratios of DP-Tfh cells ($CD3^+CD4^+CD8^+CXCR5^{hi}PD\text{-}1^{hi}$) to total GC-type Tfh cells ($CD3^+CD4^+CXCR5^{hi}PD\text{-}1^{hi}$) in SMG lesions of IgG4-RD (n = 22). **(B)** Flow cytometry profiles showing selected windows and gating strategies applied to identify B cells ($CD3^-CD19^+$) and B-cell subsets (naïve B cells, $CD3^-CD19^+IgD^+CD27^-$; memory B cells, $CD3^-CD19^+IgD^-CD27^+$; GC B cells, $CD3^-CD19^+CD20^+CD38^+$; regulatory B cells, $CD3^-CD19^+CD24^{hi}CD27^+$; plasmablasts, $CD3^-CD19^+CD24^-CD38^+$; plasma cells, $CD3^-CD19^+CD138^+$) in SMG lesions of IgG4-RD. **(C)** Scatter plots indicating the relationships between the ratios of various B-cell subsets and ratios of DP-Tfh cells to total GC-type Tfh cells in SMG lesions of IgG4-RD (n = 22) measured by flow cytometry. **(D)** Scatter plots indicating relationships between the ratios of various B-cell subsets and ratios of DP-Tfh cells to total GC-type Tfh cells in tonsils (n = 14) measured by flow cytometry. **(E)** Flow cytometry profiles showing selected windows and gating strategy for IgG4-expressing memory B cells of lymphocytes of tonsils and SMG lesions of IgG4-RD. **(F)** Naïve and memory B cells in SMG lesions of IgG4-RD (n = 14) and tonsils (n = 12) analyzed by flow cytometry. **(G)** High frequency of memory B cells expressing IgG4 (IgG4+ memory B cells) in SMG lesions of IgG4-RD analyzed by flow cytometry (IgG4-RD, n = 4; tonsil, n = 4). Data in **(A, C, D)** were analyzed by the Spearman's rank correlation test, and data in **(F, G)** were studied by the Mann–Whitney U test.

(IFN)-γ and interleukin (IL)-10, were highly expressed in DP-Tfh cells rather than SP-Tfh cells. Of note, expression profiles of authentic GC-type Tfh (i.e., SP-Tfh)-related genes, such as IL-4, IL-21, Bcl6, and Pou2af1, appeared to be shared with DP-Tfh cells (Figures 3C, D). Among genes that regulated the interaction of T cells with B cells, DP-Tfh cells expressed the costimulatory molecule CD70 on their cell surface (Figures 3C, F). CD70 is a binding partner of CD27, which is highly expressed on class-

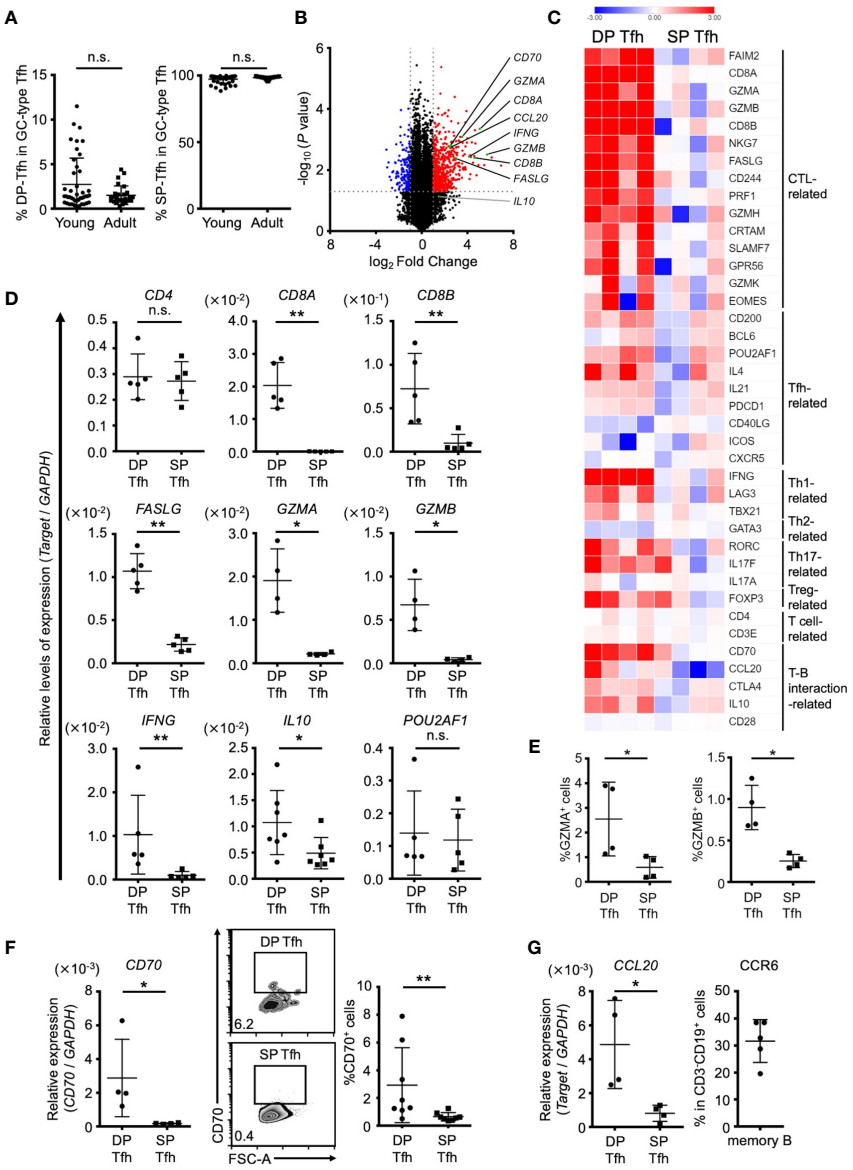

FIGURE 3
Different characteristics of DP-Tfh and SP-Tfh cells in tonsils. **(A)** Ratio of DP-Tfh cells ($CD3^+CD4^+CD8^+CXCR5^{hi}PD\text{-}1^{hi}$) to total GC-type Tfh cells ($CD3^+CD4^+CXCR5^{hi}PD\text{-}1^{hi}$) and ratio of SP-Tfh cells ($CD3^+CD4^+CD8^-CXCR5^{hi}PD\text{-}1^{hi}$) to total GC-type Tfh cells in young (n = 44) and adult (n = 27) tonsils assessed by flow cytometry are shown in the left and right, respectively. **(B)** Volcano plot showing differentially expressed genes ($p < 0.05$) with more than two-fold expression in DP-Tfh cells versus SP-Tfh cells ($CD3^+CD4^+CD8^-CXCR5^{hi}PD\text{-}1^{hi}$) derived from tonsils. The red and blue dots indicate the upregulated and downregulated genes, respectively, in DP-Tfh cells. Data were obtained from microarray analysis of four tonsil specimens in each experimental group studying DP-Tfh and SP-Tfh cells. **(C)** Heat map representing the relative abundances of transcripts identified in **(B)** for selected genes regulating helper $CD4^+$ T cells and CTLs. Relative values of gene expression are indicated by color. **(D)** Relative levels of expression of genes in DP-Tfh and SP-Tfh cells of tonsils (n = 4-7) as indicated in **(C)** are shown assessed by RT-qPCR analysis. GAPDH was used as a control. **(E)** Expression levels of granzyme A (GZMA) and granzyme B (GZMB)-positive cells in the DP-Tfh and SP-Tfh cells of tonsils (n = 4) as assessed by flow cytometry. **(F)** Expression level of CD70 in DP-Tfh and SP-Tfh cells of tonsils (n = 4) as examined by RT-qPCR analysis (left). FACS analysis of CD70 expression in these cells (middle, representative profiles, and right). **(G)** Expression level of CCL20 in DP-Tfh and SP-Tfh cells of tonsils as examined by RT-qPCR analysis (tonsil, n = 4; left). Expression of the CCL20 receptor, CCR6, on memory B cells assessed by flow cytometry (tonsil, n = 5; right). Data in **(A, D–G)** were analyzed by the Mann–Whitney U test.

switched B cells such as memory B cells, and the CD27/CD70 interaction usually facilitates memory B cells to differentiate into antibody-secreting cells (21, 22). CCL20 was highly expressed in DP-Tfh cells, but not in SP-Tfh cells, which is a ligand for the CCR6 presented on memory B cells (Figure 3G). These data suggest the possible engagement of DP-Tfh cells and memory B cells in lymphoid tissues (23).

DP-Tfh cells exert a regulatory effect on memory B cells

We next investigated the specific cellular effects of DP-Tfh cells on B-cell subsets by co-culture experiments using autologous tonsillar lymphocytes. After T cell activation using anti-CD3 and anti-CD28 Abs, the SP-Tfh cells stimulated whole B cells and B-cell subsets, including naïve B cells and memory B cells, to produce antibodies (Figure 4A). In contrast to SP-Tfh cells, DP-Tfh cells induced a weaker antibody-producing effect in whole B cells and naïve B cells, but not memory B cells (Figure 4A). This was also indicated by the results from further experiments that analyzed the ratio of the IgG level from B cells induced by DP-Tfh cells to that of the IgG level from B cells induced by SP-Tfh cells (IgG DP-Tfh cells/IgG SP-Tfh cells, Figure 4B). The corresponding ratios for whole B cells and naïve B cells were comparable, whereas the ratio for memory B cells was markedly lower than that of whole and naïve B cells (Figure 4B). Together with evidence showing that DP-Tfh cells indeed secreted the cytotoxic molecule of granzyme B after CD3 and CD28 stimulation (Figure 4C), these data suggested that DP-Tfh cells induced memory B-cell death. After co-culture experiments of memory B cells and DP-Tfh cells or SP-Tfh cells as a control, a cytotoxic assay to measure lactate dehydrogenase (LDH) in the supernatants suggested that the regulatory activities of DP-Tfh cells strongly influenced memory B-cell fate (Figure 4D). Collectively, these results imply that DP-Tfh cells can act as an unidentified regulator of memory B-cell responses.

Eomes^{hi}CD70^{hi} DP-Tfh cells in lesions of IgG4-RD

To further characterize the features of DP-Tfh cells in lymphoid tissues, we investigated the transcriptomes of DP-Tfh cells in SMG lesions of IgG4-RD in comparison with those of DP-Tfh cells in tonsils (Figure 5A). The results showed that DP-Tfh cells in IgG4-RD lesions expressed more CTL-related genes, such as Eomes and granzymes, than DP-Tfh cells in tonsils (Figures 5B–D, Supplementary Table S7). Notably, the CD70 level of DP-Tfh cells in IgG4-RD lesions was higher than that of tonsillar DP-Tfh cells (Figure 5E). Genes related to Tfh cell functions appeared to be expressed in DP-Tfh cells of tonsils rather than in DP-Tfh cells of IgG4-RD lesions (Figure 5C). DP-Tfh cells in tonsils potentially promoted IgG production from B cells, albeit with lesser ability than SP-Tfh cells, whereas DP-Tfh cells in IgG4-RD lesions did not (Figures 4A–B, 5F). Considering the results of GSEA, DP-Tfh cells in IgG4-RD lesions may show greater cytotoxic capability than DP-Tfh cells in tonsils (Figure 5G). Taken together, these findings indicate that IgG4-RD lesions favorably contained DP-Tfh cells expressing Eomes and CD70 at high levels (Eomes^{hi}CD70^{hi} DP-Tfh cells), with a high capacity to regulate memory B cells. Because Eomes and CD70 are usually upregulated in activated T cells in inflammatory tissues (24), they are also considered to be signature molecules of DP-Tfh cells induced during the persistent inflammation of IgG4-RD lesions. In our experiments, we could scarcely detect DP-Tfh cells in peripheral blood specimens of the participants (data not shown).

SP-Tfh cells are the possible origin of DP-Tfh cells

Finally, we investigated the possible origin of tissue-resident DP-Tfh cells. In pathway analysis of upstream genes identified by transcriptome analyses of GC-type Tfh cells in IgG4-RD lesions and tonsils (Figures 1B, 3B), a series of cytokines, including IL-2, IL-1β, CCL2, and IL-10, were postulated to be possible driver molecules related to the phenotype of lesional Tfh cells in IgG4-RD (Supplementary Table S8A). Further pathway analysis of transcriptomes in DP-Tfh cells and SP-Tfh cells of tonsils revealed that cytokines such as IL-2, IL-7, TNF, IL-6, IL-3, IL-1β, and IL-10 were involved in the maintenance of tonsillar DP-Tfh cells (Supplementary Table S8B). The gene expression profile of DP-Tfh cells was partly shared by authentic CD4$^+$ Tfh cells (SP-Tfh cells) in tonsils; therefore, we performed *in vitro* analysis of tonsillar SP-Tfh cells under stimulation with different combinations of these cytokines. The results showed that consecutive stimulation by IL-2 and IL-7 efficiently induced CD8 expression in SP-Tfh cells (Figure 6A). IL-7 expression was highly detected in the inflammatory SMG lesions of IgG4-RD (Figures 6B, C). A receptor complex specifically bound to IL-7 (IL-7 receptor) is a heterodimer of the IL-7 receptor α chain (CD127) and common γ chain, which regulates T cell activation (25). We examined CD127 expression in DP-Tfh and SP-Tfh cells and found that the level of CD127$^+$ Tfh cells in IgG4-RD lesions was relatively lower than that in tonsils (Figure 6D). However, we observed no significant differences in the mean fluorescent intensity (MFI) of CD127 (Figure 6D). Despite the primary structural differences in tonsils and inflamed SMGs of IgG4-RD lesions, which are secondary and tertiary lymphoid tissues, respectively, the lesions in IgG4-RD and tonsils seemed to contain CD127$^+$ Tfh cells ready to receive IL-7. Therefore, SP-Tfh cells may be a potential origin of DP-Tfh cells in IgG4-RD

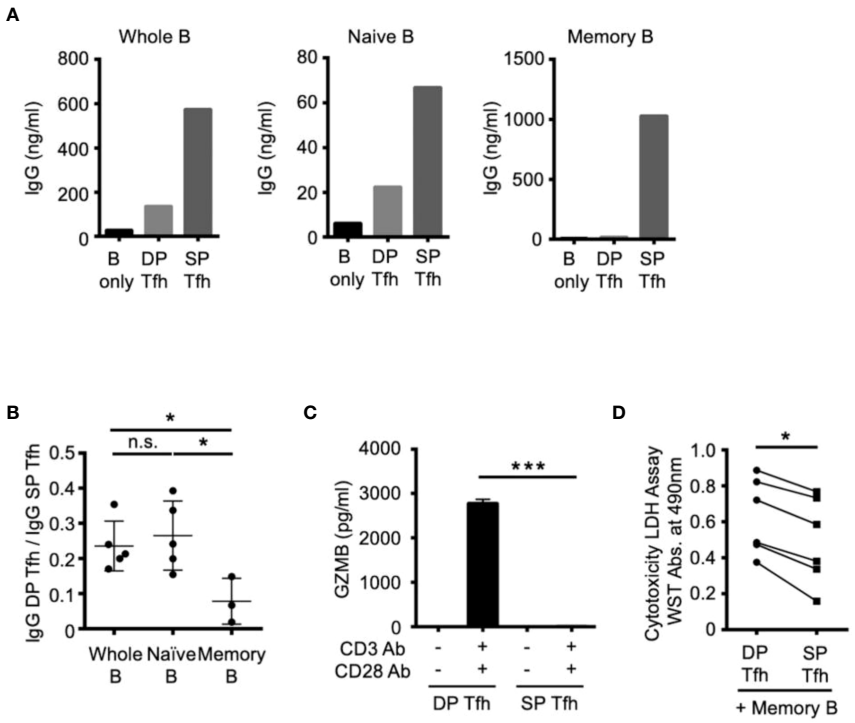

FIGURE 4
Functional effects of DP-Tfh cells on B-cell regulation. **(A)** Representative graphs of co-culture experiments to examine antibody production using autologous Tfh and B cells sorted from tonsil specimens (n = 3-5). DP-Tfh cells ($CD3^+CD4^+CD8^+CXCR5^{hi}PD\text{-}1^{hi}$) or SP-Tfh cells ($CD3^+CD4^+CD8^-CXCR5^{hi}PD\text{-}1^{hi}$) were co-cultured with whole B cells ($CD3^-CD19^+$), naïve B cells ($CD3^-CD19^+IgD^+CD27^-$), or memory B cells ($CD3^-CD19^+IgD^-CD27^+$) under the stimulation of CD3, CD28, and CD40L. After incubating cells for 7 days, IgG levels in the supernatants were analyzed by ELISA. **(B)** Effects of DP-Tfh cells on B cells investigated by co-culture experiments as demonstrated in **(A)**. Data indicate ratios of IgG levels from whole B cells, naïve B cells, or memory B cells in the presence of DP-Tfh cells to those in the presence of SP-Tfh cells (IgG DP-Tfh/IgG SP-Tfh). Data were obtained from three to five independent experiments using autologous tonsillar lymphocytes. **(C)** Increased capacity of DP-Tfh cells to secrete granzyme B (GZMB) under CD3 and CD28 stimulation in comparison with SP-Tfh cells of tonsils. After incubating cells for 7 days, GZMB levels in culture supernatants were analyzed by ELISA. Data were obtained from four independent experiments using autologous tonsillar lymphocytes. **(D)** Cytotoxicity of DP-Tfh cells for memory B cells in comparison with SP-Tfh cells. Co-culture supernatants of memory B cells and DP-Tfh cells or SP-Tfh cells derived from autologous tonsillar lymphocytes were analyzed by a cytotoxicity LDH/WST assay. The absorbance values indicating the cytotoxic activities of DP-Tfh and SP-Tfh cells in each experiment (depicted as a closed circle and rectangle, respectively) are connected by a line to evaluate their differences. Data were obtained from six independent experiments. Statistical significance in **(B–D)** was determined by the Mann–Whitney U test.

lesions and tonsils. On the bases of the data obtained in this study, we proposed a model for DP-Tfh cells in the regulation of B cells in IgG4-RD lesions (Figure 7).

Discussion

In this study, we describe $CD4^+CD8^+$ DP-Tfh cells that were defined as GC-type Tfh cells with possible cytotoxic activity to regulate the function of memory B cells in chronic inflammatory lesions. This follows the findings of a previous study that showed the abundance of tissue-resident Tfh cells in IgG4-RD lesions (13). Our findings are probably consistent with recent reports, which suggested the active involvement of cytotoxic $CD4^+$ T cells in the pathogenesis of IgG4-RD (26, 27). Our study also suggests a potential transition of SP-Tfh cells to DP-Tfh cells under stimulation by the common γ-chain cytokines of IL-2 and IL-7. In line with this, GC-type Tfh cells, especially under persistent inflammation, coordinate humoral immunity by instructing a wider range of B cells than previously thought. IL-2 primes the activation of effector T cells and induces the expression of CD127 to enable T cells to bind to IL-7 for the formation of the memory T cell pool (25, 28). In addition to their general importance in the functional modulation of effector T cells, IL-2 and IL-7 have well-recognized roles in influencing the fate of Tfh cells (29–31). IL-2 controls the gene expression profile of Tfh cells during their initial development, whereas

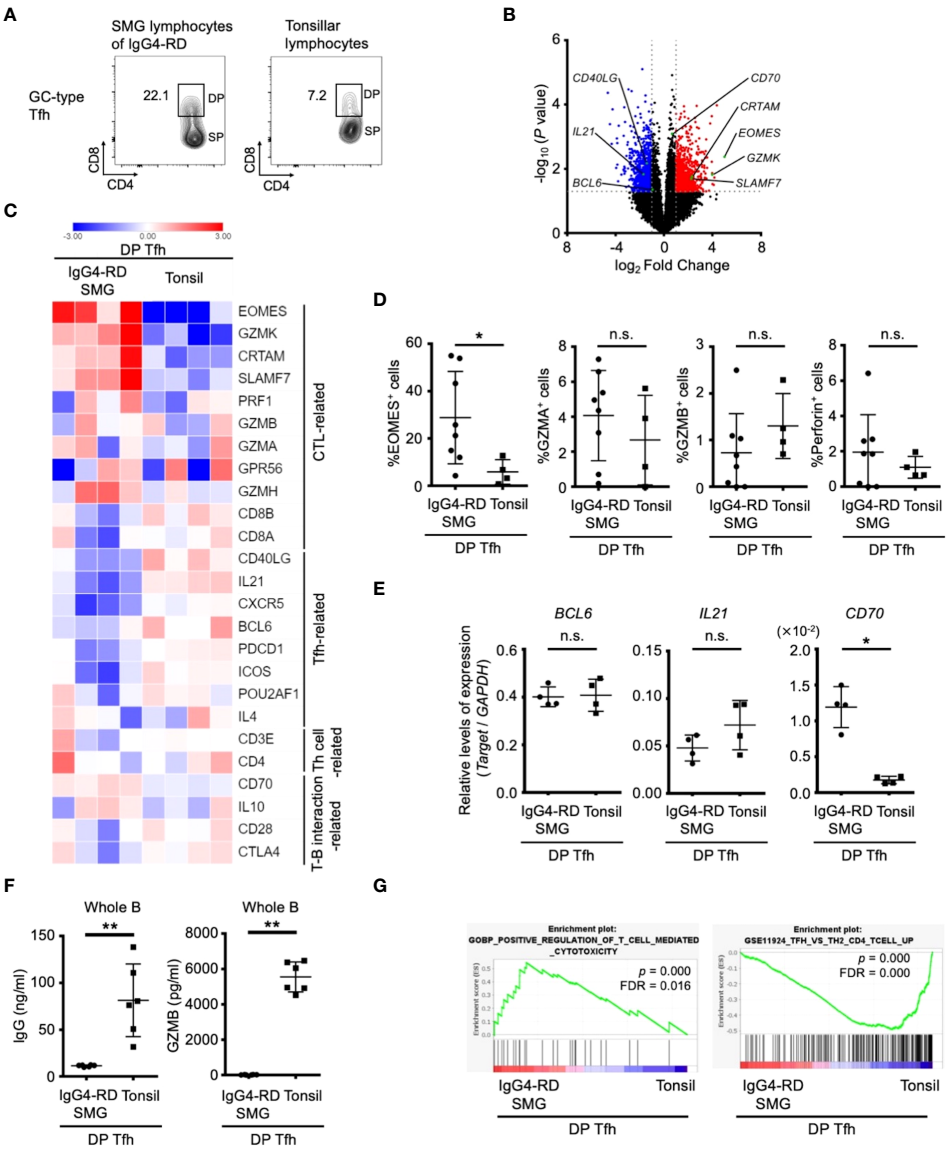

FIGURE 5
Features of DP-Tfh cells in inflammatory lesions of IgG4-RD. **(A)** Representative flow cytometry profiles of DP-Tfh cells and SP-Tfh cells in GC-type Tfh cells in the lymphocytes of tonsils and SMG lesions of IgG4-RD. **(B)** Volcano plot showing differentially expressed genes ($p < 0.05$) with more than two-fold expression in DP-Tfh cells localized in SMG lesions of IgG4-RD versus those in tonsils. Red and blue dots indicate the upregulated and downregulated genes, respectively, in the DP-Tfh cells of IgG4-RD. Data were obtained from microarray analysis of four specimens in each experimental group. **(C)** Heat map representing the relative abundances of transcripts identified in **(B)** for selected genes involved in the functioning and regulation of helper $CD4^+$ T cells and CTLs. Relative values of gene expression are indicated by color. **(D)** Expression of CTL-related molecules in DP-Tfh cells of SMG lesions of IgG4-RD (n = 8) and tonsils (n = 4) as assessed by flow cytometry. **(E)** Relative levels of gene expression in DP-Tfh cells of SMG lesions of IgG4-RD (n = 4) and tonsils (n = 4) as indicated in **(B)** analyzed by RT-qPCR. GAPDH was used as a control. **(F)** Lower B-cell helper capacity of DP-Tfh cells in IgG4-RD lesions than in tonsils. The levels of IgG and granzyme B (GZMB) in supernatants from co-cultures of DP-Tfh cells with autologous whole B cells ($CD3^-CD19^+$) under stimulation of CD3 and CD28 were analyzed by ELISA on day 7 after initial incubation (IgG4-RD, n = 6; tonsil, n = 6). **(G)** Gene set enrichment analysis (GSEA) of genes identified in **(B)** showing transcriptomes of DP-Tfh cells. Results from gene sets associated with cytotoxic T cells or Tfh cells are shown on the left and right, respectively. Data in **(D–F)** were analyzed by the Mann–Whitney U test.

IL-7 signaling represses the functional expression of Bcl6 and induces cell surface expression of CD70 and PD-1 for efficient interactions between Tfh and B cells. In our findings, the ratio of DP-Tfh cells expressing CD127 in IgG4-RD lesions was relatively lower than that in tonsils. This is probably due to the difference in the primary structures of tonsils and IgG4-RD lesions, which constitute secondary and tertiary lymphoid tissues, respectively. Thus, it is reasonable to consider the

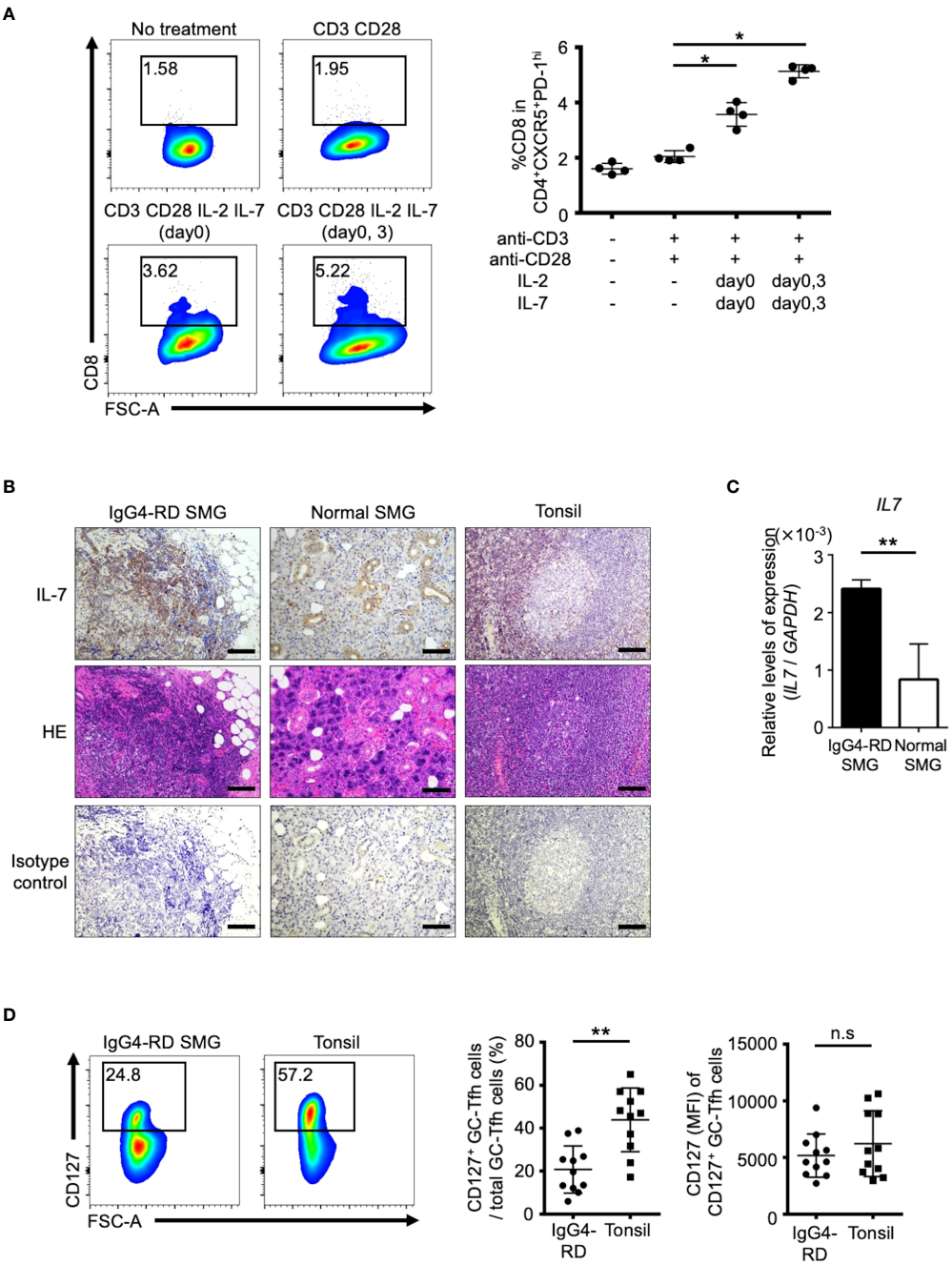

FIGURE 6
SP-Tfh cells are the possible origin of DP-Tfh cells. **(A)** Upregulation of CD8 on SP-Tfh cells ($CD3^+CD4^+CD8^-CXCR5^{hi}PD\text{-}1^{hi}$) under the stimulation of IL-2 and IL-7. SP-Tfh cells ($5x10^4$ cells/well) from tonsils (n = 4) were incubated in 200 µL of AIM-V medium with or without 2 µg/mL anti-CD3 and 2 µg/mL anti-CD28 mAbs. Under the stimulation of CD3 and CD28, 20 ng/mL IL-2 and 20 ng/mL IL-7 were added once on day 0 or twice on days 0 and 3. On day 7, cells were analyzed by flow cytometry. Representative flow cytometry profiles are shown on the left. Data obtained from four independent experiments are summarized in a graph on the right. **(B)** Histological examination of IL-7 expression. Immunohistochemistry of IL-7 in SMG lesions of IgG4-RD, normal SMG tissues, and tonsils are shown in the upper panels. The HE-stained images and isotype controls for immunohistochemistry in the corresponding tissue areas are shown in the middle and lower panels, respectively. Scale bar: 100 µm. **(C)** Relative expression levels of IL-7 in SMG lesions of IgG4-RD (n = 4) and normal SMG (n = 4) assessed by RT-qPCR analysis. GAPDH was used as a control. **(D)** Representative flow cytometry profiles showing the presence of CD127 on GC-type Tfh cells ($CD3^+CD4^+CXCR5^{hi}PD\text{-}1^{hi}$) in IgG4-RD lesions of SMGs and tonsils (left). The percentages of $CD127^+$ GC-type Tfh cells among the total GC-type Tfh cells in IgG4-RD lesions and tonsils (right). Values of the mean fluorescence intensity (MFI) of CD127 on GC-type Tfh cells in the two groups are also shown (IgG4-RD, n = 11; tonsil, n = 11). Data in **(A, C, D)** were analyzed by the Mann–Whitney U test.

transition of excess SP-Tfh cells to DP-Tfh cells in response to aberrant IL-7 and IL-2 concentrations in inflammatory lesions (32). According to our study, the inflammatory milieu in tertiary lymphoid tissues is suggested to allow the generation of DP-Tfh cells. Thus, in such immune settings, adaptive immunity depending on memory B cells may be regulated by DP-Tfh cells. Since DP-Tfh cells efficiently target $IgG4^+$ memory B cells within the tertiary lymphoid lesions of IgG4-RD, these cells might have a unique capability to regulate the resolution of pathological immune responses. This regulatory mechanism is postulated to be a previously unidentified mechanism of immune tolerance mediated by Tfh cells. In instances where $IgG4^+$ memory B cells reside in the lymph nodes, spleen and/or bone marrow of the patients with IgG4-RD, the regulatory function of DP-Tfh cells in memory B cells might be inhibited due to the lack of access of DP-Tfh cells to these tissues. This probably leads to high levels of serum IgG4 in patients with the IgG4-RD in comparison with healthy subjects.

Tfh cells expressing perforin and granzymes, which characterize a cytotoxic function (cytotoxic Tfh cells), are frequently detected in hospitalized patients with coronavirus disease 2019 (33, 34). Notably, cytotoxic Tfh cells are negatively correlated with the serum level of antibodies bound to the SARS-CoV-2 spike protein, implying the cardinal role of cytotoxic Tfh cells in the production of antigen-specific antibodies (33). Cytotoxic Tfh cells are further suggested to target GC B cells within tissues of chronic tonsillitis caused by periodic infection with group A Streptococcus (35). While the expression level of CD8 in such cytotoxic Tfh cells remains unclear, memory B cells may be regulated by cytotoxic Tfh cells, such as DP-Tfh cells, during viral and bacterial infections. In our study, $Eomes^{hi}CD70^{hi}$ DP-Tfh cells in IgG4-RD lesions potentially showed a robust cytotoxic function in comparison with tonsillar $Eomes^+$ DP-Tfh cells. Thus, the cell lytic activities of DP-Tfh cells appear to be associated with their expression level of Eomes, which is a homologous T-box transcription factor T-bet and establishes a cytotoxic effector profile of natural killer cells and $CD8^+$ CTLs (16, 36). A recent study focusing on $Eomes^+CD4^+$ CTLs has expanded and documented their roles in anti-tumor immunity and pathological responses in inflamed conditions such as rheumatoid arthritis and multiple sclerosis (24, 37). Investigations focusing on the mechanisms regulating the expression of Eomes in DP-Tfh cells may improve our understanding of the functional significance of DP-Tfh cells in various immune settings. DP-Tfh cells of mice immunized with ovalbumin did not seem to be associated with the MHC Class I tetramer specific to ovalbumin, which could certainly bind to CD8 cytotoxic T cells (unpublished observation), suggesting that

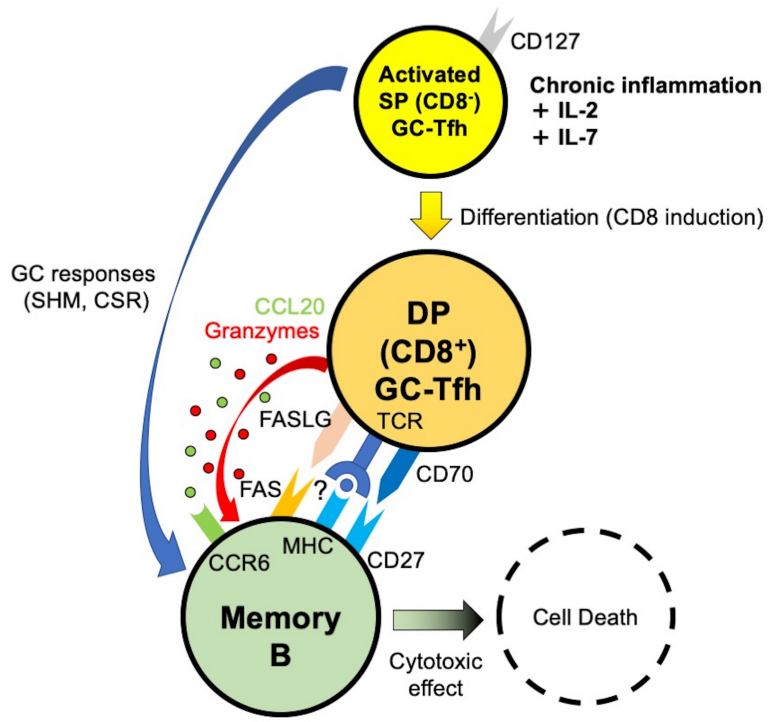

FIGURE 7
Model of DP-Tfh cell function in chronic inflammatory lesions of IgG4-RD. SHM, somatic hypermutation. CSR, class switch recombination.

the involvement of MHC Class I molecules in DP-Tfh cell function may be minimal.

Polarized Tfh-cell subsets are identified among blood lymphocytes probably affected by the surrounding cytokine milieu, which influences the differentiation of the helper $CD4^+$ T cells to distinct subsets (38). Conversely, the DP-Tfh cells are detected in tissues, but not in blood specimens, implying that their primary function may be limited to the local lymphoid tissues where they are produced. In our study of tonsillar lymphocytes, the percentages of cells expressing cytotoxic molecules like granzymes and perforin in non-Tfh DP cells were significantly lower than those of DP-Tfh cells (unpublished observation), further implying the cytotoxic capability of DP-Tfh cells in tissues. Peripheral extrathymic $CD4^+CD8^+$ T cells have been well studied across species (39). Human $CD4^+CD8^+$ T cells have been suggested to show an anti-tumor capacity that is hampered by the major histocompatibility complex molecules in tumor tissues (40). $CD4^+CD8^+$ T cells are known to include a subpopulation of cells sensitive to IL-2 and IL-7 with cell lytic enzymes, as seen in healthy subjects, thereby supporting the critical role of these cytokines in the regulation of DP-Tfh cells (41). $CD8^+$ follicular T cells ($CXCR5^+PD\text{-}1^+$) are a subset of $CD8^+$ T cells in mice and regulate GC B cells (42). A similar phenotype of $CD8^+$ follicular T cells has also been reported in human blood and suggested to be involved in the pathogenesis of viral hepatitis and Sjögren's syndrome (43, 44). Currently, the relationship between DP-Tfh and $CD8^+$ follicular T cells remains elusive. Nonetheless, studies focusing on the diversity and plasticity of Tfh cells are warranted to illustrate the functional properties of Tfh cells in secondary and tertiary lymphoid tissues as well as in peripheral blood and thereby to improve our understanding of the pathogenesis of immune-related disorders.

In summary, we report DP-Tfh cells as a heterogeneous subpopulation of GC-type Tfh cells that are enriched in IgG4-RD lesions. DP-Tfh cells may regulate IgG4 production of memory B cells in IgG4-RD, and DP-Tfh cells are a potential target to improve the pathological immune settings of IgG4-RD. As an active interaction of GC B cells and SP-Tfh cells ($CD4^+$ GC-Tfh cells) proceeds, further interaction of memory B cells and DP-Tfh cells may rationally lead to efficient regulation of humoral responses, especially in inflammatory conditions. This hypothesis may be further supported by evidence suggesting a similar expression level of CXCR4 orchestrating GC reactions in SP-Tfh and DP-Tfh cells of tonsils (unpublished observation). Considering the importance of Tfh cells in protective immune responses, future research based on the present study could also provide an efficient modality to induce vaccine-specific antibodies for preventing infections caused by harmful pathogens.

Data availability statement

Microarray data in this manuscript are available from Gene Expression Omnibus repository hosted by the National Center for Biotechnology Information (accession numbers GSE202615, GSE202616, and GSE202617 for the data presented in Figures 1B, 3B, 5B, respectively). The original contributions presented in the study are included in the Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Experiments using clinical materials were approved by the Institutional Review Board of Sapporo Medical University Hospital (IRB#25-39, IRB#292-83). All participants provided written informed consent in accordance with the Declaration of Helsinki.

Author contributions

KM, II, RK, and HS performed experiments and analyzed data. M. Yanagi, SK, and TS assisted with the experiments. AS, KS, M. Yamamoto, HT, and KT discussed data. SI designed the experiments and prepared the manuscript. All authors approved the final version of the submitted manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.941385/full#supplementary-material

SUPPLEMENTARY TABLE 1
Study participant characteristics.

SUPPLEMENTARY TABLE 2
Types of organ involvement in patients with IgG4-RD.

SUPPLEMENTARY TABLE 3
List of antibodies for flow cytometry and immunohistochemistry, and reagents used for *in vitro* experiments.

SUPPLEMENTARY TABLE 4
PCR primers used in this study.

SUPPLEMENTARY TABLE 5
Differentially expressed genes between Tfh cells in IgG4-RD lesions and Tfh cells in tonsils assessed by microarray analysis.

SUPPLEMENTARY TABLE 6
Differentially expressed genes between DP-Tfh and SP-Tfh cells in tonsils assessed by microarray analysis.

SUPPLEMENTARY TABLE 7
Differentially expressed genes between DP-Tfh cells in IgG4-RD lesions and DP-Tfh cells in tonsils assessed by microarray analysis.

SUPPLEMENTARY TABLE 8
Genes selected by iPathwayGuide analysis to predict regulators of DP-Tfh cells based on the transcriptomes of GC-Tfh cells, including DP-Tfh cells and SP-Tfh cells. **(A)** IgG4-RD Tfh cells vs. tonsillar Tfh cells. **(B)** Tonsillar DP-Tfh cells vs. tonsillar SP-Tfh cells

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(Not) Home alone: Antigen presenting cell – T Cell communication in barrier tissues

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Priming of T cells by antigen presenting cells (APCs) is essential for T cell fate decisions, enabling T cells to migrate to specific tissues to exert their effector functions. Previously, these interactions were mainly explored using blood-derived cells or animal models. With great advances in single cell RNA-sequencing techniques enabling analysis of tissue-derived cells, it has become clear that subsets of APCs are responsible for priming and modulating heterogeneous T cell effector responses in different tissues. This composition of APCs and T cells in tissues is essential for maintaining homeostasis and is known to be skewed in infection and inflammation, leading to pathological T cell responses. This review highlights the commonalities and differences of T cell priming and subsequent effector function in multiple barrier tissues such as the skin, intestine and female reproductive tract. Further, we provide an overview of how this process is altered during tissue-specific infections which are known to cause chronic inflammation and how this knowledge could be harnessed to modify T cell responses in barrier tissue.

KEYWORDS

skin, T cells, antigen-presenting cells, female reproductive tract, tissue-resident T cells, intestine, barrier tissue

Introduction

T cells are highly specialized executors of immune responses against pathogens and play important roles in maintaining tissue homeostasis. During infection or acute and chronic inflammatory responses, effector T cells (T_{eff}) can infiltrate from the periphery and establish residency and subsequent memory, involving a switch in transcriptional program using different transcription factors and signaling hubs (1–6). This explains why the majority of the T cell population found in tissues are memory T cells

(7), subdivided into central memory T (T_{cm}), effector memory T (T_{em}), and resident memory T (T_{rm}) cells. T_{em} and T_{cm} were first identified in the peripheral blood (8). T_{em} were found to be the predominant subset in non-lymphoid tissue while their T_{cm} counterparts are mainly found in secondary lymphoid organs (9–17). Later, a long-lived memory population with little to no recirculatory capacity was identified and termed T_{rm} (12–18). Another prevalent T cell subset in tissues are regulatory T cells (T_{regs}), particularly important for maintaining a tolerogenic tissue environment, preventing excessive immune responses to harmless antigens often found at barrier tissues [reviewed in (19, 20)]. T_{regs} usually refer to $CD4^+$ T cells with the unique ability to suppress pro-inflammatory effector functions in other T cells as well as contribute to tissue homeostasis (21, 22). Tissue T_{regs} can also be subdivided by the central and effector memory cell classification based on the expression of CD44 and CD62L (23–25), with central T_{regs} being able to recirculate through secondary lymphoid tissues, while effector T_{regs} exhibit a more resident phenotype, representing the predominant T_{reg} population in nonlymphoid tissues (23). Non-conventional T cells can also be found in barrier tissues. An example of this are γδT cells, which are mainly found in epithelial tissues and are particularly abundant in the intestine (26). In homeostatic conditions, γδT cells have been described to exhibit a pre-activated memory phenotype (27), being able to exert direct cytotoxic functions (28, 29). As for other T cell subsets in tissues, roles in wound healing and tissue homeostasis have also been attributed to γδT cells (30, 31). A broad overview of T cell subsets found in tissues and surface markers most commonly associated with each subset is depicted in Figure 1. It should be noted that these markers are not absolute and their expression is often changed in different tissues. However, these figures aim to give a broad overview over the most common and widely distributed markers of each subset and highlight commonalities and differences between mice and humans.

Priming by antigen presenting cells (APCs) is crucial for T cells to exert their correct functions and home to tissues. For example, the presence and function of T_{regs} in tissue has been directly linked to the presence of dendritic cells (DCs) (32). Tissue-patrolling DCs are of an immature phenotype and internalize antigens by endocytosis or phagocytosis, which are

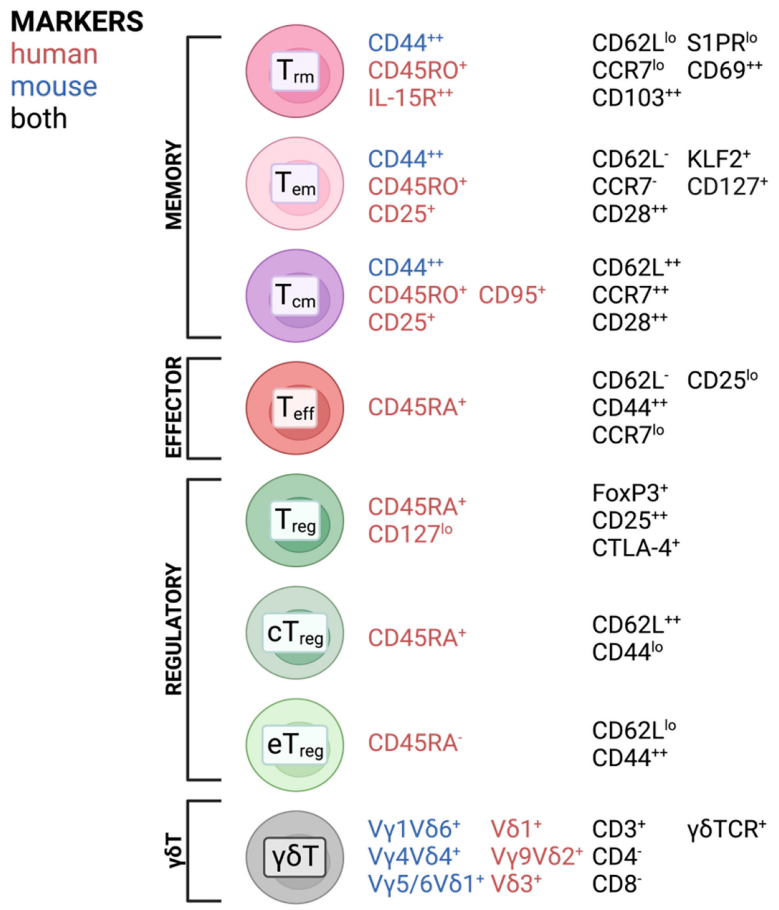

FIGURE 1
T cell subsets and commonly associated markers in mice and humans found in barrier tissues discussed in this review. Created with BioRender.com.

loaded to major histocompatibility complex class II (MHC-II) for CD4 T cell presentation *via* endosomal pathways (33). However, DCs are also efficient in cross-presenting extracellular antigens *via* MHC-I to CD8 T cells, by which exact mechanism is still under debate (33, 34). Apart from antigen uptake, DCs need to receive additional stimuli in order to mature and upregulate CCR7, by which they interact with the ligands CCL19 and CCL21 guiding them to the lymph nodes to meet naïve T cells (35, 36). Under homeostatic conditions, DCs mainly collect non-hazardous antigens from food or commensal bacteria in the intestinal tract and skin or paternal antigens of fetal cells within the female genital tract during pregnancy (37–39). On the other hand, DCs are highly sensitive against pathogen-associated molecular patterns (PAMPs), which they detect *via* their toll-like receptors or C-type lectin receptors and they sense cytokines produced by other cell types during infection (33, 40). Mature DCs upregulate molecules necessary for co-stimulation of T cells like CD86 and CD80 (41).

Classically, DCs are divided into several subclasses: conventional DCs (cDCs), monocyte-derived DCs (mo-DCs) and plasmacytoid DCs (pDCs) (42). Langerhans cells were previously also classified as DC population; however, they developmentally originate from yolk sac progenitors, which identifies them as member of the tissue-resident macrophage family. In contrast to macrophages, they can efficiently present antigens and possess a migration potential to the lymph node (43). Therefore, they are often mentioned along with other DC subsets inducing T cell responses. Conventional DCs are subdivided into type 1 classical DC (cDC1), which are known to cross-present antigens *via* MHC-I to CD8 T cells but also polarize CD4 T cells towards T_h1, while type 2 classical DCs (cDC2) mainly present antigens *via* MHC-II to CD4 T cells. The cDC1 subset in mice is $CD11b^{lo}$ and shows CD8a and CD103 on their surface, while human cDC1 express and XCR1 and CD141 (33). cDC2 express CD172a and depending on murine or human origin they highly express CD11b or CD1c, respectively (33). Especially cDC2 comprises a very heterogenous immune cell population which can acquire quite contrary immune functions depending on the context. For examples, in human there exists a cDC2 subset which expresses at the same time monocyte-related genes like CD163 and CD14, which was termed DC3 (44, 45). LCs are a population patrolling the epidermis as well as the epithelial layer of the vagina and cervix and are characterized by expression of a specific lectin receptor, langerin (CD207) and CD1a (46). Monocytes express CD14 and can be differentiated *in vitro* to monocyte-derived DCs (mo-DCs) by addition of GM-CSF and IL-4 and are a widely used model for priming T cells *in vitro* (47). However, the existence of mo-DCs *in vivo* remains under debate, but several mouse (48, 49) and human (50) studies observed that monocytes can differentiate into DC-like cells, especially under inflammatory conditions (45, 51). With the evolving of single-cell sequencing technology, more and more DC subsets are discovered and it now appears that the discrimination between DC and monocyte subsets is not that black and white, with mo-DCs in comparison to DC3 being just one example (44, 45, 52, 53). APC subset composition varies widely throughout tissues and we are still far from understanding which subset contributes to immunity and tolerance under certain conditions (54–57). DCs are in general $CD45^+$ cells, expressing HLA-DR and lacking other linage markers, such as CD3 or CD19 (52). In Figure 2, a simplified overview of the most important DC subsets in human can be found with the markers for those respective subsets in mice included.

In this review, we discuss the different subsets of T cells and APCs present in the skin, intestine and female reproductive tract (FRT) and how their interplay contributes to maintaining a homeostatic tissue environment as well as how this composition shifts during chronic inflammatory diseases and infection. While the term "immune homeostasis" is widely used, we refer to "homeostasis" as the balance between immune activation and suppression in tissues and organs in contribution to maintaining a healthy state of an organism under normal physiological conditions. This review aims to focus on the human system wherever possible; however, some insightful mechanistic studies in different animal models are included as these contribute greatly to our understanding of tissue immunity where human studies are not yet possible. To give a more comprehensive view of already described mechanistic studies not yet discovered in humans we also included animal studies when appropriate. Therefore, unless otherwise stated, findings summarized were done in humans and deviation to animal models is indicated.

Skin

The skin is one of the largest organs in the human body and essential for protection against external injury and pathogens. Next to its role in physical protection, the skin also houses a vast landscape of resident and recirculating immune cells which are poised locally to respond to tissue damage and infection. The skin is comprised of three layers: the outermost epidermis, an intermediate layer termed dermis, and the innermost layer called hypodermis (Figure 3). The epidermis is mainly comprised by structural cells such as keratinocytes, as well as melanocytes. The main immune cells found in this layer are $CD8^+$ T cells and LCs, skin-resident macrophages which originate from the fetal liver and the yolk sac, and exhibit DC-like characteristics (58). Next to structural fibroblasts, the dermis contains the majority of immune cells, including DCs, macrophages, natural killer (NK) cells, innate lymphoid cells (ILCs), as well as $CD4^+$ and $CD8^+$ T cells. Further, this layer is also supplied with lymphatic and blood vessels which allow immune cell trafficking in and out of the tissue. The lowest layer, the hypodermis, is mainly comprised of adipocytes responsible for thermoregulation (59, 60).

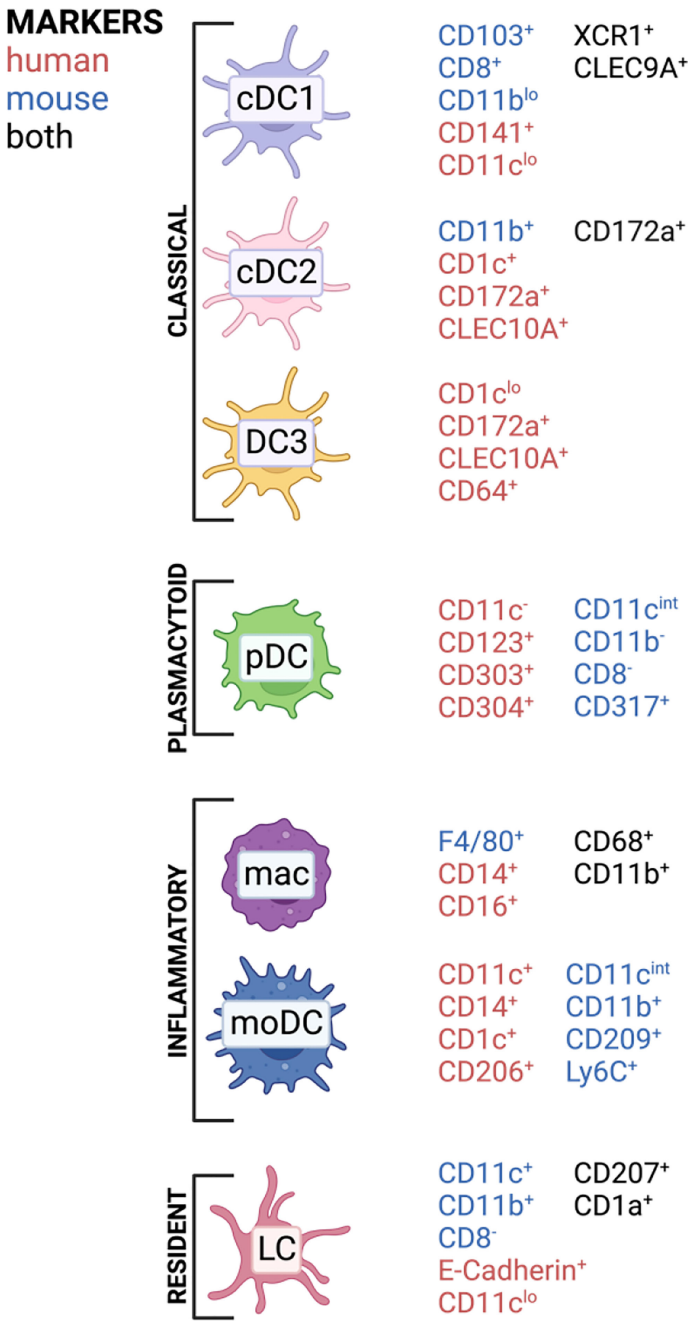

FIGURE 2
APC subsets and commonly associated markers in mice and humans found in barrier tissues discussed in this review. Created with BioRender.com.

However, recently an immunological role has been attributed to adipose tissue as it has been shown to house multiple types of immune cells (61–66). Additionally, structures such as hair follicles and nerve endings are major players in regulating immune responses in the skin. Hair follicles represent unique structures in the skin, as many studies in mice have shown that they are the primary site for T_{reg} maintenance, which are in turn essential for establishing the stem cell niche at the hair follicle (67–69). In human skin, the hair follicle is also the major site of T_{reg} localization (70). Further, the hair follicle is also of importance for DC function in the skin of mice (68, 71). Besides the hair follicle, nerve endings in the skin have been

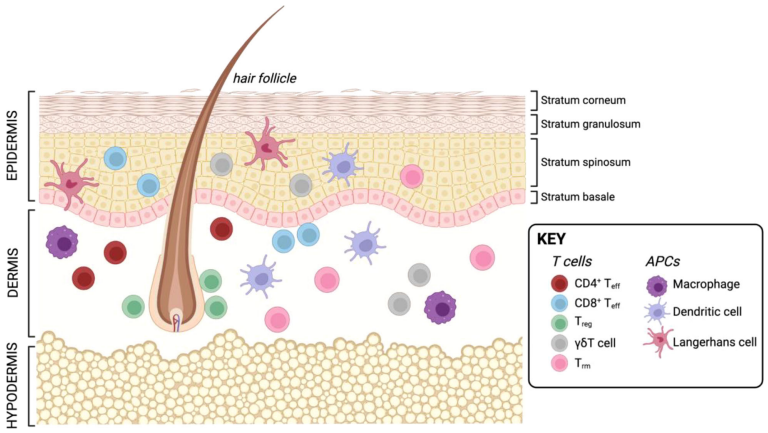

FIGURE 3
Resident T cells and APCs in the human skin. Created with BioRender.com.

shown to play an important role for $CD8^+$ T cell mediated immunity (72) as well as create a special environment for specific macrophage subsets (73) as demonstrated in mouse models.

Upon encountering pathogens or injury to the epidermal layer, LCs are the first to initiate an immune response. These cells constitute approximately 2-4% of all cells in the epidermis and are specialized macrophages with DC characteristics, expressing the surface markers CD1a and Langerin/CD207 (46), whose dendrites can extend through the stratum corneum to sample antigen without disturbing the epithelial barrier (74, 75). LCs preferentially recognize mannosylated ligands on surfaces of pathogens *via* C-type lectins and pattern recognition receptors (PRRs) (76). Binding of these receptors leads to receptor-mediated endocytosis thereby activating the LC (77). Like their conventional DC counterpart, LCs have been found to be able to traffic to the skin-draining lymph nodes (LNs) and activate naïve T cells (78–80) as well as activate skin-resident T_{regs} (81). LCs have also been described to be highly efficient at inducing a neutralizing IgG response against *S. aureus* from B cells (82). While LCs have their primary role in immune surveillance of the skin, macrophages are mainly responsible for initiating inflammatory responses in response to infection or injury as well as to tissue regeneration (83–85).

Apart from the acute role of innate immune cells in clearing infection, APCs also play a major role in activating an adaptive immune response. As in other tissues, dermal APCs expressing CD1c (86, 87) can be divided into multiple subsets. In healthy human skin, the main subsets at steady-state are $CD1a^{++}CD207^{+}$ $^{+}$ LCs, $CD1a^{+}CD1c^{+}$ DCs, $CD141^{++}CD14^{-}$ DCs, as well as two populations of macrophages that can be, in part, distinguished by their autofluorescence (AF) created by their high scatter properties: $CD14^{+}AF^{-}$ monocyte-derived macrophages (mo-Mac), and $FXIIIA^{+}CD14^{+}AF^{++}$ macrophages (88). Upon antigen encounter in the skin, dermal DCs (DDCs) become migratory and act as APCs in the lymph node where they activate and polarize different adaptive immune cells, such as naïve T cells (88, 89). It was shown in mice that the constant travel of skin APCs to the LN during homeostasis is only dependent on the CCR7 ligand CCL21, whereas CCL19 presence is dispensable for the trafficking (90, 91). However, CCL19 deficient mice exhibit lower T cell numbers due to decreased cell survival (91). However, DCs in the skin have also been shown to locally activate memory T cells within the skin, bypassing the need for tissue egress (81) and thereby enabling a rapid adaptive immune response locally.

Specifically, T cells play a major role in the cutaneous immune system, with a large tissue-resident population being found throughout the dermis and epidermis. In healthy skin, this population can comprise up to $2x10^{10}$ cells, which is nearly two times as many as found in circulation (17). Differences in T cell composition between murine and human skin have made studies using mouse models difficult. In mice, the majority of resident T cells are γδT cells with a limited T cell receptor (TCR) repertoire (92), while in human skin most resident T cells are αβT cells with a much greater TCR diversity (17). Overall, T cells in the epidermis are less proliferative but have increased capacity to produce cytokines such as IFN-γ and TNF-α (93). While αβT cells rely on antigen presentation *via* MHC molecules, γδT cells have a restricted TCR repertoire, with their receptors recognizing unconventional antigens such as phosphoantigens, stress molecules, as well as non-peptide metabolites (94–96). Human γδT cells express the Vδ1, Vδ2, and Vδ3 chains, with each subtype having a preferential distribution across the body (97). A murine-specific γδT cell subset, called dendritic epidermal T cells (DETCs), have also been shown to significantly contribute to immune homeostasis in mouse skin (98), but don't have a human counterpart. How different T cells subsets contribute to maintaining homeostasis

and how this paradigm is shifted during the inflammatory response and infection will be discussed below.

DC-T cell composition in homeostasis

Memory T cells

While the T cell subsets above mainly describe different effector states of activated T cells, a central part of T cell function is the capacity to develop long-lived immunological memory. T_{eff} cells primed in the lymph nodes by an APC are maintained in the skin as memory T cells, whose survival is supported by keratinocytes producing growth factors as well as other tissue resident (immune) cells (99, 100). These resident memory cells are crucial for maintaining tissue homeostasis as they contribute to immune surveillance and supply a rapid, specific response when re-encountering pathogens. As with all other immune cell subsets, memory T cells in the skin can be divided into two major groups: resident and recirculating. Using a human skin xenograft model with nude NSG mice, four distinct memory populations in the skin have been identified using the resident vs. recirculating paradigm. In human and mouse skin, the primarily resident subsets are T_{em} and T_{rm}. Recirculating subsets can further be subdivided into migratory memory (T_{mm}) and T_{cm} (8, 93, 101). Cutaneous lymphocyte antigen (CLA) is a marker that specifically distinguishes memory T cells originating from the cutaneous immune system as well as skin-infiltrating T cells. CLA binds to chemokine receptors, E-selectin which together with Very late antigen 1 (VLA-1)/Vascular cell adhesion protein 1 (VCAM-1) and Lymphocyte function-associated antigen 1 (LFA-1)/Intercellular adhesion molecule 1 (ICAM-1) enables skin tropism of these cells (102–105).

T_{em} are thought to be the first responders, expressing high levels of CD44 but lacking migratory and homing receptors such as L-selectin and CCR7 (8, 106, 107), making them incapable of recirculating. As their name suggests, they provide immediate effector function, which is underscored by their production of IFN-γ as well as other pro-inflammatory cytokines (93). While T_{em} are crucial for immediate adaptive responses, this population undergoes significant contraction after an infection is resolved and their niche has been found to be replaced by T_{cm} which enter from the circulation over the course of an acute inflammatory response (13). T_{cm} express high levels of homing receptors that are lacking on T_{em} (CCR7, LCA, CCR4) (17, 108, 109). Contrary to T_{em}, their reactivation primarily takes place in the local LNs. There, they undergo extensive proliferation and adopt a T_{em}-like phenotype (8, 110). The other circulating subset, T_{mm}, was described by Rei et al. (93) and shows a population of cells high in skin-homing receptors such as CLA and CCR7 but are defined by the absence of L-selectin. This lack of L-selectin has raised suspicion that these cells are able to remain in the skin after infection, where they contribute to immune homeostasis as these cells are not high producers of pro-inflammatory cytokines (93). Another, more recently discovered, family member are T_{rm} which express high levels of the integrin CD103 as well as the glycoprotein CD69. While their overall phenotype is similar to that of T_{em}, they have been shown to be maintained long-term even after an infection, as well as being significantly more potent in their effector response while also being limited in their proliferative capacity (13, 111). An essential tool in understanding the migratory behavior of T_{rm} is two-photon intravital microscopy. Multiple studies in mice have revealed that, in the skin, these cells are relatively stationary and confined in and close to the epidermis where they surveil their environment and are responsible for regulating secondary recall responses after primary challenge (112–114). Together, these memory subsets contribute to long-lasting immune memory and surveillance in the skin.

Effector T cells

While T cells in the skin at steady-state are mostly memory T cells, effector T cells (T_{eff}) can also be found. These are activated by APCs in the skin-draining lymph nodes and traffic to the skin, where they further encounter cutaneous APCs presenting their cognate antigen, which leads to T cell activation and production of effector cytokines (115, 116). Most studies on T_{eff} cells have described essential roles for $CD8^+$ T_{eff} cells in maintaining tissue homeostasis in the skin. $CD8^+$ T cells can be found in both the dermis as well as the epidermis. $CD8^+$ T cells have been shown to have increased migratory capacity within different skin compartments, albeit with slower kinetics than migration in the lymph node (117). In a sophisticated *ex vivo* imaging system of whole skin to observe T cell migration, Dijkgraaf et al., could demonstrate that human $CD8^+$ skin-resident T_{rm} in the epidermis migrate along the stratum basale, close to the basement membrane and preferentially localize below aggregations of stationary LCs. In contrast, $CD8^+$ T cells in the papillary dermis were observed to accumulate in collagen I rich regions as well as collagen I-poor dermal vessels. These observed migration dynamics highlight an important function of $CD8^+$ skin-resident T cells in tissue patrol, possibly enabling immediate cytotoxic response to antigen presentation by co-localized LCs at the epidermal-dermal junction (118). While $CD8^+$ T cell co-localization with LCs at the epidermis-dermis interface may hint at increased priming capacity by local epidermis-patrolling APCs, observed changes in morphology of $CD8^+$ T_{rm} to a more dendrite-like shape (7, 117, 119, 120) could also suggest that these memory cells can act, at least in part, independently of APCs when confronted with their respective antigen, which has been described to be the case in mice (121–123). However, it is known that specialized $CD141^+CD103^+$ DCs are especially effective at cross-presentation for $CD8^+$ T cells in the skin (124, 125).

Regulatory T cells

Similar to other immune cell populations, T_{regs} can reside in non-lymphoid tissue (NLT) such as the skin. Specific residency transcriptional programs in these organs have been described, mediating T_{reg} adaptability to different tissues in mice (126). In human skin, T_{regs} represent between 5% and 20% of all resident T cells under homeostatic conditions (127, 128), where they are known to interact with LCs and fibroblasts (81, 127). Most circulating T_{regs} found in peripheral blood express skin-homing markers which indicates that these cells are constitutively recruited to the skin over other organs (129). Similar to their effector memory counterparts, T_{regs} from the skin are also able to elicit a memory response and have been shown to persist in the skin and induce tolerance to autoantigens in a mouse model (130). In human skin, the function of skin-resident T_{regs} remains elusive, with few studies investigating their function under homeostatic conditions. Other than the canonical transcription factor FoxP3, skin T_{regs} express CLA, as well as the chemokine receptors CCR6, high levels of CCR4, a skin homing marker, high levels of L-selectin and HLA-DR. Similar to their blood counterparts, they express GITR and high levels of intracellular CTLA-4. Contrary to other skin-resident T_{eff} cells, skin T_{regs} tend to express much lower CD103 (127). Seneschal et al. demonstrated that the function of skin-resident T_{regs} is highly dependent upon the context under which they are activated by local LCs. Under steady-state, LCs appear to preferentially activate and expand $CD4^+CD25^+FoxP3^+CD127^-$ T_{regs}, which were functionally competent in suppressing autologous skin resident T_{em} cells. Further, it was suggested that this effect is MHC-restricted, showing that under steady-state conditions, LCs act in concert with T_{regs} to induce and maintain tissue homeostasis (81). While reports of antigen-specific responses by T_{regs} do exist, it is well-established that skin T_{regs} have a high proliferative capacity in response to non-antigen dependent stimuli, such as contact with dermal fibroblasts in combination with IL-15 (127). Other than their immediate immunological function, cutaneous T_{regs} are known to be involved in wound healing (131, 132), where their primary role lies in inhibiting IFN-γ production by other T cells and inflammatory macrophages (132), as well as and modulating hair follicle stem cells (133).

γδT cells

In human skin, 1-10% of all resident T cells are estimated to be γδT cells (134), with the majority expressing the Vδ1 TCR (135, 136). One known ligand for this TCR is CD1d which is able to present lipid antigens on DCs (137). CCR6 on γδT cells is thought to be an important receptor mediating recruitment of activated γδT cells *via* CCL20 expression by keratinocytes, DCs as well as endothelial cells (138). CCL20 secretion by keratinocytes is especially upregulated during acute injury, suggesting an important role for γδT cells in response to injury (139). Cytokines important in αβT cell maintenance in the skin have also been found to play key roles for γδT cell maintenance and development in this organ. IL-7R signaling, for example, has been shown to induce rearrangement and transcription of the TCR γ-chain, and IL-15 is also involved in the expansion of γδ epidermal T cell precursors as well as their survival, while IL-4 signaling has been shown to promote growth of the epidermal γδT cell compartment (140–142). The skin residency marker CD103 has also been implicated to play a role in establishing γδT cells in the murine epidermis, with CD103-deficient mice showing significant reduction in γδT cell numbers in the skin as well as abrogated morphology in the γδT cells present (143). Further, murine $CD103^-$ DETCs share a competitive niche in the epidermis with $CD103^+$ T_{rm}, indicating that CD103 is an important determinant in establishing tissue residency in the murine epidermis (113). If CD103 expression by γδT cells is also vital in human skin remains to be uncovered. Co-stimulation for γδT cells is less understood than for their αβ counterparts. However, in mice CD27 has been shown to contribute to the function of Vγ2Vδ2 T cell activation and promote IFN-γ production by these cells (144). Further CD2 and ICAM-1 have been suggested as costimulatory molecules for Vδ1 T cells (145–147). Specific functions of γδT cells in human skin are known to include regulation of keratinocyte proliferation and homeostasis through the production of insulin-growth factor 1 (IGF-1) and other keratinocyte growth factors (98, 148). Further, γδT cells are also able to contribute to skin homeostasis by recognizing damaged cells and exhibit cytotoxic activity *via* the NKG2D receptor (149), as well as perforin secretion and Fas-mediated cell lysis (150).

DC-T cell composition in infection and inflammation

Chronic inflammatory diseases

A skewed composition in terms of T cell numbers and function of skin-resident T cells has been described in a plethora of chronic inflammatory skin diseases. Accordingly, the populations of APCs in inflamed skin also shift, with the dominant subsets being $FcER1^+CD1a^{lo}$ (inflammatory dendritic epidermal) DCs, $CD1c^+CD14^{+/-}$ DC (inflammatory), $TNF\text{-}\alpha^+iNOS^+CD14^-CD11c^+CD1c^-$ (TNF-α and iNOS producing) DCs, and $CD123^+$ pDCs depending on the nature of the disease (88). Further, in a mouse model of skin inflammation, Chow et al. demonstrated that specifically usually resting T_{regs} become highly motile during both adaptive and innate inflammation, highlighting the importance of these cells to control local inflammation (151).

One prominent example of such a disease is psoriasis, which affects 2-3% of the population (152). Skin lesions in psoriasis are thought to be caused by dysregulated cross-talk between APCs and T cells, which leads to an increased production of pro-inflammatory cytokines such as IL-17, IL-12, IFN-γ, TNF-α, and IL-23 (153, 154). This creates a positive feedback loop by recruiting more lymphocytes, neutrophils and myeloid cells to the lesion ultimately causing chronic cutaneous inflammation and epidermal hyperplasia (155). Blocking of TNF-α significantly reduced expression of the DC migration marker CCR7 and its ligand CCL19, thereby supporting clinical remission of patients (156). Dermal $CD3^+$ T cells in these skin lesions are often increased by up to 15%. The composition of αβ and γδT cells in psoriasis also shifts, with some studies observing more than 40% of $CD3^+$ T cells also expressing γδ TCRs and secreting the pathogenic cytokines IL-17 and IL-23 (157). Other studies have observed CLA^+ Vγ2Vδ2 T cells homing to the skin to be increased in patients with psoriasis (158). Further, LCs have been described to preferentially utilize the MAPK-p38α signaling pathway, which has been linked to psoriasis susceptibility in humans (159). This has been shown to specifically promote production of IL-17 in $CD4^+$ T cells by promoting the expression of IL-23 and IL-6, both of which are essential for T_h17 differentiation and known to drive psoriasis pathogenesis (160). Additionally, LCs are able to induce a peripheral T cell response by priming immature $CD4^+$ T cells in the lymph node to produce IL-22 which then acts on epithelial cells, further promoting tissue inflammation *via* alarmins such as the antimicrobial peptide HBD3 (161).

While many chronic inflammatory diseases are of unknown etiology, some have been correlated to dysbiosis of the skin microbiota. An example of this is atopic dermatitis (AD), a chronic T_h2-dominated disease characterized by eczematous lesion and severe pruritus caused by immune cell infiltration of inflammatory DCs, macrophages and eosinophils (162). Further, AD is often found to be associated with transepidermal water loss due to a mutation in the filaggrin gene which leads to enhanced susceptibility to overgrowth of pathogenic *S. aureus* (163, 164). Further, patients with acute flares of the disease have been found to have an acute expansion of the cutaneous *S. aureus* population and significant loss of diversity in the cutaneous microbiome. Conversely, resolution of lesions has been association with a more diverse microbiome composition and contraction of the *S. aureus* population (165). Chronic inflammatory skin disorders still represent a major subset of disease with little mechanistic understanding of how T cell responses are shifted to cause disease.

Infection

It is becoming clear that the capacity of LCs in activating T cells in human skin is highly context dependent with their homeostatic role being more regulatory rather than activating T_{eff} cells. However, it has been demonstrated that LCs are indeed able to activate skin-resident T_{em} in the context of *C. albicans* infection, driving them to produce effector cytokines such as IFN-γ and IL-17 (81).

As the skin is constantly exposed to pathogens, the pool of T_{rm} in this and other organs is thought to reflect previous infections and exposures. In humans, many $CD69^+$ T_{rm} have been shown to recognize prevalent viruses such as influenza A (166, 167), and respiratory syncytial virus (RSV) (168) in the lung. Further, viruses that cause latent and re-activating infections such as herpesvirus (HSV)-1 and -2 (72, 169, 170), Eppstein-Barr virus (171–173), and cytomegalovirus (174) are also known to elicit a strong T_{rm} response. This is further corroborated by the correlation between presence of virus-specific T_{rm} and increased immune protection and ability to control infections, which was shown to be the case for RSV (168), hepatitis B virus (175), and HSV-2 (170) infection. Specifically, in HSV infections, $CD8^+$ T_{rm} seem to play a crucial role in resolution and protection. HSV-specific $CD8^+$ T_{rm} have been found at the dermal-epidermal junction, close to sensory nerve endings which connect the latently infected ganglia to the skin as well as the genital mucosa (72, 170, 176). These cells have been shown to rapidly produce perforin and pro-inflammatory cytokines upon asymptomatic HSV-2 shedding. Further, cluster formation around virally infected epithelial cells and recruitment of $CD8^+$ T cells from the dermis (170) emphasize that $CD8^+$ T_{rm} are at the forefront of the immune response against acute and latent HSV. While it is now possible to also study T_{rm} in humans, it is worth mentioning that the great majority of current knowledge of T_{rm} behaviour during infection was acquired using murine models of HSV infection which greatly contributed to our understanding of these cells in mucosal tissues (11, 123, 177–180).

Intestine

Similar to the skin, the intestine is constantly exposed to exogenous triggers such as food or microbiota-derived antigens. These antigens are prevented from triggering a pathogenic immune response by cellular barriers. Physically, the intestine is protected by a layer of mucus and glycocalyx which coats the epithelial layer (181) and contains high concentration of secreted IgA (182, 183). In the small intestine, this is composed of a single unattached layer, while the large intestine has two layers of protective mucus, respectively relating to the bacterial burden in each location (184). The intestine is also home to intraepithelial lymphocytes (IELs), other immune cells resident in the lamina propria (LP) and gut-associated lymphoid tissue (GALT), comprising Payer's patches (PP), cecal patches, and colonic patches distributed along the small and large intestine (185). There are differences in immune cell composition between the

small and large intestine which have been extensively reviewed elsewhere (186, 187). A simplified overview of the architecture of the small and large intestine including resident T cells and APCs is shown in Figure 4.

At the bottom of the intestinal crypts, Paneth cells are the main producers of antimicrobial products such as defensins (188) and lysozyme (189), which are secreted into the mucus at the opening of the crypt. Goblet cells, responsible for the production of intestinal mucus, have the ability to take up antigen from the intestinal lumen and deliver these antigens to DCs in the LP *via* a process called goblet cell-associated antigen passage (GAP) (190). Antigens delivered *via* this process have been shown to be taken up by $CD103^+CD11c^+$ DCs which preferentially present to T_{regs}, suggesting that this way of antigen delivery significantly contributes to induction of oral tolerance (191). While this mechanism is not well-understood yet, the more accepted route of antigen delivery from the lumen to the epithelium is *via* M cells on lymphoid follicles (e.g. on Payer's Patches), which can transport whole bacteria (192, 193) that can then be taken up by DCs in the epithelium. This continued sampling of the microbiota by the immune system is crucial to maintaining homeostasis and resistance to pathogens. For example, expression of the chemokine receptor CX3CR1 in mice is essential for APCs to extend their dendrites between epithelial cells and take up intestinal bacteria from the lumen (194) which are then transported to the mesenteric LNs, where production of secretory IgA by plasma cells is induced (195–197). While originally being described as DCs due to their functional properties (194), CX3CR1+ APCs were classified as macrophages by others as they also express the macrophage markers CD64 and F4/80 and derive from monocytes (198, 199). Specifically, DCs in the intestine have the major responsibility in establishing tolerance to oral and microbiota-derived antigens. The gut-draining LNs as well as the GALT are the primary sites of T cell priming by intestinal DCs. As in other tissues, many DC subsets have been identified in the human intestine, with specific subsets more prevalent at specific anatomic locations. In humans, intestinal cDCs are divided into subgroups based on the expression of CD103 and SIRPα (200, 201), with $CD103^-$ $SIRP\alpha^+$ cDC2 further subcategorized based on the expression of the chemokine receptor CCR2 (202).

Intestinal cDCs are the only DCs expressing the enzyme RALDH2, which is required for metabolizing Vitamin A to all-trans retinoic acid (RA) (203). This metabolite is required for imprinting gut-homing receptors on T cells, namely α4β7 and CCR9 (204–207). Both $CD103^+$ and $CD103^-$ cDCs in humans have been found to express RALDH2 (208), which is reinforced by expression of RA by stromal cells in the mesenteric LNs (209, 210). In humans, the majority of IELs are T cells, with the highest proportion of non-T immune cells in the colon (211). The highest number of IELs are found in the proximal small intestine, decreasing in the distal small intestine, and lowest numbers in the colon (212). In the adult jejunum, the majority of IELs are $CD8^+$ αβT cells with a tissue-resident T_{em} phenotype [reviewed in (213)], while the ileum and colon have higher numbers of $CD4^+$ αβT cells, with a minor population of γδT cells (212). In the LP, $CD4^+$ T cells dominate over $CD8^+$ T cells, with the majority of cells exhibiting T_{reg}-like or T_{em} phenotypes (214–217). IL-17 producing $CD4^+$ T cells are most common in the LP of the colon and ileum, with lowest numbers in the jejunum (216), which is inverse to the distribution of T_{reg}:non-T_{reg} T cells observed in mice (215, 217).

DC-T cell composition in homeostasis

Memory T cells

In contrast to skin, sustained CD69 expression is not necessary for T_{rm} formation in the small intestine (7). Further, in the human intestine CD103 is also not necessary for T_{rm}

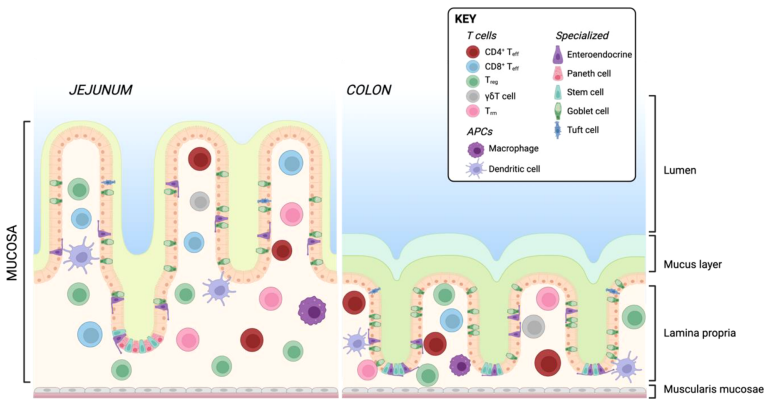

FIGURE 4
Resident T cells and APCs in the human small and large intestine. Created with BioRender.com.

persistence (218, 219), and is higher expressed on $CD8^+$ T_{rm} than $CD4^+$ T_{rm} (216, 220, 221). Human intestinal T_{rm} specifically express CD161, a C-type lectin-like receptor (222, 223), and they share the classic T_{rm} phenotype of downregulating LN homing receptors CD62L and CCR7 as well as the upregulation of adhesion molecules CRTAM and chemokine receptors CXCR6 and low expression of CX3CR1 (224). In the human small intestine, both $CD4^+$ and $CD8^+$ T_{rm} have been described to survive years, with $CD4^+$ T_{rm} exhibiting a T_h1 phenotype upon reactivation (218, 225). In the gut, it has yet to be elucidated if T_{rm} are continuously replenished from circulating T_{cm} under homeostatic conditions or whether the local population proliferates *in situ*, which has so far not been described. The TCR repertoire of $CD8^+$ $CD103^+$ vs. $CD103^-$ T_{rm} has been described to have low clonal overlap, however overlap between $CD103^-$ $CD8^+$ T_{rm} was shown to be similar to that of T cells from the peripheral blood, indicating that $CD103^-$ T_{rm} are recruited from the periphery and represent an intermediate state between circulatory and resident T cells (218). A study utilizing two-photon laser scanning microscopy revealed that intestinal T_{rm} have restricted mobility (226), indicating that intestinal T_{rm} are able to remain at the site of primary infection.

In mice, memory precursor cells expressing low levels of KLRG1 have been identified as a T_{rm} precursor, whose development is accelerated by DC-derived TGF-β (227). Inflammatory monocytes expressing IL-12 and TNF-β have been shown to suppress TGF-β-induced CD103 expression, leading to an increased population of $CD103^-$ LP T_{rm} (228). Additionally, intraepithelial $CD103^-$ T_{rm} appear to preferentially develop from $KLRG1^+$ T cells over T cells that never express KLRG1 (229). Lastly, while IL-15 is critical for T_{cm} and T_{em} maintenance, this cytokine is not necessary for T_{rm} retention in the intestine (230).

Overall, T_{rm} biology and contribution of antigen presenting cells to T_{rm} generation and maintenance in the human intestine still have many open questions. More detailed reviews on intestinal T_{rm} can be found elsewhere (231, 232).

Effector T cells

While at steady-state, DCs in the gut preferentially induce T_{regs}, with T_{eff} cells being primarily induced during infection or inflammation, which has mostly been studied in mice. Intestinal cDCs "escaping" regulatory conditioning in the gut at homeostasis have, however, been shown to induce tonic protective T_{eff} responses. This escape has been proposed to be mediated by early exposure to TLR ligands and pro-inflammatory cytokines, reducing residency time of cDCs and pDCs in the epithelium and thereby limiting exposure to regulatory-inducing factors (233, 234). Another example of this is p38-MAPK signaling in mouse $CD103^+$ DCs, which has been shown to regulate fate-decision between T_{reg} and T_h1 cells from infiltrating naïve T cells by influencing RALDH2 expression required for T_{reg} induction (235). Further, specific TLR5 signaling activating $CD103^+CD11b^+$ cDCs induces IL-6 and IL-23 production which promotes T_h17 development and antimicrobial peptide production (200, 236).

The local microbiota is also essential in inducing T cell subset differentiation and polarization in the gut. In mice, it has been shown that monocolonization with segmented filamentous bacteria (SFB), which are members of the order Clostridiales, can induce the development of LP-resident $CD4^+$ T_h17 cells (237). This selective T_h17 induction is MHC class II-dependent and requires presentation of SFB antigens by resident intestinal $CD11c^+$ DCs (238). The relationship between SFB and T_h17 has further been demonstrated in mice engineered to express the human antimicrobial peptide HBD5. These mice exhibited loss of SFB which subsequently correlated to a lower percentage of T_h17 cells in the lamina propria (239).

Regulatory T cells

T_{regs} are central components of establishing tolerance in the intestine and crucial for maintaining homeostasis. Specifically in the gut, T_{regs} are necessary for controlling pro-inflammatory responses to commensal pathogens as well as establish tolerance to food antigens (240–242). Both thymus-derived (t)T_{regs} and periphery-induced (p)T_{regs} have been described in the gut, with pT_{regs} being thought to play the main role in establishing oral tolerance (243, 244), having been shown to control dysregulated T_h1 responses to food antigens (245). In the colon, the predominant subset of pT_{regs} expresses the T_h17 master transcription factor ROR-γt, the expression of which is dependent on the microbiota (245–248). The ROR-γt^- pT_{regs} conversely are critical for homeostasis maintenance in the small intestine (245). In mice, $Helios^+$ tT_{regs} in the gut express GATA3 and exhibit a tissue-repair phenotype (246, 249, 250). This $GATA3^+$ T_{reg} subset has not, however, been described in humans so far.

TGF-β is an essential cytokine for pT_{reg} differentiation and is, unsurprisingly, present at high concentrations in the intestine (251). DC-derived TGF-β in the gut is essential for local T_{reg} differentiation, which has been demonstrated in mice by ablating expression of the integrin responsible for activation of latent TGF-β ($\alpha_v\beta_8$) on DCs which lead to impaired induction of T_{regs} in the mesenteric LNs (252). Contrarily, deletion of the TGF-βR1 on T_{regs} resulted in normal T_{reg} numbers in the gut (253). However, the authors did not analyze T_{reg} subsets in this study, therefore it cannot be excluded that compensatory T_{reg} expansion was the underlying cause for this observation. Other than cytokines, the metabolite RA is an important contributor to T_{reg} differentiation in the gut. Together with TGF-β, RA has been shown to induce pT_{reg} characterized by upregulation of CCR9 and $\alpha_4\beta_7$ (254–256). Particularly $CD103^+$ DCs are crucial for this induction, as they show a high expression of RALDH2, the enzyme metabolizing vitamin A to RA (257, 258). Particularly development of ROR-γt^+ pT_{regs} is dependent on DC-derived RA (247, 259), further emphasizing that local T_{reg}

induction is crucial to intestinal homeostasis. Other than RA, DCs play a role in T_{reg} induction *via* TLR signaling in the gut. For example, TLR2-mediated recognition of polysaccharide A on the commensal *Bacteroides fragilis* has been shown to trigger induction of T_{regs} and their production of the anti-inflammatory cytokine IL-10 (260).

γδT cells

Intestinal intraepithelial γδT cells play an extensive role in tissue surveillance, having a high migratory capacity and moving through the intestinal epithelium using occludin-mediated cell-cell contact (261). The majority of γδT cells in the human intestine express V7δ TCR (262) and have been associated with intestinal homeostasis *via* the production of keratinocyte growth factor 1 (KGF1) (263). Their significant contribution to gut homeostasis has been shown in γδT cell deficient mice, showing that mice lacking these cells have reduced intestinal epithelial cell turnover (264), increased susceptibility to dextran sulfate sodium (DSS)-induced colitis (263), and increased gut permeability (265). In humans, intestinal γδT expressing NKG2A have been shown to express TGF-β1, thereby dampening IFN-γ and granzyme B production by co-cultured αβT cells from patients with coeliac disease (266). Together, studies so far indicate that intestinal γδT cells have an important role in regulating tissue homeostasis and contribute to controlling inflammatory responses in the gut. However, a lot of open questions about their effector functions and interplay with other cells, such as APCs, in humans still remain.

DC-T cell composition in inflammation and infection

Chronic inflammatory diseases

Inflammatory bowel disease (IBD) is a well-known and well-studied chronic inflammatory condition in the intestine and covers ulcerative colitis and Crohn's disease. IBDs have been linked with multiple exogenous factors such as environmental factors, microbiota dysbiosis, and genetic background (267, 268), which culminate in an overall inappropriate immune cell activation in the gut. In IBD, DCs are known to contribute to disease pathology *via* TLR2/4-induced production of IL-12, IL-6, and IL-23 (269, 270), which further impacts T cell polarization and drives T_h17-mediated disease phenotypes. $CD103^+CD141^+CD1c^+$ cDCs are reduced in inflamed intestinal lesions, showing functional impairments such as decreased RALDH2 activity (271). Further, some findings have indicated that intestinal inflammation, such as seen in Crohn's disease, impairs normal DC trafficking which consequently leads to dysregulated T cell responses in the gut. For example, CCR7 expression on $CD83^+$DC-$SIGN^+$ intestinal cDCs is lower in patients with Crohn's disease (272). Further, it has been observed that leptin production in mesenteric fat is increased in early Crohn's disease patients (273), which has been associated with upregulation of CCR7, maturation and migration of cDCs (274). Whether CCR7 expression is timepoint dependent and what effect this has on T cell priming in Crohn's disease remains to be elucidated.

In recent years, the role of T_{rm} in IBD has become apparent. For example, $CD69^+CD103^+$ T_{rm}-like cells in the LP have been described to be increased in patients with ulcerative colitis and Crohn's disease. Further, the authors could show that increased levels of $CD4^+$ T_{rm} are associated with early IBD relapse (275). Along the same line, Bishu et al. described these $CD4^+$ T_{rm} as functionally competent TNF-α producers in inflamed tissue of patients with Crohn's disease (276). $CD8^+$ T_{rm} have also been implicated in IBD pathogenesis. Bottois et al. described two distinct subsets of $CD8^+$ T_{rm} expressing KLRG1 and CD103, showing that $CD103^+$ $CD8^+$ T_{rm} in Crohn's disease patients exhibit a T_h17-like phenotype, while highly proliferative $KLRG1^+$ $CD8^+$ T_{rm} present with increased cytotoxic effector function and are overrepresented during acute inflammation (277). Single-cell RNA-sequencing studies of ulcerative colitis also showed transcriptional changes in the $CD8^+$ T_{rm} compartment, with an increased inflammatory signature (278, 279). In a recent publication using mass spectrometry, HLA-DR^+CD38^+ $CD4^+$ T_{em} were found to be enriched in lesions of Crohn's disease patients. The authors could further use imaging mass cytometry of tissue sections to show co-localization of memory $CD4^+$ T cells together with HLA-DR^+CD11c^+ DCs located below the epithelial layer in the inflamed regions of the intestine (280). T_{rm} with a regulatory signature have also been described to be reduced in IBD, characterized by $CD103^+Runx3^+$ and expression of the regulatory-associated molecules CD39 and CD73 together with IL-10 production (281). Furthermore, studies revealed a decrease in both the $CD103^+$ $CD8^+$ and $CD4^+$ T_{rm} compartment during active IBD, which recovered during remission phases, whereas the opposite observation was made for $CD103^-$ T_{rm} (282). These studies further demonstrate the heterogeneity of intestinal T_{rm} and are likely a reflection of T_{rm} plasticity during different phases of the inflammatory response.

Infection

While the physical barriers like the intestinal mucus protect against food-borne pathogens and harmful commensals (known as pathobionts), many microbial organisms have evolved to evade host defense and cause infections. Infections with such enteric pathogens are most commonly associated with diarrhea, which is a major cause of death worldwide (283). The most frequent enteric infections are with *Salmonella* spp. and *Campylobacter* spp (284)., with other examples being *Vibrio cholerae*, *Shigella* spp. and certain strains of *Escherichia coli* (285). The most common pathobiont infections are caused by *Enterococcus* spp (286). and *Clostridium difficile* (287).

While TLR2 is important in inducing T_{regs} (see above), TLR5, the receptor for bacterial flagellin (288), has been implicated in the host response to invasive pathogens such as *Salmonella* spp. $CD11c^+$ LP-resident DCs express TLR5, which is important in modulating DC movement, as TLR5-deficient mice have increased survival and lower dissemination when infected orally with *Salmonella* spp. whereas no difference was observed when mice were infected intraperitoneally (289). This observation indicates that trafficking to the mesenteric LN by DCs is impaired thereby preventing dissemination of the infection. Another important consequence of TLR-mediated activation of DCs is cytokine production. A crucial cytokine in the gut produced by DCs in response to infection is IL-23, which has been linked to infection with pathogens like *Salmonella* spp (290)., *C. rodentium* (291), and *C. jejuni* (292). The receptor for IL-23 in the gut is expressed on multiple immune cells such as T_h17, NKT, γδT cells and ILCs (293, 294). IL-23 receptor signaling in turn triggers production of IL-17 and IL-22. IL-17 appears to have time-dependent effects during intestinal infection. During early *Salmonella* spp. infection, IL-17 produced in the caecum is primarily mediated by T_h17 cells and to a lesser extent γδT and NKT cells (295, 296). Another example of the importance of T_h17-mediated immunity during infection has been shown in rhesus macaques where SIV-induced depletion of T_h17 cells leads to erosion of the mucosal barrier and increased dissemination of *S. enterica* Typhimurium to the mesenteric LNs (296). IL-17A or IL-17F deficiency in mice lead to increased pathology in response to *C. rodentium* infection (297).

The IL-23-T_h17 axis is also important in human intestinal infection. Patients suffering from *C. jejuni* infection show increased percentages of T_h1 and T_h17 cells, as well as increased levels of the respective effector cytokines. The authors could show that when intestinal epithelial cells were treated with IL-17A or IL-17F, intracellular survival of *C. jejuni* was significantly decreased, emphasizing the importance of these cytokines in human infection (292). Further, IL-17 expression was also detected in the duodenum of patients recovering from *V. cholerae* infection, the causative agent of cholera. Kuchta et al. observed that in patients suffering from acute cholera, IL-17 expression was increased compared to later disease stages or healthy subjects, suggesting that *V. cholerae* infection also induces an immediate mucosal T_h17 response (298).

The other IL-23-induced cytokine important in intestinal infection is IL-22. In general, IL-22 is associated with tissue repair and is known to be a major inducer of antimicrobial peptide production by mucosal epithelial cells (299, 300). In the context of infection, IL-22 has been found to increase colonization resistance to the pathobiont vancomycin-resistant enterococci (236). Similar to IL-17, IL-22 has also been shown to have time-dependent effects. During early infections, IL-22 is primarily produced by ILCs and only later on by T cells. This was demonstrated by Ahlfors et al, showing that during infection with *C. rodentium* IL-22 is initially produced by ILC3s and then by $CD4^+$ T cells (301).

Overall, it has become clear that the DC-induced IL-23-T_h17 axis is particularly important in response to intestinal infection by modulating epithelial microbial peptide expression and preventing dissemination of intestinal infection.

Female reproductive tract

The immune system in the FRT has a dual role as it protects the barrier tissue against pathogens transmitted during sexual intercourse, and promotes tolerance to foreign antigens necessary to allow fertilization and embryo development. As these two diametrical roles are important at specific times during the menstrual cycle, the composition of immune cells undergoes major fluctuations. During menstruation, a much higher density of $CD1a^+$ DCs was observed in the human uterus compared to proliferative and secretory phase (302). Uterine macrophages increase constantly in numbers during secretory phase and peak at menstruation, while the total number of T cells remains constant (303–305). The sex hormone progesterone does not only inhibit activation of DCs (306), but also causes polarization of T cells into T_h2 and T_{reg} direction (307, 308). Moreover, subsets of immune cells do not only change during the menstrual cycle, but also differ when comparing tissues from pre- and postmenopausal females (309). There are substantial differences between the structure and physiology of the female genital tract between the most frequently used animal model of mice compared to humans, as the murine uterus contains two uterine horns and also the estrous cycle has a length of around 5 days compared to 28 days in humans. However, due to the previously low interest in female reproductive health, scientists started only recently to investigate immune cell populations in large scale in the FRT of humans. Therefore, most knowledge on the female genital immune system was obtained in mice (310). With this section, we aim to shed light on specific features of antigen uptake and presentation as well as T cell responses in the female genital tract and raise awareness for inflammatory conditions and chronic infections.

The female genital tract is structured in several parts: the lower reproductive tract lined with multilayered stratified epithelia forming vagina and ectocervix, the endocervix as an interphase and the upper genital tract with single columnar epithelium forming the uterus, adjacent to the fallopian tubes stretch connecting the ovaries with the uterus which are composed of secretory and ciliated columnar epithelial cells (Figure 5). The main APC subsets in the human vaginal tissue are, similar to skin, in the epithelial layer LCs characterized by CD207 expression and in the lamina propria DCs characterized by expression of CD1c as well as CD14 on a specific subset (311, 312). In addition to DCs, another frequent APC subsets in the vagina are $CD1c^-CD14^+$ macrophages additionally having

CD163 on their surface (311, 312). In the cervix, the most frequent immune cell population are macrophages which make up more than 25% of all $CD45^+$ immune cells (55). $CD11c^+CD14^+$ DCs accounting for another approx. 20% of immune cells are the most common DC subset and a large proportion also express DC-SIGN. Other DC subsets such as $CD11c^+CD14^-$ myeloid DCs and $CD123^+$ plasmacytoid DCs were described in low numbers (55). The percentage of APC subsets within $CD45^+$ immune cells is quite similar in cervix and uterus, however, the APC compartment in the uterine endometrium shows some substantial differences. There are less DC-$SIGN^+$ DCs and DCs expressing $CD103^+$ involved in antigen sampling and migration were almost exclusively found in the endometrium (313). In the murine endometrium, both $CD103^+$ and $CD103^-$ DCs migrate to the local lymph nodes upon antigen challenge. The $CD103^+$ DCs preferentially present antigens to T_{regs}, whereas their $CD103^-$ counterparts were shown to stimulate an effective CD4 T cells response (314). In the murine uterus, DC in the decidua of pregnant females were shown to be trapped in the tissue, despite keeping responsiveness to pro-inflammatory stimuli and migration capacity towards CCL21 (315). This indicates that by preventing DC trafficking to the draining LN, T cell tolerance to fetal antigens is promoted. Single-cell sequencing of human uterine samples during secretory and proliferative phase revealed presence of myeloid cells during both phases, being composed of DCs as well as M1- and M2-polarized macrophages (316). $CD11c^+$ DCs can be further divided into $CD11b^{++}$ and $CD11b^{lo}$ DCs, with the $CD11b^+$ expressing DCs being the most abundant subset in all tissues of the FRT and correlating with CD14 expression (313). In the vagina, the ratio of CD4 to CD8 cells is almost equal, with an increasing ratio towards endocervix and ectocervix (55, 317). In the uterus, however, CD8 T cells represent the predominant subset (55). B and NK cells make up less than 5% of immune cells in the human ectocervix and are not in focus of this review (55).

DC-T cell composition in homeostasis

Memory T cells

In general, most T cells in the female reproductive tract are T_{rm} being $CCR7^-CD45RA^-$. More than 80% of cervical T cells express CD69 within both stroma and epithelium (317, 318). The marker CD103 being associated with a T_{rm} phenotype in other tissues is in the cervix almost exclusively present on epithelial CD8 T_{rm} (318), but also enriched on vaginal CD4 T_{rm} (317). These vaginal $CD103^+CD69^+$ CD4 T_{rm} show a T_h17 signature including high expression of RORC, IL-17A, IL-17F and IL-22 (317). A recent publication used T_{rm} derived from human cervix to assess antigen-specific CD4 and CD8 response against HSV-2 (319). An elegant mouse study using parabiosis models revealed that CD8+ T_{rm} in the mucosa undergo proliferation *in situ* after mucosal rechallenge independently of CD11c+ DCs (114). On the other hand, bystander memory CD8 T cells consisting of T_{cm} and T_{em} are recruited during local challenge without antigen recognition and develop a T_{rm}-like phenotype by upregulating CD69, but not CD103 (114). To investigate how the recruitment of bystander memory cells to sites of infection as well as tissue autonomous amplification of local T_{rm} contributes to immunity in the human FRT, it is important to apply functional models with human cells and validate other experimental approaches in the future. To date, the T_{rm} subset is the best studied immune cell subset in the FRT and will be discussed further in the sections about the respective infectious diseases.

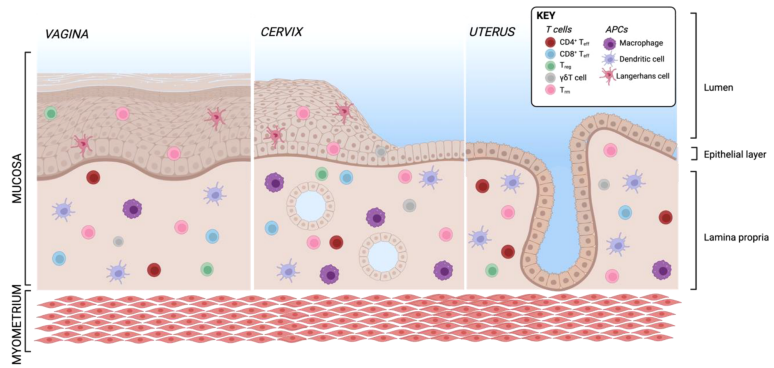

FIGURE 5
Resident T cells and APCs in the human FRT. Created with BioRender.com.

Regulatory T cells

Recently, T_{regs} were shown to make up around 15% of the CD4 population with comparable percentage within all tissues from the lower FRT, including vagina, endocervix and ectocervix (317, 320). It is reported that T_{regs} are induced in the decidua of mice and humans to protect the developing embryo from the immune system of the mother, nicely summarized in the following reviews (321–323). However, T_{regs} can also have an unfavorable role if they dampen the immune response against sexually transmitted infections such as human immunodeficiency virus (HIV), human papilloma virus (HPV) or chlamydia. In a mouse model of intravaginal *N. gonorrhea* infection TGF-β^+ T_{regs} were induced in cervix-draining lymph nodes, thus evading the immune response and enabling pathogen survival (324). The occurrence of T_{regs} in the mucosal tissue is described for several pathogens and conditions, while the mechanisms of their induction still need to be elucidated.

γδT cells

Human studies revealed a γδT cell percentage ranging from 5% to 10% of $CD3^+$ T cells depending on tissue sampling during the proliferative phase or secretory phase. The majority of them expressing Vδ1 (325, 326), but CCR5 can be found on the surface of both Vδ1 and Vδ2 (327). HIV infection significantly reduces the number of γδT cells in the cervix (327). Abnormal vaginal flora due to bacterial vaginosis was shown to change the composition of vaginal γδT cells to higher levels of Vδ2 (328). Beside their role during infection, γδT cells seem to be involved in tolerance induction during pregnancy. The decidua of women with spontaneous abortions showed increased numbers of γδT cells with an additional upregulation of $V\delta2^+$ cells (325). In the murine female genital tract, γδT cells represent a much higher proportion of immune cells and express preferentially IL-17A under steady state (329). As IL-17A was described to be essential for resistance against fungal infection, a murine study revealed that TCRγδ deficient mice are more susceptible to *C. albicans* growth in the FRT (330). To date, our knowledge about γδT cells in the FRT is still limited and remains to be addressed in different disease settings.

DC-T cell composition in infection

Viral infections

CD4 and the chemokine receptors CCR5 and CXCR4 are hijacked by HIV. Beside T cells, this repertoire of receptors is found on all four APC subsets in the vagina in different quantities, indicating a role of these cells during HIV acquisition and transmission to other cell types (311). It was shown that exclusively $CD14^+$ DCs take up HIV virus-like particles and express CCR5 ligands (313). The type-I interferon inducible lectin Siglec-1 expressed on $CD14^+$ DCs was identified to play an indispensable role in HIV uptake and transmission to CD4 T cells which can be blocked by anti-Siglec-1 antibodies (331). As $CD14^+$ DCs are most frequently occurring in the ectocervix, this tissue is highly relevant to study HIV transmission (313, 332). CD4 T_{rm} from the ectocervical region expressing CD69 are characterized by high CCR5, thereby function as a primary target for HIV infection and persistence (333, 334). Numbers of CD4 T_{rm} are significantly decreased in cervix tissue of infected individuals, but increased activation can be observed (333). In the same lines, $CXCR3^+$ T_{rm} in the skin and anal mucosa of HIV infected individuals starting antiretroviral therapy late remain constantly depleted, thereby creating an optimal environment for HPV related cancer development (335). HIV-infected individuals show increased T_{regs} and reduced T_h17 cells, the ratio between these two cell types can be restored by anti-retroviral therapy (ART) (336). The percentage of T_{regs} remained increased even under ART and was associated with a skewed ratio of CCL17/CCL20 in the ectocervix samples of these women (336), indicating that APCs as major source of those cytokines, are causing the disbalance of T cells in these conditions.

Infections with HPV are widespread and almost every human encounters HPV during their life time. There are several different types, with only some of them being transmitted sexually and causing infections that can lead to cancer development in the cervix. Patients with HIV infection possess an increased risk to develop HPV associated cancer with T cells as important players in the course of HPV-related malignancies (337, 338). Upon HPV infection, T cells in the cervix obtain a more activated profile by upregulation of HLA-DR, independent of HIV status of the patients (339). However, in patients with a co-infection of HIV and HPV, lower numbers of CD4 T cells were observed compared to HPV-negative HIV-infected patients (339). In individuals with HPV-associated genital warts, an accumulation of T_{regs} was reported (340). It was shown that T_{regs} are attracted by CCL17 and CCL22, which are mainly produced by $CD1a^+$ LCs and macrophages within the warts, respectively (340). Trafficking of APCs such as LCs is impaired in HPV lesions, as the chemoattractant for (CCL20) and activation pattern of LCs (CCR7, CD80 and CD86) seem to be decreased (341–343). Also, T_h17 cells seem to play a role in progression of HPV-related intraepithelial cervical neoplasia (CIN), as patients with high CIN or cervical cancer exhibit high numbers of T_h17 cell in the blood, which is correlated with high IL-17 levels in the cervix tissue (344). In a study assessing the T cell infiltration in cervical cancer patients, $CD103^+$ CD8 T cells infiltrate the tumors and are associated with good prognosis (345). These findings indicate that a T_h17 and T_{reg} response is correlated with progressive HPV infection, whereas CD8 T cells are beneficial. However, most studies focus on late stages in CIN progression/tumor development and little is known about early processes of HPV infection.

Bacterial infections

Infections with chlamydia are the most common bacterial sexually transmitted infection in humans. However, most of our knowledge of immune reactions during chlamydia infections was obtained in mice, as studying immunity against *chlamydia trachomatis* (Ct) is connected with many difficulties, such as the high number of asymptomatic cases and the development of tolerance instead of immunity when using inactivated bacteria. The later problem was addressed in a mouse model by Stary et al. showing that live and UV-inactivated Ct are taken up by either $CD103^-$ and $CD103^+$ DC subsets, causing priming of immunogenic effector T cells or T_{regs}, respectively (314). In mice, induction of T_h1 cells plays a huge role in conveying protective immunity, whereas stimulation of $CD8^+$ T cells was suggested to play a role in chronical inflammation and cause tissue destruction rather than advancing protective immunity in mice (314, 346, 347). T_h1 polarization initially relies on IL-12 production by DCs, as IL-12 deficient mice had prolonged times of chlamydia shedding (348). In fact, the most important immune mechanism for chlamydia clearance is IFN-γ, as T-bet deficient mice could not control *chlamydia* growth, but T cells shifted to a more T_h17 response, whereas IFN-γ or IFN-γ-receptor deficient mice die from systemic infection (349, 350). T_{rm} of the FRT seemed to be essential to protect against subsequent chlamydia infection (314). However, a recent publication suggests that also circulating memory T cells can protect against infection without being primed in the tissue (351). Apart from conveying protective immunity, T cells can also be involved in undesirable responses causing FRT pathology and chronic inflammation. Especially activation of non-antigen-specific CD4 as well as CD8 bystander cells can exacerbate the pathology in a mouse model of chlamydia infection (352). The presence of T_{regs} was on the one hand shown to exacerbate Ct infection (314), on the other hand, they are described to skew T cell differentiation into a T_h17 direction, which was correlated with increased pathology in a *chlamydia muridarum* mouse model of infection (353). Together, these findings suggest that the T cell response during Ct infection is highly plastic and the induction of a certain cytokine milieu is essential.

Discussion

All the same: Commonalities and differences in tissue APC-T cell crosstalk

When comparing the three different tissues summarized in this review, some overarching themes are apparent: The majority of T cells in tissues are T_{rm} cells (7), closely followed by T_{regs} (19, 20), both cell types reflecting the constant exposure to environmental compounds and antigens in barrier tissues and the need for a balance between immune tolerance and reaction. Further, DC subsets are responsible for controlling this balance, but they are often described by different markers in different tissues and their subsets appear more tissue-specific than those of T cells, whose identity is often easier to define across tissues. However, some clear differences exist also in T cells. Expression of CD69 and CD103, canonical T_{rm} markers in the skin (13, 111) and FRT (317, 318), seem dispensable for T_{rm} establishment in the intestine (7). T_{rm} are relatively stationary within the respective tissue, however, there are quite substantial differences in motility between T_{rm} in different tissues, as T_{rm} in the FRT move up to 5-times faster compared to T_{rm} in skin epidermis, probably depending on the architecture of the tissue and density of the structural cells (114). While $CD4^+$ and $CD8^+$ T_{rm} exist in all discussed barrier tissues, the skin harbors more $CD8^+$ T_{rm} than the intestine and the FRT, where the distribution of $CD4^+$:$CD8^+$ T_{rm} is approximately equal (218, 225). Further, T_{reg} induction in the intestine is highly dependent on RA produced by local DCs (254–256) and in the FRT, progesterone (307), independently of DCs, appears to take a similar role, while no hormones or metabolites are yet identified to induce T_{regs} in the skin. In general, it appears, that while all barrier tissues are continuously exposed to microbial antigens, only the intestine has dedicated DC subsets to specifically induce T_{regs} to promote tolerance against the microbiome (191). This observation fits with the fact that, in the skin, most T_{regs} respond and get activated by non-antigenic stimuli while most T_{regs} (127) in the gut are antigen specific (240–242). In general, aside from their function in maintaining immune tolerance, the function of T_{regs} in different tissues is often diverse, ranging from direct suppression of activated immune cells to aiding in tissue repair (19, 20), thereby emphasizing the need to characterize these cells and their non-canonical functions in a tissue context better. Similar to this, γδT cells exhibit both regulatory and cytotoxic functions across tissues even though their distribution is tissue-specific (Vδ1 in the skin, Vδ7 in the intestine, Vδ1 and Vδ2 in the FRT) (149).

During an immune challenge in barrier organs, such as during infection, T_{rm} are poised locally in all three tissues, reacting to previously encountered antigenic stimuli directly. Further immune responses are induced by APCs which traffic to the respective draining lymph nodes and recruit T_{eff} cells to the tissue. T_h17 responses are crucial in controlling infections, both bacterial and viral (313, 332). Interestingly, the same responses and effector cytokines are also often the ones that are pathogenic in chronic inflammatory diseases (153, 154). How and why exactly these exacerbated immune responses cannot be controlled by tissue-resident T_{regs}, which are present in barrier tissues in great abundance under homeostatic conditions, has yet to be elucidated. However, all chronic inflammatory diseases discussed in this review are characterized by a decrease in tissue T_{regs}, but whether this is cause or effect of chronic tissue inflammation and what role APCs play in this shift of T cell subsets during chronic inflammation remains a big question that should be the topic of further research.

Into the (un)known: On big data, future perspectives, and individualized therapies

Previous dogmas of dividing immune responses strictly into pro- and anti-inflammatory immune cell subsets are outdated. The more we learn about tissue-specific immune responses, the more we understand that there is not the one beneficial and harmful immune cell subset to every disease. It is more a fine-tuned balance act between APCs and T cells to enable immunity against pathogens but protect the host from autoimmunity. With current advances in single-cell RNA sequencing (scRNA-seq) and multichannel flow cytometry, we will be able to get a better insight, which players are involved in regulating immunity during homeostasis. scRNA-seq has specifically enabled much greater insight into molecular mechanisms of tissue immunity as well as led to the discovery of new immune cell subsets or new definitions of existing subsets. This is especially valuable since this approach allows for the acquisition of a large amount of data from, often limited, human material. Further, a lot of information that is derived from these big data experiments would be impossible to acquire using traditional experimental models as it is now possible to also model *in vivo* dynamics from these datasets, such as the interplay between different cell types (354, 355) and temporal dynamics across the development of organs (356–358), and tracking T cell clones across tissues (359, 360). Analyses like these have revealed novel regulatory T cell-APC interactions at the maternal-fetal interface important for embryo implantation (361), a renewed focus on pDCs in skin inflammation (362), novel Vδ1 T cell effector subsets (363), and detailed profiling of different immune niches and interactions across the human intestine (54). Further, a better understanding of tissue adaptation of different immune cells is becoming appreciated, highlighting basic principles of immune biology in barrier tissues but also appreciating that these cells have the potential to specifically adapt to the local tissue environment and how this changes in disease (126, 364–366). As highlighted in this review, communication between different immune cell types is absolutely essential in determining the outcome of an immune response and understanding this interplay at a deeper level in local tissues is an important step towards developing new therapeutic avenues that can act in a much more targeted manner than previously possible. Further, the plasticity of immune cell subtypes, especially APCs and T cells, is becoming more appreciated as having whole transcriptome data can separate cell types that were previously indistinguishable and is an important step towards understanding fundamental changes during disease development. As this knowledge progresses, it will be interesting to see if we will gain a better understanding of responses to immunotherapy and why some patients benefit while others do not. Moreover, this technical evolution will also allow to come away from animal models and help uncover tissue-specific differences as well as overarching themes in immune defense in barrier tissues. In addition, we want to emphasize the importance of investigating the interplay of different human immune cell subtypes in complex 3D model systems to further validate findings from big data-based models and how these can be translated to patient care. It will be crucial to define the function of rare DC subsets, T_{regs} or γδT cells as they seem to have a major role in immune balance despite their low frequencies. Especially the mechanisms balancing different γδT cell subset or T_{regs} and T_h17 cells will be an important focus for further studies. In the future, integrating different large datasets will be highly valuable in better understanding more complex disease systems, such as metabolic dysregulation as well as epigenetic modifications. Together, these data will yield a clearer picture of biological networks and how they are perturbed in different diseases. Currently, we are at the start of a new era of understanding biological mechanisms that lead to disease and disease progression. In the future, insights gained from these basic studies will in turn re-shape how therapeutics are developed and most likely emphasize the importance of more patient-specific approaches to health care.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Immune and inflammatory mechanisms of abdominal aortic aneurysm

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Abdominal aortic aneurysm (AAA) is a life-threatening cardiovascular disease. Immune-mediated infiltration and a destruction of the aortic wall during AAA development plays significant role in the pathogenesis of this disease. While various immune cells had been found in AAA, the mechanisms of their activation and function are still far from being understood. A better understanding of mechanisms regulating the development of aberrant immune cell activation in AAA is essential for the development of novel preventive and therapeutic approaches. In this review we summarize current knowledge about the role of immune cells in AAA and discuss how pathogenic immune cell activation is regulated in this disease.

KEYWORDS

abdominal aortic aneurysm, inflammation, immune cells, cytokines, microbiota, vascular immunology, tissue microenvironment

Introduction

Cardiovascular diseases (CVD) are the leading cause of death globally with an estimated ~18 millions of annual deaths (up to 32% of global deaths) (1) and high prevalence in both high and low income countries (2). Abdominal aortic aneurysm (AAA) is a CVD characterized by abdominal aorta dilatation exceeding the diameter of aorta by 50%, caused by immune cell-mediated inflammation and degradation of the medial layer; eventually followed by aortic rupture and bleeding that is often sudden and fatal. AAA affects about 5% of the population and represent 15th most frequent cause of mortality in the US, where each year ~200,000 people are diagnosed with AAA. Smoking, age (> 60 years old), hypertension, atherosclerosis, and male gender are established AAA risk factors (3–8). Although new potential therapies have been recently proposed for AAA treatment, including nanoparticles loaded with antihypertensive drugs, statins or inhibitors of vascular endothelial growth factor receptor (VEGFR) (9, 10), the current standard of care is still mostly limited to surgery at late stages of the disease (11, 12). Despite significant progress in the

understanding of pathophysiology of AAA (3, 13–16), immune and inflammatory mechanisms controlling this disease pathogenesis only recently started to come to light as a mainstream and pivotal players. Nowadays, chronic inflammation caused by the infiltration and activation of various immune cells is an important driver of AAA (3, 5, 6). Yet, factors regulating immune cell recruitment and activation in AAA remains incompletely understood. Here we discuss recent data on immune and inflammatory mechanisms implicated to the control of AAA development and briefly highlight local and systemic factors impacting immune cell activation in this disease.

Immune cells in abdominal aortic aneurysm

Innate immune cells

Myeloid cells, including neutrophils, monocytes, macrophages and dendritic cells (DC) play diverse and important roles in inflammation, immunity and tissue repair (17). They also contribute to the aortic inflammation and vessel destruction during AAA (6, 16, 18, 19). Early myeloid cell infiltration in the aortic wall is considered to be a hallmark of AAA development both in mice and humans (20, 21), suggesting that these cells could contribute to initial steps of aortic wall destruction (Figure 1).

Neutrophils

Neutrophils, cells of bone marrow origin, are the most abundant circulating leukocytes in the human immune system and the first effector cells to be recruited to the site of injury, infection or inflammation. Neutrophils represent one of the most prevalent cell populations found in the aneurysm and are detected even in early lesions (19). Neutrophils are capable to release different types of granules containing various bioactive molecules such as myeloperoxidase (MPO), neutrophil elastase (NE), defensins, cathepsin G, azurodicin, and endotoxin-neutralizing proteins (22), NADPH oxidase (NOX) and matrix metalloproteinases (MMPs) (23–25). The latter are highly abundant in human and mouse AAA tissues (26–28). Activated neutrophils produce extracellular traps (NETs), a web-like defense structures to trap foreign cells, growth factors, cytokines, proteases

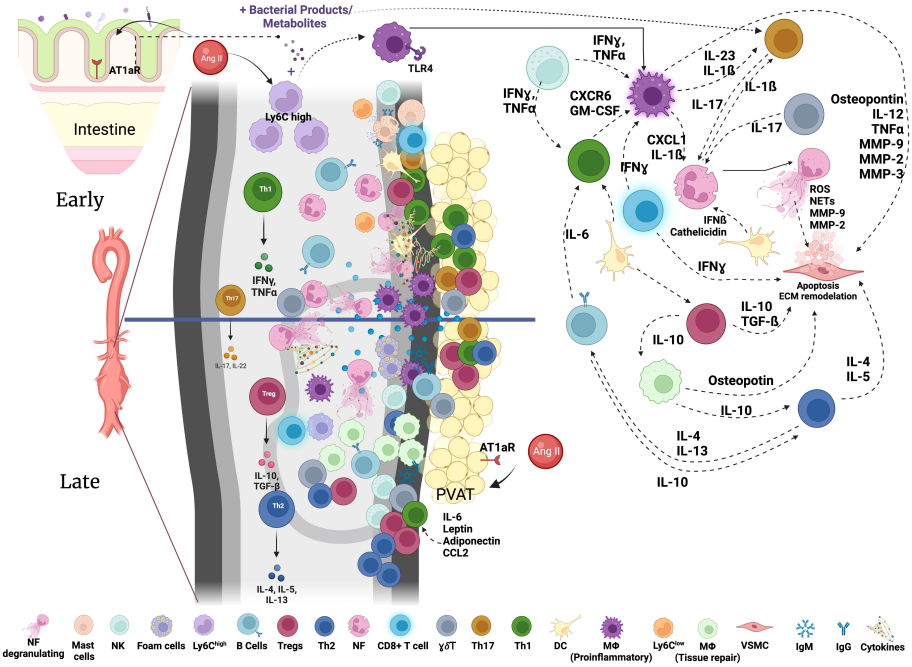

FIGURE 1
Immune networks in aortic abdominal aneurysm. Various immune cells are found in the aorta with AAA. The composition and activation status of immune cells infiltrating the aortic wall during AAA development is dynamic and changes through the course of disease development. Activated immune cells contribute to the inflammatory environment in the aortic wall and VSMC apoptosis resulting in the destruction of the aorta and progressive growth of AAA eventually leading to rupture. NK, Natural Killer; MF, macrophages; Tregs, T regulatory cells; NF, neutrophils; γδ T cells, Th17, T helper 17 cells; Th1, T helper 1 cells; Th2, T helper 2 cells; DC, dendritic cells; VSMC, vascular smooth muscle cells; IgM, immunoglobulin M; IgG, immunoglobulin G; MMPs, matrix metalloproteinases; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, Interleukin; ECM, Extracellular Matrix; AT1aR, Angiotensin II Receptor Type 1; TLR, Toll-like Receptor; PVAT, perivascular adipose tissue; Ang II, Angiotensin II.

and expose them to effector protein (29). NETs are long intersecting fibers consisting of released neutrophil DNA, and histones 3 and 4 (H3 and H4) as well as cytoplasm-derived effector molecules MPO, NE, and cathepsin G (29). Neutrophils play essential and diverse roles in CVD (5, 18, 30–32). NETs production in atherosclerosis is triggered by inflammatory stimuli including LPS and cholesterol crystals (29, 31, 33, 34). Hypochlorous acid generated by MPO oxidizes circulating LDL, contributing to the activation of macrophages and foam cell formation (22). Neutrophil NETs promote inflammation facilitating the activation of Th17 cells and macrophages regulating the release of IL-1β, IL-18 and other pro-inflammatory cytokines (29). Moreover, NETs induce apoptosis of vascular smooth muscle cells (VSMC) leading to the thinning of the fibrous cap and eventual plaque rupture (29). In myocardial infarction (MI), the recruitment and infiltration of neutrophils has been also associated with cardiac damage, but nevertheless neutrophils are needed for healing processes after MI. Neutropenic mice were characterized by increased fibrosis and heart failure because of altered macrophage polarization skewed to a highly inflammatory state with low phagocytic capacity (29).

NLR (Neutrophil to Lymphocyte Ratio) was recently suggested as a prognostic marker for AAA patients, where high frequency of neutrophils in circulation in comparison with lymphocytes predicted a poor prognosis and mortality in patients with ruptured aneurysm (35–38). It has been shown that neutrophils, neutrophil-derived IL-8 and NETs elements were elevated in plasma and tissue of patients with AAA suggesting enhanced neutrophil activation (18, 33, 34). The recruitment of neutrophils is facilitated by CXCL1, CCL2, CCL5 and CXCL8 chemokines (22, 39) that are mainly produced by pro-inflammatory macrophages and are elevated in serum and aorta from humans (40–42) and mice with AAA (43). The depletion of neutrophils or genetic ablation of neutrophil-specific genes (e.g., MPO, MMP-9) attenuates AAA development, suggesting overall pathogenic role for these cells in AAA (3, 19, 44, 45). Several types of neutrophil-derived effector molecules including NETs were detected in the intraluminal thrombus of patients with advanced AAA (23, 31). In elastase mouse model, NETs citrullinated (cit-) H3 and neutrophil elastase were found in the adventitia and at the border of intima and media; and depositions of cit- H3 and H4 were found in the intraluminal thrombus where they co-localized with IL-1β (18). Both Angiotensin (Ang) II and NADPH oxidase-derived ROS were also shown to stimulate NETs formation (38) and inhibition of NETosis by altering the function of PAD enzyme family significantly limits aneurysm development (46, 47). Altogether, these studies imply neutrophils as important inflammatory regulators of AAA. Nevertheless, mechanisms triggering NETosis as well as interaction of neutrophils with other cell types within AAA lesions require further investigation. It also remains to be determined whether neutrophils initiate the destruction of the aortic wall in AAA or simply work as a first responders to the injury driven by some other factors.

Monocytes

Monocytes, originated from the bone marrow, play crucial roles in host defense and contribute to various chronic inflammatory diseases, including CVD (6, 48–60). In humans, three populations of monocytes have been described based on CD14 and CD16 surface expression. Classical monocytes represent up to 90% of circulating monocytes and are characterized by $CD14^{++}$ and $CD16^{-}$ expression, high surface expression of CCR2, CD62L (L-selectin), and low levels of CX3CR1. Non-classical monocytes are characterized by $CD14^{+}$ $CD16^{++}$ surface phenotype, high levels of CX3CR1 and low CCR2. The third population has an "intermediate" phenotype of $CD14^{++}$ $CD16^{+}$ and was suggested to have pro-inflammatory and enhanced phagocytic properties (6). In mice, two homologous populations $Ly6C^{high}$ (classical) and $Ly6C^{low}$ (non-classical) had been described. $Ly6C^{high}$ monocytes are equivalent to human classical monocytes, and were shown to promote inflammatory responses and perform antimicrobial and phagocytic functions. $Ly6C^{low}$ monocytes, corresponding to human non-classical monocytes, are involved in vessel patrolling, immune surveillance and tissue repair. Monocytes had been implicated to the pathogenesis of various CVD, and elevated numbers of circulating monocytes had been associated with atherosclerosis, myocardial infarction and AAA (6, 20). Several studies described changes in circulating monocytes in patients with AAA. While one study showed a reduction of classical monocytes in circulation and augmented proportion of intermediate monocytes, an increase of classical monocytes or no alterations in their presence had been also reported (16, 61–63). In mice Ang II infusion, which heightens blood pressure and drives AAA development, was shown to increase number of circulating $Ly6C^{high}$ monocytes (6, 16), while the administration of angiopoietin 2 reduced circulating $Ly6C^{high}$ monocytes and attenuated AAA (64). Moreover, mice lacking CCR2 were protected from AAA due to the limited recruitment of monocytes to the aorta along with low IL-6 and CCL2 expression (65). The role of CD11b, an integrin subunit expressed on monocytes, but also on macrophages and facilitating the recruitment of immune cells to the site of inflammation, had been investigated in AAA. Higher levels of CD11b on circulating monocytes from patients with AAA compared to healthy subjects have been reported (6, 26). *In vitro* experiments showed that monocytes from patients with AAA are more capable for adhesion and transmigration. However, the knockout of CD11b ($Itgam^{-/-}$) did not significantly affect the incidence of AAA, but nevertheless reduced maximum abdominal aortic diameter, macrophage infiltration, MMP-9 and IL-6 expression, as well as elastin and collagen degradation (66).

Monocytes differentiate from hematopoietic stem and progenitor cells (HSPC); and Ang II was shown to activate

those cells and stimulate myelopoiesis in the bone marrow (20). Another source of monocytes is a spleen where extramedullar hematopoiesis occurs (67, 68). Spleen-derived monocytes were shown to contribute to atherosclerosis, myocardial infarction and AAA (16, 69–71). Recent study demonstrated that in AAA acute mobilization of monocytes from the spleen to the circulation was dependent on Triggering Receptor Expressed on Myeloid Cells (TREM)1 and driven by Ang II *via* AT1R (21). Moreover, TREM 1 was also shown to regulate CD62L expression thereby facilitating monocyte infiltration into the aortic wall during AAA development (21).

The role of non-classical Ly6C^{low} monocytes in AAA had been also suggested in studies utilizing NR4A1 (Nuclear receptor subfamily 4 group A transcription factor) deficient mice. The reduction of Ly6C^{low} monocytes in these animals was associated with augmented AAA and elevated elastin destruction, suggesting potentially protective role of this monocyte subset (72). While these data imply that circulating monocytes play important roles in AAA, a detailed contribution of monocyte subsets, their cooperation with neutrophils and mechanisms controlling monocyte output in AAA remains to be elucidated.

Macrophages

Monocytes recruited to the aortic tissue are capable to further differentiate into macrophages or dendritic cells (DC) (73). Monocytes-derived macrophages are generally classified into "inflammatory" and "tissue repair" subsets, both of which had been implicated to AAA development (3, 6). While the localization of macrophages in AAA had been established (61, 74), macrophage polarization at different stages of the disease requires further investigation. Single-cell RNA sequencing of aortas showed about 5-fold early expansion of pro-inflammatory macrophages in $CaCl_2$-induced AAA, while tissue-repair subset was not affected, suggesting the predominance of inflammatory macrophages (75).

Inflammatory macrophages acquire their phenotype upon activation with a vast variety of stimuli, including LPS, ROS, fatty acids, inflammatory cytokines or local hypoxia in the aortic wall (76–79). Inflammatory macrophages are characterized by elevated expression of pro-inflammatory mediators such as IL-1β, IL-6, TNF, IL-12, IL-23, MMPs, NOS2 and chemokines including CCL2 and CXCL1, in turn regulating the recruitment and activation of other immune cells as well as VSMC apoptosis (6). The expression of these pro-inflammatory molecules is particularly prominent at the advanced stages of AAA. Moreover, Ang II was suggested to promote macrophage activation *via* upregulation of TLR4 (80).

The role of various macrophage-derived cytokines and bioactive molecules had been investigated in multiple studies using pharmacological or genetic approaches, however many studies reported the conflicting results depending on the model used. For example, pharmacological blockade or knockout of IL-1β was shown to reduce AAA in $CaCl_2$ model (81). However, recent study by Batra et al. using the same mouse model of AAA came to the opposite conclusions and showed that *Il1*β$^{-/-}$ *or* *Il1r*$^{-/-}$ mice were not protected from the disease development, and *Il1r*$^{-/-}$ mice develop even larger AAA (82). Serum IL-1β levels were elevated in patients with AAA, which particularly was linked to rs35829419 polymorphism of NLRP3 common allele (83). Indeed heightened expression of NLRP3 inflammasome had been detected in AAA tissue (83). The genetic inactivation of NLRP3, or other inflammasome components (caspase-1 or ASC) reduced the incidence of AAA and ECM degradation in mice infused with Ang II (83). Activation of TLR4 can induce MMP9 expression in VSMC and macrophages, while expression of these entities was reversed in *Tlr4*$^{-/-}$ mice (80, 84). Similar results were observed with TLR4 antagonist, Eritoran (80). Recent study also documented higher TLR4 and MMP9 expression in lymphocytes rather than macrophages in human AAA (85).

Extracellular matrix degradation mediated by MMPs is a hallmark of AAA. Elevated serum MMP9 served as a prognostic marker for AAA (34), and it is known that genetic ablation of MMP9 and MMP2 halts AAA development in $CaCl_2$ model (86, 87). Adoptively transferred WT macrophages promoted AAA growth in *Mmp9*$^{-/-}$ but not *Mmp2*$^{-/-}$ mice, suggesting the importance of MMPs in macrophages and collaborative action between MMP2 and MMP9 (86, 87).

TNF, a major macrophage-derived cytokine (88) was suggested to contribute to AAA in calcium chloride model (82), and its genetic ablation or pharmacological inhibition of TNF limited AAA development (89). At the same time the ablation of its main receptor TNFR1 (p55) in *Ldlr*$^{-/-}$ mice subjected to Ang II infusion did not significantly affect AAA formation, but strongly reduced atherosclerosis (90).

Elevated levels of IL-6, which is presumably myeloid cell derived, had been detected in serum and aortic tissue from patients with AAA (91). The production of IL-6 in aneurysm tissue is directly regulated by Ang II signaling (92); and IL-6 ablation protects from endothelial dysfunction induced by Ang II (93).

The role of IL-12 and IL-23 cytokines in AAA was suggested but different studies reported conflicting results. Antibody-mediated blockade of IL-12p40 at early stages of the AAA reduced aortic diameter and limited macrophage infiltration in elastase perfusion model (75). However, knockout of IL-12p40 resulted in augmented AAA development in Ang II model (94). While the observed difference in phenotypes may be due to different models used, it is important to note that p40 is a shared subunit between IL-23 and IL-12, and therefore genetic inactivation likely affects both cytokines. These data suggest that the results using neutralization or genetic knockout of one of the subunits of heterodimeric cytokines should be interpreted with caution. Moreover, both IL-12 and IL-23 are implicated in the regulation of microbiota, the effect of which has to be considered.

Recent studies identified among $CD11b^+CD68^+Adgre1^+$ macrophages a unique subset marked by Netrin 1 expression. Netrin 1 (Ntn1) is a protein of the laminin family, which was suggested to be involved into the axon guidance and cell migration (95). *Ntn1*-positive macrophages expressed high amounts of pro-inflammatory and pro-angiogenic markers including MMP3, while macrophages with lower levels of *Ntn1* exhibited anti-inflammatory phenotype and expressed high level of macrophage mannose receptor 1 (*Mrc1*) and *Scd1, Cd36, Cydec, Dgat2, Apoc1* genes. Hematopoietic cell-specific Netrin-1 deficiency, meanwhile, prevented AAA formation (95).

Exosomes are lipid bilayer nanoparticles containing RNA and proteins that mediate cell-cell communication. They are produced by macrophages and other cell types as communication tools (96). Increased presence of exosomes has been associated with CVD, including AAA where exosomes were detected in the adventitia, mostly in areas of macrophage accumulation (97). *In vitro* experiments suggest that macrophage exosomes mediate VSMC migration and metabolism by modulating the expression of MMP2 in JNK- and p38-dependent manner. Inhibition of exosome formation by GW4869 reduced AAA progression, preserved elastin integrity and decreased MMP2 expression in a mouse model (96).

Tissue repair macrophages, known to perform tissue surveillance and tissue repair functions, are also implicated in AAA development (98). This subset of macrophages becomes more abundant at the late stages of the disease development, which might represent a compensatory mechanism to prevent further AAA expansion or rectify tissue injury. While Ang II stimulates $Ly6C^{high}$ monocyte infiltration, it was also suggested to regulate the switch from pro-inflammatory to tissue repair macrophage phenotype (99). Also, coagulation factor XIIIa was shown to promote macrophage differentiation toward tissue repair phenotype in the aneurysm (6). Cytokines produced by this subset of macrophages, such as IL-10 and TGFβ, had been shown to play an important protective role in AAA. Increased IL-10 systemic level correlated with reduced AAA diameter and dissection in elastase model in rabbits (100) and $Apoe^{-/-}$ mice infused with Ang II (101). Infusion of recombinant IL-10 promoted smooth muscle cells proliferation in the aorta (100), and systemic induction of IL-10 by its overexpression increased accumulation of $FoxP3^+$ Tregs in aortic tissue reducing the inflammation and diameter of AAA (101). Transforming growth factor (TGF-β) was shown play a protective role in AAA, since antibody neutralization of TGF-β augments AAA severity accompanied by macrophage accumulation in the aortic wall and enhanced ECM degradation (102). VSMC specific deletion of TGFβR2, however, seems to protect from the development of thoracic but not abdominal aneurysms, implying that TGFβ could act through different cell types at different part of the aorta (103).

Tissue resident macrophages. Aortas also harbor tissue resident macrophages, which originate from yolk sac during development (104, 105). These macrophages are also heterogeneous and can be polarized toward anti-inflammatory or tissue repair subsets. In cardiac repair, they play an important role in tissue regeneration and were shown to remove debris, regulate extracellular matrix (ECM), and stimulate cardiomyocytes proliferation (106), but their exact role in AAA has not been fully dissected yet. Single cell RNAseq analysis of elastase-driven AAA and healthy vessels revealed that $CX3CR1^+$ (yolk-sac derived) macrophages are the most abundant subset in healthy aorta representing 62.5% of total macrophage population, while bone marrow derived macrophages ($CCR2^+Ly6C2^{low}F4/80^{low}CD11b^{low}H2\text{-}Aa^{low}$) start to dominate in AAA lesions (107). Another tissue resident subset of $Flt3^+$ macrophages is increased in AAA and expresses pro- and anti-inflammatory cytokines such as CCL3, IL-1β and IL-10 (107), suggesting their contribution to cell recruitment and activation. A trans-differentiation of VSMCs toward a "macrophage-like" phenotype was demonstrated in atherosclerotic disease (108), however the relevance of this mechanism to AAA remains to be determined.

The interplay between monocytes, macrophages and neutrophils could also be implicated to their reciprocal activation during AAA pathogenesis. Early monocytes infiltration in the aortic wall in AAA and differentiation toward inflammatory macrophage subset with the subsequent production of CXCL1 may further facilitate neutrophil recruitment, contributing to the aortic wall destruction. Conversely, neutrophils produce IL-6 known to contribute to pro-inflammatory macrophage activation (6). Moreover, macrophage macropinocytosis was linked to the engulfment of NETs, and a negative correlation between the density of macrophages and NETs in AAA was observed (109).

Dendritic cells

Dendritic cells (DC) are professional antigen presenting cells that link innate and adaptive immune responses (6). DC activate T cells and also contribute to innate immune responses *via* secretion of pro-inflammatory cytokines, including TNF, IL-12, IL-23 and others as well as chemokines (105, 110). Dendritic cells can be divided on conventional DC, plasmacytoid DC, lymphoid DC and inflammatory DC subsets. The latter differentiate from the recruited monocytes at the site of inflammation (111). DC had been detected in AAA (45, 112) and depletion of $CD11c^+$ DC using DTR-driven approaches led to the reduction in maximum diameter of AAA in Ang II-driven model (112). Depletion of DC lowered numbers of circulating $CD44^{high}$ $CD62L^{low}$ effector CD4 T cells, $CD44^{high}$ $CD62L^{low}$ effector CD8 T cells and B cells. Moreover, DC depletion also attenuated SRA matrix degradation by limiting neutrophil elastase activity, resulting in limited elastin degradation and

heightened collagen content (112). Plasmacytoid DC activation in AAA has been linked to NET formation due to their ability to produce cathelicidin and type I IFNs (45). Therefore, DC were suggested to promote lymphocyte and neutrophil infiltration and activation, and regulate matrix content and organization. Nevertheless, the putative self-antigens presented by DC in AAA are not known and mechanisms driving initial DC accumulation and activation in AAA remains to be elucidated.

Mast cells

Mast cells had been detected in AAA lesions in outer media and adventitia, and their number correlated with AAA diameter (113). Mast cells are known to produce proteases such as tryptase and chymase, inhibition of which is explored as a therapeutic approach for AAA in animal models (114). Immunoglobulin E (IgE) is a signature molecule of allergic responses activating FcεR1 on mast cells. *Apoe*$^{-/-}$*Ige*$^{-/-}$ mice infused with Ang-II or treated with $CaCl_2$ were protected from AAA and neutralization of IgE by antibodies reduced AAA formation and inflammation in the aorta (115, 116). Amelioration of the disease was accompanied by limited recruitment of neutrophils and lowered expression of MIP-2a and CXCL5 in AAA tissue (116). One of the suggested mechanisms was *via* TNF produced by mast cells, which was regulated by metalloendopeptidase Meprin-α (Mep1A). Mast cell-derived TNF regulated MMP2 production and VSMC apoptosis in AAA; and the Mep1A deficiency ameliorated the disease (117). These observations provide an important largely unexplored link between allergic inflammation and AAA development and warrant detailed investigation in future studies.

NK and ILC cells

Both Natural killer (NK) and Innate lymphoid cells (ILC) are professional innate cytotoxic cells capable of producing cytokines, such as IFNγ, or cytotoxic molecules, such as FasL (CD95L), perforin and granzymes. They typically act to eliminate infected, stressed, senescent or transformed cells (118). NK cells represent a potent source of inflammatory IFNγ, and their pathologic role in atherosclerosis had been previously suggested (119). Immunohistochemistry and microarray analysis of human AAA tissue revealed elevated presence of NK cells in AAA tissue along with upregulated granzyme B and other cytotoxic markers (120, 121). Hematopoietic deficiency of CD95L, a transmembrane protein regulating cell death or pro-survival pathways (122), significantly reduced AAA formation in $CaCl_2$ model, which was associated with lowered infiltration of macrophages and T cells along with limited MMP-2 and MMP-9 expression (123).

Innate lymphoid cells (ILC) comprise of three major populations (ILC1, ILC2 and ILC3) which are characterized by distinct functions and spectrum of produced cytokines (124). ILCs can be typically found at mucosal surfaces, in the adventitia of arteries, pericardium, adipose tissue as well as liver (124, 125). While ILC1 are known producers of IFNγ, ILC2 represents a critical source of type 2 cytokines such as IL-4, IL-5, IL-9 and IL-13 (125). ILC2 were implicated to the regulation of metabolic homeostasis, obesity, helminth infection and allergic lung inflammation (126–128). In atherosclerosis-prone mice fed with high fat diet (HFD) ILC2 cells were found in para-aortic fat tissue and were characterized by pro-inflammatory gene expression profile (124). Also, NK cells expressing IL-4, IL-5 and IL-13 were associated with the development of AAA in early studies, nowadays would be probably classified as ILC2 (120). NK cell mediated IL-13 production can induce MMP-2, -9, -13 and -14 in pulmonary diseases (129), thereby hinting at its potential role in AAA progression *via* similar mechanisms. ILC3 are RORγt-dependent cells, which produce IL-17A and IL-22 cytokines (130). The role of these cells in CVD only recently attracted attention and was discussed elsewhere (131), while their role in AAA have not yet been examined.

iNKT

Invariant Natural Killer T (iNKT) cells express TCRβ and NK1.1 surface markers. NKT cells recognize non-classical antigens, including lipids, presented in the context of MHC-I and MHC-I-like molecules, including CD1d (132). In vascular diseases, NKT cells has been implicated in the progression of atherosclerosis (57, 133). In human AAA tissue, an increased proportion of activated Vα24Jα18^{+}NKT subsets in the media was reported (134). Elevated presence of iNKT cells in AAA had been also found in *Apoe*$^{-/-}$ mice infused with Ang II, especially after the treatment with α-galactosylceramide (αGC), a synthetic glycolipid that activates iNKT cells *via* CD1d. That correlated with increased incidence of AAA. Histopathological, immunofluorescent staining and RNAseq results also showed more severe infiltration by inflammatory cells in the Ang II+ αGC group (134). Interestingly, opposite results were found in another study, where activation of iNKT cells by αGC attenuated Ang II-mediated AAA in obese ob/ob mice *via* induction of anti-inflammatory macrophage polarization (135). Overall this points out to possible iNKT role in AAA development but complimentary "loss-of-function" experiments are still missing.

Adaptive immunity

T cells

T cells represent a key arm of adaptive immunity and are composed of CD4^{+}TCRβ^{+} (helper) and CD8^{+}TCRβ^{+} (cytotoxic) subsets. Depending on environmental cues CD4 T cells can differentiated toward Th1, Th2, Th17, Th22, regulatory T (Treg,

$CD4^+FoxP3^+CD25^+$) and more recently described Tfh lineages (136), most of which have been found in AAA (93, 105, 137). T helper subsets are characterized by the production of subset-specific cytokines impacting the inflammatory environment at the site of inflammation (138). Degradation of ECM proteins such as elastin and collagen progressing during AAA development is accompanied by $CD4^+$ T cells infiltration (116). Recent study utilizing RNAseq on sorted "bulk/conventional" CD4 T cells revealed that CXCR6/CXCL16 axis is necessary for the recruitment of CD4 T cells to AAA. CD4 T cells were shown to produce GM-CSF, which in turn controls the recruitment and polarization of pro-inflammatory monocytes to the aortic wall through upregulation of CCL2 and activation of IRF5 (interferon regulatory factor 5) (139).

Th subsets: Th1 and Th2

Th1 cells are characterized by production of IFNγ, which plays a pro-inflammatory role in atherosclerosis (140–142). In AAA, however, the role of IFNγ is not clearly defined. Early studies showed that administration of recombinant IFNγ into mice lacking $CD4^+$ T cells promotes aneurysm development (143). However, IFNγ deficiency was also associated with augmented AAA in Ang II–induced mouse model, suggesting a protective role for this cytokine in AAA (7). The proposed mechanism suggests that IFNγ is a regulator of CXCL10 expression in AAA, which in turn controls the recruitment of protective effector T cells (7). However, CXCL10 can also attract NK cells, which are considered pathogenic in AAA because of the production of so-called "type 2" cytokines (IL-4, IL-5 and IL-13) in antigen-independent, innate immune mode manner. Moreover, the neutralization of IFNγ by antibodies did not protect mice from AAA (144). Overall while these observations put IFNγ as an important player in AAA, they warrant further studies of its role at different stages of this disease, mechanisms of its induction, cell specificity of IFNγR signaling as well as cell type specific mechanisms of IFNγ production.

Th2 helper subset is characterized by the production of "type 2 cytokines" such as IL-4 and IL-5; and in that capacity these cells are similar to ILC2 and NK cells. These cytokines contribute to the control of B cell activation and clonal expansion (136, 145). They were shown to suppress early atherosclerotic lesions, however IL-4 deficiency only slightly alters the course of the disease (146, 147). IL-5 deficiency was shown to accelerate atherosclerosis (148). However, in AAA Th2 cells producing IL-4 and IL-5 were suggested to be pathogenic, particularly due to the ability to induce VSMC apoptosis (149, 150). The shift from Th1 to Th2 was associated with AAA augmentation (151). In humans, however, large AAA were characterized by Th1 cytokines profile whereas Th2 response was a predominant in patients with small aneurysms (152). The difficulty to assign a specific role for Th2 cells in AAA is related to the fact that type II cytokines can be also produced by NK cells and ILC2 (153, 154). The specific cellular source of type 2 cytokines had not been explicitly studied in AAA and future studies addressing cell specificity will be important.

Th17 cells

Th17 helper subset is regulated by the transcription factor RORγt and known to produce characteristic cytokines IL-17A, IL-17F and IL-22. Th17 cells are dependent on IL-23, IL-6 and IL-1β cytokines derived from myeloid and epithelial cells (155). Th17 cells play a pro-inflammatory, disease-promoting role in many inflammatory pathologies, including atherosclerosis (156–158) and had been implicated to AAA. IL-17A genetic deletion in elastase model of AAA attenuated the disease development and limited inflammatory cell infiltration (8). Similar phenotype was also observed in the Ang II-infusion model, where genetic and pharmacological neutralization of IL-17 or use of RORγt antagonist limited the disease (91, 159). Conversely, SOCS3 (suppressor of cytokine signaling 3) overexpression and reduction of IL-17A expression accelerated AAA (160). It is important to note that SOCS3 has multiple downstream targets beside IL-17A, for instance IL-10, which has its own, protective function in AAA. As Th17 cells expansion is driven by IL-23, its genetic and pharmacological ablation mitigated AAA, which was associated with reduced IL-12p40 production and lowered MMP expression (94).

Overall, more mechanistic studies better dissecting cell type specific responses are needed to elucidate the relative contribution of Th1 versus Th2 versus Th17 or other subsets of CD4 T cells in comparison with other cell types producing similar cytokines in AAA (161).

Regulatory T cells

Tregs are professional suppressors of immune and inflammatory responses known to inhibit the activation of other T cells and innate immune cells, thereby controlling the inflammation, autoimmunity, and anti-tumor immunity (162). Tregs had been detected in aortic tissue and their protective role in atherosclerosis had been demonstrated in multiple studies (163, 164). Tregs were also implicated to AAA pathogenesis by suppressing inflammatory cell accumulation (mainly macrophages and T cells) and proinflammatory molecules expression including CCL2, IL-6 and ICAM-1 (165, 166). Prostanoids and eicosanoids are essential inflammatory mediators associated with AAA development, and cyclooxygenase COX2, an enzyme regulating the conversion of arachidonic acid to prostanoids and eicosanoids, expression is upregulated in patients with AAA (167). Tregs can suppress COX2 expression by myeloid cells, thereby limiting AAA (168).

T cell co-inhibitory molecule cytotoxic T lymphocyte associated antigen-4 (CTLA4) is known to act as a potent negative regulator of immune responses (169). Overexpression of CTLA4 in CTLA-4 transgenic *Apoe*$^{-/-}$ mice fed with WD and

infused with Ang II limited AAA incidence by 66%, reduced the diameter of abdominal aorta and mortality by 26% (110). These effects led to lowered number of accumulated CD4 T cells and downregulated expression of CD80 and CD86 (ligands for CTLA-4) on $CD11c^+$ dendritic cells in lymphoid tissues. CD11c depletion led to reduced accumulation of macrophages and CD4 T cells, attenuating aortic inflammation, preserved vessel integrity, and decreased AAA and aortic rupture (110). In atherosclerosis Tregs in the aorta were shown to lose their suppressing anti-inflammatory properties converting to pro-inflammatory subsets. The conversion was mediated by the environment in atherosclerotic plaque characterized by local hypoxia, dyslipidemia and overproduction of pro-inflammatory cytokines. During atherosclerosis development Tregs were shown to lose FoxP3 (Treg specific transcription factor) expression, thereby switching to exTregs and upregulating transcription factors typical for other Th subsets, for instance to Th1 or Tfh (follicular helper cells) (170–173). It remains to be determined whether such conversion also takes place in AAA.

Pharmacological treatment with statins has been widely used in CVD, in part due to their immunomodulatory properties. Simvastatin and Treg depletion with anti-CD25 antibody in *Apoe*$^{-/-}$ mice subjected to Ang II infusion lowered the incidence and severity of AAA accompanied by reduced VSMC apoptosis and ROS production in the aortic wall (174). In patients with AAA receiving simvastatin, the levels of Ang II signaling marker caveolin-1 and Nrf2 activation were decreased (175), while protective eNOS expression was increased (176), suggesting its beneficial effect in AAA.

T follicular helper cells and T follicular regulatory helper cells

T follicular helper cells (Tfh) are localized in the germinal centers of secondary lymphoid organs where they regulate antibody class switching in B cells thereby controlling humoral immunity. Tfh are characterized by the expression of transcriptional factor Bcl-6 (B cell lymphoma 6) as well as CXCR5 and PD-1 (programmed death-1) (177, 178). A circulating subpopulation is characterized by CXCR3 and CCR6 expression and can be divided on cTfh1 ($CXCR3^+CCR6^-$; producing IFNγ), cTfh2 ($CXCR3^-CCR6^-$; secreting IL-4, IL-5, and IL-13), and cTfh17 ($CXCR3^-CCR6^+$; producing IL-17A and IL-22) subsets (179). Tfh cells have been implicated to the regulation of autoimmune and inflammatory diseases including CVD. Their presence had been detected in the aortic wall (180) and CXCR3+ Tfh cells were found elevated in atherogenic environment (177, 178). Moreover, decreased frequency of cTfh1 and increased frequency of cTfh2 and cTfh17 had been described in patients with atherosclerosis compared to healthy controls (179). The genetic ablation of Bcl-6 in $CD4^+$T cells slightly reduce atherosclerotic plaque size in *Apoe*$^{-/-}$ mice (178).

T follicular regulatory helper cells (Tfr) are Tfh cells that also express FoxP3 as well as IL-10 and TGFβ. Tfr cells were shown to suppress the activation of Tfh cells upon adoptive transfer to *Apoe*$^{-/-}$ mice causing marked decrease of Thf population along with atherosclerotic plaque size (181). However, the role of these cells in AAA is yet to be investigated.

CD8 T cells

Much less is known about the contribution of CD8 cytotoxic T cells to AAA development. Early studies found $CD8^+CD28^-$ IFNγ producing T cells in AAA tissue and in circulation. Besides, a population of CD8 T cells lacking CD27 (that allows accumulation of CD8 T cells in tissues) was detected in human AAA lesions but not peripheral blood, suggesting a potential unique role of this subset of CD8 T cells in AAA (182). More recently, the role of CD8 T cells was assessed in the elastase model of AAA utilizing *Cd8*$^{-/-}$ animals and transgenic CD8 T cells. The study suggested that CD8, but not CD4 T cell derived IFNγ activates MMP9 and MMP2, thereby enhancing AAA development (183).

γδ T cells

γδ T cells is a subset of T lymphocytes that can directly recognize antigen without APC and produce IL-17 and IFNγ. γδ T cells were detected in atherosclerotic aortas and were suggested to regulate neutrophil activation in IL-17 dependent manner (184). In humans, no difference in proportions of CD4, CD8, and $γδ1^+$ T cells were detected between aneurysm tissue and PBMCs (185). At the same time $γδ2^+$ T cells were found in greater numbers in aorta, and the frequency of Tregs was significantly lower in AAA compared to PBMCs (185). The number of CXCR5 expressing $Vδ2^+$ T cells was significantly increased in aneurysm tissue compared to normal aorta or PBMCs from patients with aneurysm. Moreover, the frequency of IL-$17A^+$ cells in AAA was significantly higher among $γδ2^+$ T cells compared to CD4 or CD8 T cells. Importantly, IL-17A-producing $γδ2^+$ T cells were found only in the aortic tissue, implying their potential role in the progression of AAA (185). In experimental model, γδ T cell deficiency inhibited the inflammatory response in the aorta and attenuated AAA, suggesting overall pro-inflammatory AAA-promoting role for γδ T cells (184).

B cells

B cells represent another key arm of adaptive immunity performing their functions *via* antibody and cytokine production as well as antigen presentation. Two types of B cells had been described: B1 and B2 (186). B1 cells originate from fetal liver and in adult organisms mostly reside in the abdominal

cavity. They are active producers of IgM antibody with predominant specificity to different components of bacterial products and phospholipids; their activation is mostly T-cell independent. B1 cells control atherosclerosis by the production of LDL specific IgM antibodies, which suppressed inflammatory macrophages polarization and foam cell formation (186). B2 cells develop in bone marrow and differentiate to antibody-producing plasma cells upon antigen exposure and help from T cells. B2 cells produce different flavors of antibodies, including IgG, IgA and IgE, as well as cytokines IL-10 and IL-6 (186), and can further promote Th1 and Th17 cell responses. IgE was shown to activate CD4 T cells and macrophages through FcεR1 receptor recognizing IgE (4). In atherosclerosis, high fat diet (HFD) was shown to enhance the activation of T cells by augmenting cDC production, a mechanism in part mediated by B cell derived GM-CSF (186). In addition, atherosclerotic plaque is a source of endogenous TLR ligands that might promote B cell activation (186).

The role of B cells in AAA had been also suggested (187, 188). B cells had been detected mostly in adventitia in both human and mouse AAA (5, 187, 189). Ablation of B cells by knockout of muMT (IgG heavy chain) or cell depletion by anti-CD20 reduced AAA development in elastase and Ang II infusion models, which was associated with an increased presence of Tregs (187, 189, 190). B cell-deficient muMT mice were presented with reduced expression of MMP9. Furthermore, inhibition of key B cell receptor signaling molecule Syk suppressed AAA growth, reduced inflammatory response and limited immunoglobulin deposition in AAA (190). This overall suggests that B2 cells promote AAA. B cell accumulation in $CaCl_2$-induced AAA at various stages of the disease progression does not significantly change during the disease progression, however, IgG and IgM had been detected in AAA with a peak at 1 week after the $CaCl_2$ perfusion (190). Interestingly, B cell activation and production of autoantibodies such as anti-Hsp70, anti-Hsp65, or anti-AT1R occurs in humans and rodent models of hypertension (191), suggesting a potential role of Ang II in B cell activation. BAFF, a member of the tumor necrosis factor family of cytokines, drives the differentiation of B cells and is a critical survival factor for mature B cells. Recent studies demonstrated that blockade of BAFF receptors in elastase perfusion model attenuated AAA (170). The antagonist of BAFF depleted most of mature B cell subsets in spleen and circulation, decreased infiltration of B cells to the aorta, along with proinflammatory macrophages, and reduced number of apoptotic cells in AAA. The study also reported that in AAA tissue, B cells and macrophages were found in close contact (188, 190), suggesting a possible role of B cell-macrophage communication in AAA pathology. Future studies will be needed to elucidate the mechanisms regulating B cell activation in AAA, role of specific antibodies, including IgA as well as contribution of B1 cells and IgM to AAA pathogenesis.

Taken together, most of the immune subsets have well established or suggested function in AAA which is further summarized in Table 1, along with the information of genetic or pharmacological tools helpful to ascertain the role of various cells in AAA promotion or inhibition.

Mechanisms that regulate immune cells in AAA

While various immune cells had been detected in aortic tissue and multiple study attempted to address the mechanistic role of these cells in AAA, the upstream mechanisms controlling immune cells activation and accumulation in AAA are still under investigation. Despite previously established roles of listed below factors in immune cell activation in other diseases including CVD, one can hypothesize and extrapolate it to AAA to suggest how these factors influence immune activation and function in AAA and where spatially the activation of immune cells occurs (Figure 2).

Angiotensin II

High blood pressure is one of the key risk factors for AAA. RAS (Renin-Angiotensin-Aldosterone System) controls vasoconstriction and blood pressure. Moreover, RAS was also implicated in the regulation of cell growth and vascular wall integrity influencing many cellular processes (192). Ang II is one of the key enzymes of RAS. Ang II acts through its receptors AT1R and AT2R to regulate cardiovascular remodeling. Ang II also shares some of the signaling pathways with growth factors, promoting growth of cardiac myocytes, fibroblasts and vascular smooth muscle cells (VSMCs) *via* MAPKKK pathway (193, 194). AT1Rs are expressed by vascular, endothelial cells and various immune cells (13) suggesting Ang II involvement in their activation. Multiple studies demonstrated the effect of Ang II on immune cells. For example, Ang II was shown to induce a pro-inflammatory program in THP-1 macrophages *in vitro* (195). Moreover, interaction between Ang II and core clock gene Rev-erbα in macrophages had been proposed, which through the AT1R/LXRα pathway was implicated to the control of MMP9 expression (196). Furthermore, Ang II was shown to control not only mature immune cells but also hematopoietic stem and progenitor cells (HSPC) leading to enhanced myeloid differentiation and myeloid cells production (197).

Recent work identified cytokine-dependent mechanisms that cooperate with Ang II to induce stress myelopoiesis and AAA (198). Specifically, IL-27R signaling was shown to potentiate the response of HSPC in the bone marrow to Ang

TABLE 1 The role of immune cells in AAA.

Cell type	Target	Intervention	Effect on AAA	AAA model	Ref
Neutrophils	Neutrophil depletion	Anti-PMN	⬇	Elastase	(19)
	Inhibition of NETosis	Losartan	⬇	*Ex vivo*	(38)
		Cl-amidine, YW3-56	⬇	Elastase	(46, 47)
Monocytes /Macrophages	Ly6C^{high} monocytes	Ang II infusion	⬆	Ang II	(6, 16)
		Angiopoietin-2	⬇	Ang II	(64)
	CCR2	*Ccr2*$^{-/-}$	⬇	Ang II	(65)
	Ly6C^{low} monocytes	*Nr4a1*$^{-/-}$	⬆	Ang II	(72)
	CD11b^{+} cells	*Itgam*$^{-/-}$	⬇	$CaCl_2$	(66)
	MMP9	*Mmp9*$^{-/-}$	⬇	$CaCl_2$	(87)
	MMP2	*Mmp2*$^{-/-}$	⬇	$CaCl_2$	(87)
	TNFα	*Tnf*$^{-/-}$, TNFα antagonist (infliximab)	⬇	$CaCl_2$	(89)
	TNFR1	*Tnfr1*$^{-/-}$	⬇	Ang II	(90)
	Macrophage exosomes	GW4869 (exosome inhibitor)	⬇	$CaPO_4$	(96)
	IL-23	*Il123*$^{-/-}$, anti-IL-23 antibody	⬇	Ang II	(94)
	Netrin 1	*Ntn1*$^{-/-}$ in hematopoietic cells	⬇	Ang II	(95)
	NLRP3, ASC or Caspase-1	NLRP3$^{-/-}$, ASC$^{-/-}$ or caspase-1$^{-/-}$	⬇	Ang II	(83)
Macrophages/Neutrophils/DC	IL-1	*Il1β*$^{-/-}$, anti IL-1*β*	⬇	$CaCl_2$	(81)
		Il1β$^{-/-}$, *Il1r*$^{-/-}$	⬆	$CaCl_2$	(82)
Macrophages/DC	IL-12p40	Anti IL-12p40 antibody	⬇	Elastase	(75)
			⬆	Ang II	(94)
Monocyte/Macrophages/ CD4T Th1	TLR4	*Tlr4*$^{-/-}$	⬇	Ang II	(80)
		Eritoran (drug), TLR4 signaling suprresion	⬇	Ang II	(80)
Macrophages/ Treg	TGF-β	*Tgfbr*$^{flox/flox}$ Acta2-CreER (smooth muscle specific)	⟷	Ang II	(103)
		AntiTGFβ antibody	⬆	Ang II	(103)
	IL-10	rIL-10 infusion	⬇	Elastase	(100)
		IL-10 systemic induction with minicircle vector transfection	⬇	Ang II	(101)
Monocytes/Macrophages/ Neutrophils/ CD4T cell Th17	IL-6	*Il6*$^{-/-}$	⬇	Ang II	(93)

(Continued)

TABLE 1 Continued

Cell type	Target	Intervention	Effect on AAA	AAA model	Ref
Dendritic cells	CD11c+ cells	*Cd11c*DTR	⬇	Ang II	(112)
Mast Cells	IgE	*Ige*$^{-/-}$, anti-IgE	⬇	Ang II	(116, 117)
				$CaCl_2$	
	Meprin α	*Meprin1a*$^{-/-}$	⬇	Ang II	(117)
Th1/CD8/NK/ILC1/γδ T cells	IFNγ	*Ifng*$^{-/-}$	⬆	Ang II	(7)
		Anti IFNγ	⟷	Ang II	(150)
CD4T Th2/ILC2/NK	IL-4	Anti IL-4	⬆	Elastase	(150)
Th2/NK/ILC	IL-5	Anti IL-5	⬆	Ang II	(149)
Th17/ILC3	IL-17A	*Il17a*$^{-/-}$	⬇	Elastase	(8)
		Il17a$^{-/-}$ and anti IL-17A	⬇	Ang II	(8, 159)
Tregs	CTLA4	CTLA-4 Tg	⬇	Ang II	(169)
	CD25	anti CD25(PC61) antibody	⬇	Ang II	(174)
CD8 T cells	CD8	*Cd8*$^{-/-}$	⬇	Elastase	(183)
		*Cd8*Tg	⬆	Elastase	(183)
γδ T cells	γδ Tcells	*γδTcr*$^{-/-}$	⬇	Elastase	(184)
B cells	B2 cells	muMT$^{-/-}$	⬇	Elastase	(190)
		Anti CD20	⬇	Ang II	(190)
		Anti BAFF	⬇	Elastase	(188)

II. The ablation of IL-27R in mice infused with Ang II protected them from AAA (198). Mitigation of aneurysm development was associated with blunted accumulation of myeloid cells in the aorta due to attenuation of Ang II-induced HSC expansion. Mechanistically, IL-27R signaling was required to induce transcriptional programming to overcome HSC quiescence and increase differentiation and output of mature myeloid cells in response to stress stimuli to promote their accumulation in the diseased aorta (198). It is conceivable that other cytokines (such as IL-1 and IFNγ) may also conspire with Ang II to enhance emergency myelopoiesis from BM during AAA development.

Spleen was shown to play an important role as a reservoir for extramedullar hematopoiesis (199), that had been previously linked to atherosclerosis development (200). The mobilization of both Ly6C^{high} and Ly6C^{low} monocytes from the spleen in response to Ang II had been reported (16). B cells were suggested to regulate early monocyte mobilization and promote macrophage accumulation in the AAA through mediation of extramedullary hematopoiesis. Splenectomy prior to Ang II infusion inhibited early monocyte mobilization and protected from AAA (16).

Cells of adaptive immunity also express ATRs. It has been reported that Ang II stimulation activated inflammatory phenotype in T cells and facilitates their infiltration to adventitia and perivascular adipose tissue (PVAT) as well as into the heart (191). The modulation of adaptive immune activation in hypertension has been attributed to target organ oxidative stress and was suggested to be sex dependent (191). Although effects of Ang II on T cells have been explored, the role of Ang II in the regulation of B cells in AAA is poorly understood despite AT1R being expressed on B cells.

Ang II was suggested to affect the composition of gut microbiota, and therefore a possible crosstalk between microbiota-inducing cytokines and Ang II during AAA development should be taken into the consideration (Figure 2). Systemically, Ang II was shown to modify plasma and fecal metabolites in conventionally raised mice vs germ-free

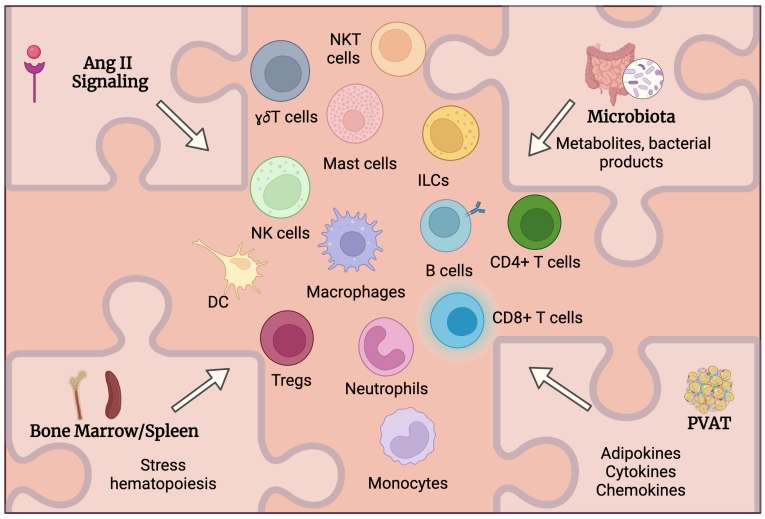

FIGURE 2
Potential mechanisms regulating immune cells activation in AAA. Multiple factors may systemically and locally regulate immune cells activation in AAA. Angiotensin (Ang) II receptors are expressed on various immune cells and can directly regulate their activation and function. Alteration of intestinal barrier will impact microbiota composition and function leading to changes in circulating metabolites and microbial products that in turn may regulate immune cell activation in AAA. Ang II as well as microbiota-derived products may stimulate immune cell mobilization from bone marrow and spleen. Perivascular adipose tissue (PVAT) may contribute to the inflammation in the aortic wall *via* the production of adipokines, cytokines and chemokines regulating immune cells accumulation in PVAT.

(GF) animals, suggesting the role of Ang II in the regulation of intestinal epithelial cells and microbiota (201), possibly in a sex-dependent manner (201). Despite multiple studies convincingly demonstrating an important role of Ang II in AAA (13), additional work is needed to better understand the cell specificity of Ang II signaling in this disease.

Microbiota in CVD

Diet, inflammation, aging and increased bone marrow (BM) myeloid cell output all contribute to the development of CVD (102, 202). Microbiota is a common facilitator for processing and metabolizing food, inducing inflammation and regulating BM output. A connection between unhealthy diet, alterations in bacterial composition in the intestine and CVD has recently emerged (203). The relationship between dysbiosis and obesity has been suggested, supporting the emerging view that gut microbiota contribute to metabolic disease by modulating host metabolism (202, 204–206). Germ free (GF) mice are resistant to diet-induced obesity, with some mechanisms of microbiota modulating inflammation and lipid metabolism suggested. GF mice are characterized by reduced adipose tissue inflammation, while the presence of gut microbiota increases macrophage content in the fat with a polarization toward pro-inflammatory phenotype (207, 208). Metabolic alterations that contribute to atherosclerosis and possible contribution of gut microbiome to this disease development *via* altered production of microbial- or food-derived metabolites have been also reported (208–212), however more mechanistic studies are needed to demonstrate causative rather than correlative effect.

The composition of the diet was shown to regulate barrier function of the intestine. Hence, intestinal permeability and alterations in the microbial community in the gut are affected by high fat, high carbohydrate "Western diet" and can cause translocation of bacterial products and metabolites, further impacting CVD development (213–218). Moreover, microbiota-derived factors can modify activation state of intestinal epithelial cells (IEC) and immune cells. For instance, high fiber diet induced favorable changes in microbiota, and played a protective role in the development of atherosclerosis by controlling acetate (short chain fatty acid) production. Its effects were accompanied by downregulation of *Egr1*, a master regulator gene involved in cardiac hypertrophy, cardiorenal fibrosis, and inflammation (210). On the other hand, dietary serves as a substrate for microbiota-catalyzed overproduction of di- and tri-methylamines. Trimethylamine-N-oxide (TMAO) is a metabolic derivative of L-carnitine and choline; found to be upregulated in patients with CVD, and its serum levels correlate with higher risk of myocardial infarction and atherosclerosis development (211, 219).

Microbiota and its products also has been shown to influence hematopoietic stem cell (HSC) differentiation and BM output (220). At the same time, WD was suggested to impact epigenetic reprogramming of granulocyte and monocyte precursor cells in NLRP3 inflammasome dependent manner,

skewing bone marrow cell differentiation toward myeloid lineages and enhancing so-called "trained immunity" in atherosclerosis. This reprogramming is maintained even after the switch to chow diet, showing long lasting "trained immunity" induced by WD along with enhanced production of myeloid cells which are later on recruited into CVD lesion sites (52).

Single-cell RNA sequencing of human AAA tissues revealed increased expression of histone demethylase JMJD3 in aorta infiltrating monocyte and macrophages, resulting in reduction of repressive histone methylation H3K27me3 marks on promoters of inflammatory genes and concomitant upregulation of inflammatory gene expression. *Jmjd3* expression was shown to be controlled by IFNβ/JAK/STAT pathway and led to NF-κB-dependent induction of inflammatory gene transcription in aorta-infiltrating macrophages contributing to vascular inflammation (221). These results suggest that epigenetic modifications could play a modulatory role in the regulation of inflammatory environment in AAA.

Microbiota and AAA

While many studies are focused on the connection between alterations of microbiota and atherosclerosis, its contribution to AAA is far less understood. Change in gut microbiota composition is linked to hypertension in rodents and humans (202, 207, 222). The relationship between microbiota diversity and AAA severity in humans has been recently reported, and changes in microbiota composition correlated with AAA presence and size (223). Specifically, a decrease in relative abundance of Bacteroidetes and increased relative abundance of Proteobacteria had been reported in patients with AAA (223). The relation between AAA size and α-diversity index was inverse, and severity had a positive correlation with increased relative abundance of Enterobacteriaceae and decreased abundance of Veillonellaceae (223). The reduction of the Verrucomicrobia (particularly represented by *Akkermansia)* was detected in mice infused with Ang II (224).

Germ-free (GF) mice infused with Ang II were characterized by lower neutrophil infiltration into the aorta, attenuated cardiac and kidney inflammation, fibrosis, and systolic dysfunction (207). Accordingly, attenuated leukocyte adhesion, lowered infiltration of $Ly6G^+$ neutrophils and $Ly6C^+$ monocytes into the aortic wall, limited endothelial dysfunction and reduction of blood pressure were observed in GF mice subjected to Ang II infusion, indicating possible contribution of gut microbiota to immune cells activation in response to Ang II (207). A link between normal vascular function and microbiota was directly proven by microbiota depletion (225). When young GF mice were analyzed for vascular contractility and structure, males and females showed differential response: males showed a marked decrease in contraction of arteries and increased vascular stiffness, while females showed hypertrophic remodeling. Also, ROS generation by neutrophils was blunted in female GF mice and exacerbated in male GF mice (225).

While several microbiota-derived products and metabolites had been implicated in the regulation of immune cells activation in CVD as described above, most of the studies to date had been focused on atherosclerosis. It remains to be determined whether similar mechanisms are also specifically involved in the pathogenesis of AAA. Here we will briefly discuss microbial metabolites and their role in CVD and possible contribution to AAA.

LPS

LPS is an obligatory component of gram-negative bacteria wall. Elevated levels of LPS can be detected in the circulation as a result of altered gut barrier and increased gut permeability and alteration of microbiota composition. Unhealthy diets can induce gut dysbiosis and alter gut barrier function, thereby contributing to elevated LPS in the circulation. LPS promotes activation of myeloid cells *via* TLR4/MyD88 pathway, systemic inflammation and monocyte infiltration to vascular wall (226–228). The effect could be further exacerbated by combined action with other cardiovascular disease modifying factors. Hence, LPS in combination with oxLDL was shown to induce NLRP3 inflammasome activation and IL-1β production by macrophages that contribute to atherosclerosis development (52). LPS induces various pro-inflammatory cytokines production including IL-6, TNF as well as Osteopontin (Spp1) by macrophages (52, 227, 229, 230). Moreover, LPS together with oxLDL was shown to inhibit cholesterol transporters ABCA1 and ABCG1, which in turn affects reverse cholesterol transport (231). Furthermore, LPS together with TMAO had been demonstrated to enhance *Spp1, Il1b*, and *Cd36* gene expression in the aorta (229).

In vitro study comparing monocyte-derived macrophages from AAA patients and matched controls showed limited response to LPS due to TLR4 cytosolic internalization, which may be a sign of diminished inflammatory responsiveness, but also may reflect an excessive LPS signaling which resulted in "LPS tolerance" (232).

TMAO

TMAO (trimethylamine-N-oxide) is produced in the liver from choline and L-carnitine dietary-derived metabolite TMA (trimethylamine) whose production itself requires gut microbiota. TMAO production has been associated with the presence of specific bacterial taxa in the gut, for example *Prevotella spp* (202, 211, 233). Elevated serum TMAO was found in individuals with CVD and was implicated into atherosclerosis and thrombosis (218, 229, 234–240). In experimental models L-carnitine or choline supplementation to mice led to the upregulation of TMAO and augmented atherosclerosis acting by reducing *in vivo* cholesterol reverse

transport and modifying microbiota (202, 233). Moreover, no upregulation of TMAO was detected in GF mice despite L-carnitine supplementation, thus no augmentation of atherosclerosis was found (211), implying an important link between intestinal microbiota, TMAO production and CVD. TMAO had been also implicated into the regulation of platelet hyperreactivity and thrombus formation and these parameters were reduced in the absence of microbiota in GF mice (208).

The bacterial fermentation products such as lactate and acetate had been shown to regulate storage and metabolism of lipids in intestinal epithelial cells *via* control of β-oxidation and PPARα pathways (209). However, not all studies demonstrated a notable effect of microbial products on CVD. For example, one study reported that choline supplementation or WD feeding in conventionally raised and GF mice led to a minor dysbiosis in mice, but the effects on atherosclerosis were only driven by cholesterol levels in plasma (212). The role of TMAO in AAA has been recently suggested. TMAO added to drinking water promoted AAA development in Ang II and $CaCl_2$ mouse models (241). This was accompanied by heightened elastin degradation and upregulation of ROS, MMP-2 and 9 and senescence markers in the aorta (241).

Bile acids

Multiple members of gut microbiota including *Clostridium, Bifidobacterium* and *Lactobacillus* participate in bile acid metabolism through bacterial bile-salt hydrolase (BSH) activity, necessary for the bile acid deconjugation and formation of free bile acids and taurine residues (242). Decreased bile-salt hydrolase in dysbiotic conditions had been linked to enhanced foam cell formation by upregulation of hepatic FXR (Farnesoid X Receptor) and inhibition of Cyp71a (cholesterol 7 alpha-hydroxylase) and LXR, thus promoting cholesterol accumulation within the liver, intestinal cells and plaque macrophages (243). Bile acids such as deoxycholic acid (DCA), signal through G protein-coupled BA receptor 1 (TGR5), causing the activation of macrophages and the production of inflammatory cytokines. Interestingly, it was suggested that low concentrations of secondary BA may have anti-inflammatory effects, whereas high concentrations are clearly pro-inflammatory (244). Ang II infusion to conventional raised mice resulted in upregulation of taurodeoxycholate and taurodeoxycholic acid in feces, while no changes had been observed in GF mice (201). Taurodeoxycholate has been shown to lower blood pressure in rats (201), which suggests that microbiota may also exerts beneficial effects in the host as a homeostatic mechanism.

Short chain fatty acids (SCFA)

SCFA including propionate, butyrate and acetate are produced by gut bacteria from dietary fiber. These metabolites can regulate inflammation in intestinal macrophages by signaling through G-protein coupled receptors or by inhibiting histone deacetylases (244). SCFA also contribute to the expansion of Tregs and IL-10 secretion in colon (244). The reduction of butyrate producers such as *Eubacterium* and *Roseburia* in atherosclerosis had been reported and was associated with increased adhesion of monocytes to the inflamed endothelium, promoting plaque development (243). Propionate administration in hypertensive *Apoe*$^{-/-}$ mice lowered systemic inflammation and attenuated hypertension, vascular dysfunction, atherosclerosis and fibrosis. The role of SCFA in the inflammatory environment in AAA remains to be experimentally tested directly. Dietary supplementation of propionate has been suggested for AAA patients, but its actual benefits remain to be determined (217).

As the relevance of microbiota to CVD emerges the number of identified metabolites will continue to grow. For example, recently Nemet et al. identified the correlation between plasma metabolite phenylacetylglutamine (PAGln) and severity and outcomes of myocardial infarction and stroke (245). PAGln enhanced platelet activation and thrombosis signaling through G-protein coupled receptors, such as α2A, α2B and β2-adrenergic receptors (245).

Role of perivascular adipose tissue in AAA development

Perivascular adipose tissue (PVAT) is adipose tissue that surrounds the vessels as a distinct layer. As any other adipose tissue PVAT is composed of white and brown adipocytes, and fibroblasts (234). It is infiltrated by multiple immune cells (234) and heavily innervated (246, 247). In pro-inflammatory environment or under the conditions of lipid overload, adipocytes become activated and produce pro-inflammatory cytokines (including TNF and IL-6) and adipokines (such as leptin) which further facilitate immune and VSMC cell activation (234, 248–250). Increased adiposity and lipid deposition is associated with the shift toward white adipocytes and heightened accumulation of pro-inflammatory immune cell types (251), for example pro-inflammatory adipose macrophages (252). In obesity the inflammation in the visceral adipose tissue can be mediated by skewing T helper response from Tregs towards pro-inflammatory T helper subsets, with loss of a unique population of $Ly6C^+$ Tregs normally localized in "lean" adipose tissue (253). Therefore, increase in white adipose tissue accompanied by low-grade inflammation, PVAT may contribute to AAA development given that it contains higher number of immune cells compared to healthy aortic wall (254). PVAT is also implicated in the control of vascular tone, however both anti- or pro-contractile effects had been described (234, 255–259). Moreover, PVAT was also shown to play a critical role in vascular regulation by local secretion of RAS components, including Ang II, as well as production of other entities which regulate vessels, blood pressure and inflammation, including leptin, IL-6, catecholamines and prostanoids, resistin and adiponectin (234, 259, 260). Angiotensin and aldosterone are

both present in PVAT tissue and modulate endothelial dysfunction as well as immune cell infiltration (261).

The role of Ang II receptor signaling in adipocytes had been recently suggested. Adipose tissue transplantation from *Apoe*$^{-/-}$ *At1r*$^{-/-}$ mice to *Apoe*$^{-/-}$ mice infused with Ang II attenuated aortic aneurysm formation, macrophage infiltration, osteopontin expression by macrophages and gelatinolytic activity in the abdominal aorta (255). Levels of ceramides in PVAT correlated with elevated accumulation of macrophages and T cells in human AAA (262). Indeed, both CD4 and CD8 T cells had been detected in human samples, and activated CD69^{+} CD4 T cells were present in higher numbers in PVAT than in AAA lesions and their accumulation was dependent on Ang II (152, 262). B1 cells were also detected in PVAT in human and mouse aorta samples (263). The effects of microbiota and circulating metabolites on PVAT that can further transpire to affect AAA are largely unknown and will remain a subject of future studies.

Perspective

During the past decade the contribution of immune cells to the pathogenesis of AAA became evident and different immune cells had been found in AAA lesions. Some mechanistic studies provided evidence regarding the role of immune cells in AAA pathogenesis. However, the specific contribution of immune cell subsets remains poorly understood and warrants future studies using cell type specific knockouts and more physiologically relevant models. Remaining questions include the understanding of the dynamic of immune cell accumulation and their contribution at early or more advanced stages of the disease. For example, it would be very interesting to determine experimentally whether initial steps of AAA development are actually mediated by recruited neutrophils (and monocytes) or they are only responders to the injury of the aorta generated by other factors. The spatiotemporal changes in cell lineage plasticity (for macrophages, neutrophils or VSMC) will be further addressed through in depth multiproteomic phenotyping and next generation single cell RNA sequencing. These approaches are also great for in depth mechanistic studies of samples derived from human AAA patients.

Better understanding of the mechanisms regulating immune cells activation and accumulation in AAA would provide an important knowledge for therapeutic interventions and likely will allow consideration of new preventive approaches. While the role of microbiota alterations had been implicated to the control of chronic inflammatory diseases including atherosclerosis, the field still gathers and catalogues data on the roles and mechanisms of microbiota action in AAA. A particular interest may represent a focus on the effect of diets and food additives that had been demonstrated to impact microbiota composition and function as well as affect immune cells and inflammatory responses. Future mechanistic studies focusing on interplay between microbiota, metabolites and immune cells during AAA initiation and progression will be of a great interest and potential translational importance.

Several models of AAA development in rodents had been developed and widely used in experimental studies, however they are not fully reflecting human pathology and frequently provide opposite results which are likely related to the nature and limitations of the models. The immune cell responses and requirements for specific immune subsets or mediators may vary between the models. It would be important to take into the account chronic inflammatory nature of AAA as well as contribution of systemic factors such as microbiome when experiments are designed and interpreted.

Overall, understanding of novel immune mediated mechanisms regulating AAA development and factors driving pathogenic immune cell activation will pave the road for novel therapeutics and preventive approaches in AAA and other CVDs, therefore representing an exciting area of research for future studies.

Author contributions

AM-S prepared the Figures, AM-S and EK wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The CARDS toxin of *Mycoplasma pneumoniae* induces a positive feedback loop of type 1 immune response

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Background: Within the past 3-5 years, *Mycoplasma pneumoniae* has become a major pathogen of community-acquired pneumonia in children. The pathogenic mechanisms involved in *M. pneumoniae* infection have not been fully elucidated.

Methods: Previous protein microarray studies have shown a differential expression of CXCL9 after *M. pneumoniae* infection. Here, we conducted a hospital-based study to explore the clinical significance of the type 1 immune response inflammatory factors interferon (IFN)-γ and CXCL9 in patients with *M. pneumoniae* pneumonia (MPP). Then, through *in vitro* experiments, we explored whether CARDS toxin stimulated F-DCs (dendritic cells incubated with Flt3L) to promote Th-cell differentiation; we also investigated the IFN-γ-induced CXCL9 secretion pathway in macrophages and the role of CXCL9 in promoting Th1 cell migration.

Results: The CXCL9 expression level was upregulated among patients with a higher fever peak, fever duration of greater than 7 days, an imaging manifestation of lobar or segmental, or combined pleural effusion ($P<0.05$). The peripheral blood levels of IFN-γ and CXCL9, which were higher in patients than in the healthy control group, were positively correlated with each other ($r=0.502$, $P<0.05$). In patients, the CXCL9 expression level was significantly higher in the bronchoalveolar lavage fluid (BALF) than in the peripheral blood, and the BALF CXCL9 expression level was higher than that in the healthy control group (all $P<0.05$). Our flow cytometry analysis revealed that M1-phenotype macrophages ($CD16^+CD64^+CD163^-$) were predominant in the BALF from children with MPP. In *in vitro* experiments, F-DCs stimulated with CARDS toxin promoted the differentiation of $CD4^+IFN\text{-}\gamma^+$ Th (Th1) cells ($P<0.05$). Moreover, IFN-γ induced high levels of CXCL9 expression in M1-type macrophages in a dose-dependent and time-dependent manner. Additionally, macrophages transfection with STAT1-siRNA-1 downregulated the expression of CXCL9 ($P<0.05$), and CXCL9 promoted Th1 cell migration ($P<0.05$).

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Conclusions: Our findings suggest that CARDS toxin induces a type 1 immune response positive feedback loop during *M. pneumoniae* infection; this putative mechanism may be useful in future investigations of immune intervention approaches for *M. pneumoniae* pneumonia.

KEYWORDS

Mycoplasma pneumoniae, CARDS toxin, type 1 immune response, CXCL9, positive feedback

Introduction

Acute respiratory tract infections are the most common and frequently occurring childhood diseases worldwide, particularly in children aged <5 years (1). *Mycoplasma pneumoniae* is a major respiratory tract pathogen in children (2, 3). The clinical manifestations of *M. pneumoniae* infections are diverse, in terms of both respiratory disease and a wide range of extrapulmonary manifestations (4, 5). The incidence of community-acquired pneumonia (CAP) caused by *M. pneumoniae* is characterized by cyclical epidemics at intervals of 3 to 7 years (6); up to 40% of CAP cases can be attributed to *M. pneumoniae* (7). In recent years, the annual incidence of *M. pneumoniae* pneumonia (MPP) has been increasing and become more severe, with about 5% requiring intensive care unit treatment (8). Severe *M. pneumoniae* pneumonia (SMPP) is characterized by massive pleural effusion, acute respiratory distress syndrome (ARDS), pulmonary consolidation, pulmonary fibrosis, obstructive bronchitis, and even life-threatening pulmonary sequelae, such as bronchiectasis, atelectasis, occlusive bronchitis, etc. Some children present with necrotizing pneumonia (NP) (9). After treatment with macrolide antibiotics for ≥7 days, the clinical manifestations in some children with MPP (usually children with SMPP) are not relieved and the lung imaging findings gradually worsen; these patients are diagnosed with refractory *M. pneumoniae* pneumonia (RMPP) (10).

There are no specific clinical symptoms during the early stage of *M. pneumoniae* infection in children; in the absence of clear clinical signs, affected patients can develop SMPP or RMPP (11). Systemic inflammatory responses and immune disorders have important roles in the occurrence, development, and prognosis of SMPP or RMPP; the influence of macrolide antibiotic resistance is increasing (12, 13). Therefore, considerable global research attention has been focused on the immunological pathogenesis of *M. pneumoniae* infection, as well as efforts for early identification and intervention.

The network of inflammatory interactions formed by innate and adaptive immunity has an important role in MPP and is closely related to disease severity (12). It's mainly composed of types 1, 2, and 3 immune responses causing cytokine "waves". The type 1 immune response mainly involves Th1 cells, interferon (IFN)-γ, and M1 macrophages (Mφs), which exert immune effector functions. The expression level of the Th1 cytokine IFN-γ is closely associated with the severity of MPP and the degree of recovery (14). M1-type Mφs can be activated by IFN-γ (15); numerous inflammatory factors (e.g., interleukin [IL]-1β, IL-6, tumor necrosis factor-α, and CXCL9) are then released. Chemokine CXCL9, also known as the IFN-γ-induced monokine, can recruit leukocytes to sites of inflammation. CARDS toxin is a virulence factor that stimulates excessive immuno-inflammatory responses after *M. pneumoniae* infection; its functions include adenosine diphosphate-ribosylation and vacuolization (16, 17).Inhibition of CARDS toxin is expected to reduce MPP severity, and CARDS toxin may be useful as a vaccine antigen (18). Therefore, the specific regulatory mechanism of IFN-γ/CXCL9 and CARDS toxin remains unclear, which has important research implications for MPP.

In the present study, we collected peripheral blood and bronchoalveolar lavage fluid (BALF) from children with MPP, conducting a hospital-based study to explore the clinical significance of the type 1 immune response inflammatory factors IFN-γ/CXCL9; through *in vitro* assays, we explored whether CARDS toxin promote Th-cell differentiation and the IFN-γ-induced CXCL9 secretion pathway in macrophages. Our findings may be useful in early identification, guidance regarding treatment and prognosis, and future investigations of targeted treatment and immune intervention approaches for MPP.

Materials and Methods

Patients

This study included patients who were diagnosed with MPP from July 2019 to October 2021 at Children's Hospital of Soochow University (N=140); all diagnoses were confirmed by both MP-immunoglobulin (IgM) positivity and the presence of MP-DNA ($\geq 1.0\times10^4$ copies/ml) in nasopharyngeal aspirates

(NPA) or BALF, as measured by real-time quantitative polymerase chain reaction (qPCR) (19). The patients' ages ranged from 17 months to 16 years; their clinical manifestations included fever, cough, tachypnea, chest retractions, abnormal auscultatory findings, and radiologic evidence of CAP.

Among MPP patients, 117 cases were selected as experimental group A to study the correlation of CXCL9 expression level in peripheral blood; while 23 cases were selected as experimental group B and measured IFN-γ and CXCL9 in peripheral blood and BALF; to further study the correlation between IFN-γ/CXCL9 and immune inflammatory damage in the lung.

Cases met one or more of the exclusion criteria were excluded (a): co-infection (b); clinical and imaging features indicative of fungal pneumonia (c); presence of congenital respiratory diseases, and abnormalities in other systems (e.g., heart, liver, kidney, and blood) (d); >3 days of treatment with glucocorticoids (20) and/or azithromycin before admission. To exclude co-infections, seven respiratory virus antigen tests (influenza A and B; parainfluenza 1, 2, and 3; respiratory syncytial virus; and adenovirus) and other pathogen tests (human bocavirus; human rhinovirus; human metapneumovirus; and chlamydia pneumoniae) were conducted; the results were negative. The results of bacterial cultures of NPA and BALF were also negative.

Peripheral blood and BALF were also collected from 18 children who underwent emergency surgery for foreign bodies in the ENT department of our hospital during the same period with 23 cases of MPP children; these children served as a control group. The inclusion criteria were as follows: no respiratory tract infection within the previous 4 weeks, no chronic lung disease or bronchopulmonary malformation, and no history of treatment with hormones or immunosuppressive agents.

The studies involving human participants were reviewed and approved by the Ethics Committee of the Children's Hospital of Soochow University (2019LW014) on July 24, 2019. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. All participant data were anonymized prior to analysis.

Collection of clinical data

All patients' age and sex were recorded. In the experimental group A, the following clinical data were recorded: duration of fever, fever peak, and imaging manifestations. Peripheral blood samples obtained within 24 h of admission were used for measurements of CXCL9 and specific antibodies to *M. pneumoniae*.

In the experimental group B, the following clinical data were also recorded: durations of hospital stay and fever, fever peak, and laboratory test data on admission (Table 1). Peripheral blood samples obtained within 24 h of admission were also used for measurements of complete blood counts, C-reactive protein (CRP), lactate dehydrogenase (LDH), immunoglobulins (IgA/IgG/IgM), lymphocyte subsets, specific antibodies to *M. pneumoniae*, IFN-γ and CXCL9. Flexible fiber bronchoscopy and bronchoalveolar lavage were performed in accordance with existing guidelines (21). BALF was gently aspirated, collected, and prepared for detection of the protein concentrations of IFN-γ and CXCL9.

Real-time fluorescent qPCR for *M. pneumoniae* in NPA or BALF

Nucleic acid extraction and qPCR for the detection of *M. pneumoniae* 16S rDNA were performed as previously described (17, 21). Briefly, samples (NPA/BALF) were shaken for 30s, then centrifuged at 12,000×g for 5 min. Subsequently, the sediment was collected and DNA was extracted from 400-μL of each sample. The primers for *M. pneumoniae* 16S rDNA were as follows: forward: 5′-GCAAGGGTTCGTTATTTG-3′; reverse: 5′-CGCCTGCGCTTGCTTTAC-3′ (amplicon size: 380 bp).

Real-time qPCR was performed using the iQ5TM BIO-icycler (Bio-Rad, Hercules, CA, USA). The PCR conditions were as follows: 37°C for 2 min; initial denaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 10s, annealing at 55°C for 30s, and extension at 72°C for 40s. After amplification, a computer connected to the instrument automatically analyzed the results, then expressed the test results as Ct values. For samples with a Ct value <38, a quantitative reagent (Aikang Co., Ltd., Hangzhou, China) was used for further quantitative determination.

Detection of IFN-γ and CXCL9 by enzyme-linked immunosorbent assays

IFN-γ and CXCL9 were measured in peripheral blood or/and BALF samples from MPP and control groups using the appropriate commercial enzyme-linked immunosorbent assay kits, in accordance with the manufacturer's instructions. Enzyme-linked immunosorbent assay kits were purchased from Xuguang kexing Biotechnology Co., Ltd. (Suzhou, China).

Flow cytometry analysis of cell counts and Mφ phenotypes in BALF from children with MPP

Flow cytometry was used to determine cell counts of Mφs, and lymphocytes in BALF from children with MPP, using flow cytometry equipment (Beckman Coulter, Miami, FL). The forward scatter (FSC) value represents cell volume; a larger FSC value indicated greater cell volume. The side scatter (SSC)

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TABLE 1 Demographic data and clinical characteristics in experimental group B.

Clinical parameters	MPP cases (experimental group B, n=23)	Controls (n=18)	*p*	Blood-CXCL9		BALF-CXCL9	
				r	*P*	*r*	*P*
Age, years	4.90(3.80,7.00)	2.25(1.90,2.73)	0.00*	N/A	N/A	N/A	N/A
Male, n (%)	8(34.78%)	10(55.56%)	0.183	N/A	N/A	N/A	N/A
Hospital stay, days	9.13±2.26	N/A		-0.04	0.86	-0.33	0.13
Duration of fever, days	6.78±2.76	N/A		-0.43	0.040*	0.44	0.036*
Peak of fever (°C)	39.36±0.59	N/A		-0.51	0.51	0.16	0.48
WBC, 10^9/L	7.59±3.41	N/A		0.3	0.17	0.19	0.4
N (%)	53.23±17.10	N/A		0.54	0.009*	0.17	0.43
PLT, 10^9/L	334.30±137.68	N/A		0.15	0.49	0.11	0.62
EOS (%)	1.95±1.50	N/A		-0.34	0.11	0.42	0.048*
CRP, mg/L	12.71±12.66	N/A		-0.06	0.77	0.05	0.82
LDH, IU/L	384.58±125.10	N/A		0.33	0.13	0.06	0.77
IgA, g/L	1.02±0.53	N/A		-0.17	0.44	0.28	0.2
IgG, g/L	9.31±2.36	N/A		0.01	0.96	0.27	0.21
IgM, g/L	1.38±0.41	N/A		0.36	0.09	0.06	0.79
$CD3^+$, %	71.79±5.85	N/A		-0.23	0.33	0.13	0.6
$CD3^+CD4^+$, %	41.79±5.96	N/A		0.09	0.71	-0.28	0.23
$CD3^+CD8^+$, %	27.88±5.79	N/A		-0.24	0.32	0.29	0.21
$CD3^-CD(16^+56^+)$, %	8.68±4.04	N/A		-0.14	0.56	0.11	0.63
$CD3^-CD19^+$, %	18.45±5.67	N/A		0.39	0.09	-0.27	0.25

*$P < 0.05$.

value represents cell granularity; a larger SSC value indicated higher granularity. Cells with greater volume and higher granularity were considered neutrophils; cells with less volume and lower granularity were considered lymphocytes; monocytes (Mφs) were in between the two types of cells; then, Mφ phenotypes were determined by flow cytometry.

Protein microarray

Protein microarrays enable determination of the protein-binding specificities of multiple analytes in solution; they are versatile tools for high-throughput analyses of the human proteome (22). In a preliminary experiment, six blood samples from the SMPP group, four blood samples from the MPP group, and five blood samples from the control group were analyzed by Ray Biotech Co., Ltd. (Guangzhou, China), which provided all reagents and instruments.

Multiple protein molecules were immobilized on a solid support in a predetermined arrangement to form a microarray. Samples were incubated with the protein chip; after specific binding reactions occurred, the components that were not bound to the protein on the chip were removed by washing. Fluorescently labeled antibodies were used for secondary incubation; the fluorescence value of each point on the chip was analyzed using a commercial fluorescence scanning analysis instrument and software.

Animals and F-DCs (DCs incubated with Flt3L) induction

The animal study was reviewed and approved by the Ethics Committee of Soochow University (SUDA20200510A02) on May 10, 2020. C57BL/6 mice (age, 4–5 weeks; weight, 20±2g) were purchased from the Laboratory Animal Center of Soochow University (Suzhou, China). All animals were housed in separate cages under constant temperature (25±1°C) and humidity (50%) with 12-h light-dark cycles; they had free access to food and water.

Bone marrow mononuclear cells (BM-MNCs) were isolated from C57BL/6 mouse bone marrow *via* density gradient centrifugation. BM-MNCs were cultured in 24-well plates with Mouse Steady-State Dendritic Cell Culture Kit (Dongling Biotechnology Co., Ltd., Suzhou, China); the cell density was adjusted to 1.5×10^6 cells/ml. Flt3L (Jinsirui Biotechnology Co., Ltd., Nanjing, China) was added to each well at a final concentration of 200 ng/ml; we obtained suspension cultured cells after 9 days of incubation (23), which were DCs with diverse phenotypic characteristics (i.e., F-DCs). Cultures were

incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Construction of recombinant CARDS toxin

Recombinant CARDS toxin was constructed from Sino Biological Co., Ltd. (Beijing, China) and its biological function was verified as previously described (17, 21). According to the manufacturer's instructions, the recombinant CARDS toxin was expressed in insect cells, and the insect cell expression system belonged to the eukaryotic cell expression system. From the NCBI database, we obtained the full-length gene sequence and protein sequence of MPN372, which coded CARDS Toxin. The optimized MPN372 gene sequence was cloned into the pFastBac donor plasmid vector for virus packaging. High-Five cells in logarithmic growth phase were infected with high titer recombinant virus to express the target protein, which was further purified by nickel column.

Use of CARDS toxin to stimulate F-DCs for the regulation of Th cell differentiation *in vitro*

$CD4^+$T cells (1×10^5 cells/ml) were isolated from mouse spleens in accordance with the instructions of the Mouse Naïve $CD4^+$T Cell Isolation Kit (STEMCELL Technologies, Vancouver, Canada). Labeled cells were isolated using EasySep magnets without a column, and the target cells were transferred to new tubes. Naïve $CD4^+$T cells from the spleen were negatively selected using magnetic beads. Flow cytometry equipment was described as previously.

F-DCs were screened for phenotype by flow cytometry and the cell concentration was adjusted to 1×10^5 cells/well. F-DCs incubated with cell growth medium were used as the negative control group; F-DCs incubated with CARDS toxin (10 ng/ml) were used as the experimental group. Both groups of cells were co-incubated with naïve $CD4^+$T cells (1:4 ratio of DCs to T cells) for 24 h at 37°C with 5% CO_2. The numbers of $CD4^+$IFN-γ^+Th, $CD4^+$IL-4^+Th, and $CD4^+$IL-17^+Th cells were analyzed by flow cytometry.

IFN-γ-mediated induction of CXCL9 expression in M1-type Mφs

THP-1 cells in suspension (1×10^5 cells/ml) were purchased from Noble Biological Co., Ltd. (Shanghai, China). Phorbol ester (PMA) was added to the THP-1 cells in suspension at a final concentration of 50 ng/ml. Then, these cells were seeded into six-well plates (1×10^5 cells/ml), and adherent mononuclear Mφs were obtained after incubation for 24 h. The cell growth medium was then changed to complete medium without PMA; cells were cultured for 3 days to observe changes in morphology. Pseudopodia were observed, indicating successful Mφs induction.

IFN-γ has been reported to induce Mφs differentiation into M1-type Mφs (15). Here, we stimulated Mφs (1×10^5 cells/ml) with different concentrations of IFN-γ (0 ng/ml, 1 ng/ml, or 10 ng/ml) for 24 h; we used two experimental methods (i.e., real-time qPCR and ELISA), to measure the CXCL9 expression level at 3 h, 6 h, and 9 h. The primers were synthesized by Jinkairui Biological Engineering Co., Ltd. (Wuhan, China). ELISA kits were purchased from ELK Biotechnology Co., Ltd. (Wuhan, China). Primer sequences are shown in Supplementary Table S1.

The Western blot is an important laboratory technique that allows for specific identification and characterization of proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-separated proteins are electophoretically transferred to a polyvinylidene fluoride (PVDF) membrane which is then incubated with specific antibodies, then developed to show the protein of interest; β-actin is acted as an internal control. We determined the expression of protein CXCL9, STAT1 and p-STAT1 by Western Blot at different concentrations (IFN-γ 0 ng/ml, 1 ng/ml, or 10 ng/ml) and different times (3 h, 6 h, and 9 h). Reagents were purchased from ASPEN Biotechnology Co., Ltd. (Wuhan, China).

STAT1-small interfering RNA (siRNA) *in vitro* assay

STAT1-siRNA-CON (negative control group), STAT1-siRNA-1, STAT1-siRNA-2, or STAT1-siRNA-3 were transfected into Mφs (1×10^5 cells/ml) using Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA, USA); the cells were incubated for 24 h after transfection. The effect of STAT1 interference (STAT1-siRNA-1, STAT1-siRNA-2, and STAT1-siRNA-3) on the expression of STAT1 in Mφs was analyzed by qPCR. The results of STAT1-siRNA interference efficiency verification showed that the three pairs of STAT1-siRNA down-regulated the expression level of STAT1 by 43%, 70%, and 80%, respectively; it met the quality control standards (at least one pair of siRNAs had a silencing efficiency of more than 70% at the mRNA level under standard conditions of use).

IFN-γ (1 ng/ml) was used to stimulate Mφs that had been transfected with STAT1-siRNA-1; after IFN-γ stimulation for 24 h, the level of CXCL9 expression was analyzed by qPCR and ELISA; CXCL9, STAT1 and p-STAT1 protein expression were analyzed by Western blot.

Measurement of cell migration *via* Transwell assays

$CD4^+$T cells (1×10^5 cells/ml) were isolated from the peripheral blood of healthy volunteers using autoMACS columns with the Direct Human $CD4^+$T Cell Isolation Kit (STEMCELL Technologies). $CD4^+$T cells were treated with PMA (20 ng/ml) + ionomycin (1 μg/ml) at 37°C for 1 h; Breedellin A (1 μl/ml) was then added and cells were cultured for an additional 3–5 h to obtain Th1 cells.

In the lower chamber of the Transwell plate (Corning, NY, USA), Mφs (1×10^5 cells/ml) were stimulated with IFN-γ (1 ng/ml) for 24 h; one group was transfected with STAT1-siRNA-1, whereas one group was not transfected. Unstimulated Mφs were used as negative controls. In the upper chamber of the Transwell plate, Th1 cells were seeded. The suspension in the lower chamber was subjected to smear staining to analyze the cell morphology and determine the number of migrating Th1 cells.

Statistical analysis

Data were stored in a Microsoft Excel-supported database, statistical analyses were performed using SPSS statistics version 20.0 (International Business Machines Corp., NY), and graphics were prepared using GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA). Categorical data of patient characteristics were compared using the Chi-square test. Data with normal distributions were expressed as means ± standard deviations; comparisons among groups were performed by *t*-tests or one-way analysis of variance. Data with non-normal distributions were expressed as medians (interquartile ranges); comparisons between groups were performed by the Mann–Whitney *U*-test. The *Spearman* correlation coefficient was used for correlation analysis. *P*-values <0.05 were considered statistically significant.

Results

Demographic data and clinical characteristics

Demographic data and clinical characteristics were collected uniformly from the control and MPP groups (experimental group A and experimental group B). In experimental group A, there were 52 (44.44%) male and 65 (55.56%) female cases, with a male to female ratio of 1:1.25 and a mean age of 5.74 ± 3.03 years.

In experimental group B, there were 8 (34.78%) male and 15 (65.22%) female cases, with a male to female ratio of 1:2.25, while in the control group there were 10 (55.56%) male and 8 (44.44%) female cases, with a male to female ratio of 1.25:1, as shown in Table 1. There was no significant difference in terms of sex between the control group and experimental group B ($P>0.05$) and there was a difference in terms of age between two groups ($P<0.05$). Most airway foreign bodies in children occur in children under 3 years old and the peak incidence is in children aged 1-2 years. Therefore, specimens will to be collected to verify the difference level of IFN-γ/CXCL9 between the two groups in the further. The duration of fever was 6.78 ± 2.76 days, the fever peak was 39.36 ± 0.59°C, and the duration of hospitalization was 9.13 ± 2.26 days, as shown in Table 1.

Altered expression of CXCL9 protein in children with MPP

To identify differentially expressed proteins (DEPs) in patients with *M. pneumoniae* infection, we selected six blood samples from the SMPP group, four blood samples from the MPP group, and five blood samples from the control group for protein microarray analysis in a preliminary experiment. DEPs were defined as proteins with $P<0.05$ and fold change >1.2 or <0.83 (absolute $\log_2$ [fold change] >0.263).

In terms of DEPs with significant expression, the levels of CXCL9 (MIG), CXCL10 (IP-10), and IL-18 changed between the two groups (Figures 1A, D). Gene Ontology (GO) enrichment analysis between the two groups was performed at three levels: cellular component, molecular function, and biological process. DEPs were related to CXCR3 chemokine receptor binding, cytokine response, and cell chemotaxis and migration (Figure 1B). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis indicated that DEPs might be related to the JAK/STAT signaling pathway (Figure 1C). Further clinical pre-experimental research was conducted in the early stage through detecting the same samples by ELISA; only the level of CXCL9 expression significantly differed between the two groups ($P<0.05$) (Figure 1E). The level of IFN-γ expression is reportedly closely associated with MPP severity and subsequent recovery; moreover, IFN-γ can induce the production of CXCL9 (24). The above findings suggested that CXCL9 plays an important role in *M. pneumoniae* infection.

Clinical significance of IFN-γ/CXCL9 in peripheral blood and BALF from children with MPP

In experimental group A, the duration of fever was 4.52 ± 2.96 days, the fever peak was 39.05(38.70, 39.70) °C. The CXCL9 expression level was upregulated among patients with a higher fever peak, fever duration of greater than 7 days, an imaging manifestation of lobar or segmental, or combined pleural effusion (All $P<0.05$, Figure 2).

In experimental group B, the peripheral blood levels of IFN-γ and CXCL9, which were higher in patients than in the healthy control group, were positively correlated with each other ($r=0.502$,

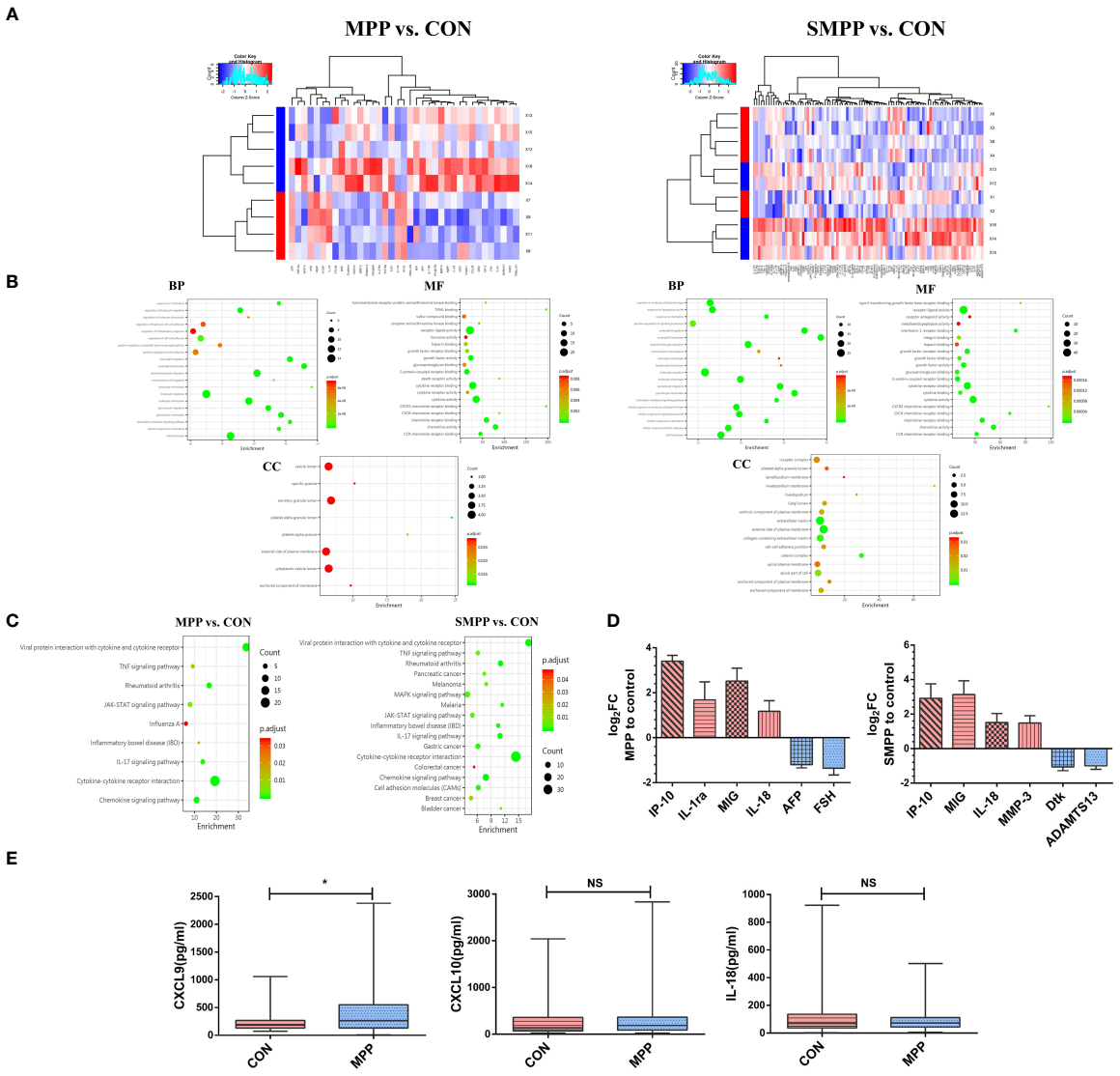

FIGURE 1
CXCL9 protein expression exhibited the greatest difference between groups (MPP vs. CON; SMPP vs. CON). **(A)** Heatmap of differentially expressed proteins (DEPs) expression abundances (MPP vs. CON: n = 94; 81 downregulated, 13 upregulated; SMPP vs. CON: n = 37; 29 downregulated, 8 upregulated). **(B)** Gene ontology analysis was used to identify the biological functions of DEPs. **(C)** Kyoto Encyclopedia of Genes and Genomes analysis of DEPs revealed enrichment in the cytokine-cytokine receptor interaction pathway. **(D)** Analysis of the first six DEPs by log_2FC between different groups (MPP vs. CON; SMPP vs. CON; red refers to the up-regulation of expression, blue refers to the down-regulation of expression; MIG: CXCL9; IP-10: CXCL10). **(E)** Comparison of CXCL9, CXCL10, and IL-18 expression between the control and MPP groups during the preliminary experiment; statistical comparison was performed using the Mann–Whitney *U*-test (**P*<0.05; NS, not significant).

P<0.05). In patients, the CXCL9 expression level was significantly higher in the BALF than in the peripheral blood, and the BALF CXCL9 expression level was higher than that in the healthy control group (All *P*<0.05, Figure 3A). The expression level of IFN-γ in BALF between experimental group B and control group was low.

CXCL9 in peripheral blood was negatively correlated with the duration of fever, whereas CXCL9 in BALF was positively correlated with the duration of fever (*r*=-0.43 and *r*=0.44, respectively; both *P*<0.05). CXCL9 in peripheral blood was positively correlated with neutrophils(N)%, whereas CXCL9 in BALF was positively correlated with eosinophils(EOS)% (*r*=0.54 and *r*=0.42, respectively; both *P*<0.05; Figure 3B). Other laboratory test data showed no significant correlations, as shown in Table 1.

Cell counts and Mφ phenotypes in BALF from children with MPP

We selected 5 children with MPP in experimental group B and 5 children in the control group to measure cell counts and Mφ

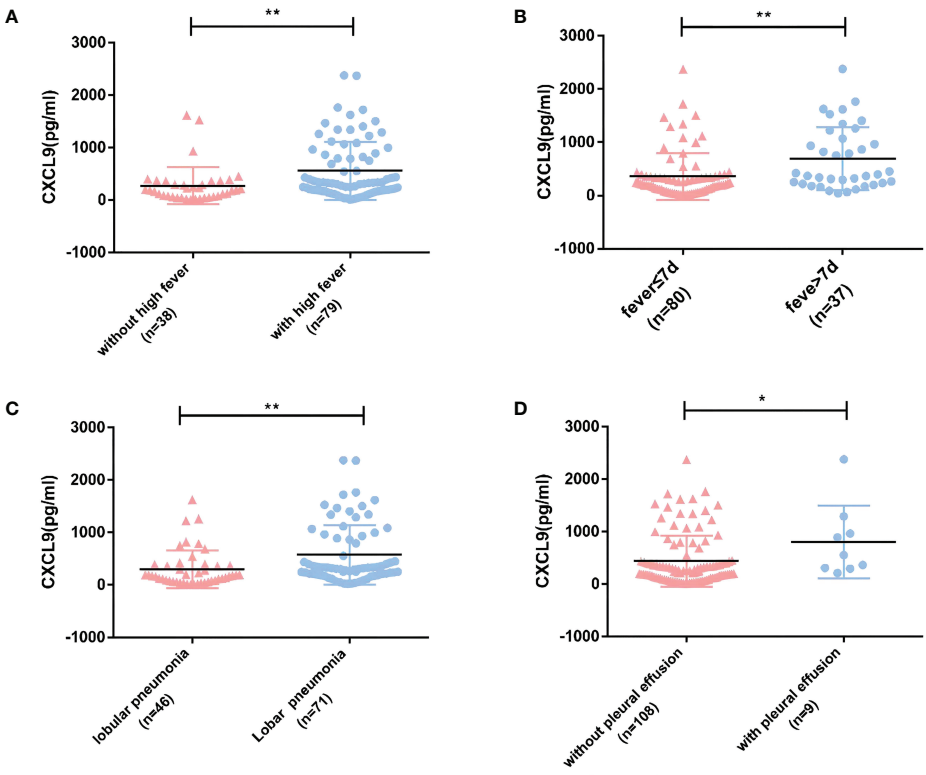

FIGURE 2
Relationships between CXCL9 expression in peripheral blood and clinical indicators in experimental group A (MPP: n=117). **(A, B)** Relationships between CXCL9 expression and the degree and duration of fever. **(C)** Relationship between CXCL9 expression and the extent of lung lesion on imaging. **(D)** Relationship between CXCL9 expression and pleural effusion. To detect statistically significant differences, the Mann–Whitney *U*-test was carried out (*P<0.05; **P<0.01).

phenotypes in BALF by flow cytometry. Results showed that the cell counts of Mφs were significantly higher than those of the control group (t=2.844, P<0.05; Figure 3C). Previous studies have shown that Mφs mainly comprise two distinct functional phenotypes: classically activated Mφs (M1) and alternatively activated Mφs (M2) (25). Our flow cytometry analysis revealed that M1-phenotype Mφs ($CD16^+CD64^+CD163^-$) were predominant (Figure 3D).

CARDS toxin-mediated differentiation of Th1 cells

Mouse BM-MNCs were isolated, then differentiated into F-DCs using Flt3L; $CD11c^+$DCs were identified by flow cytometry (Figure 4A). $CD4^+$T cells with low carboxyfluorescein diacetate succinimidyl ester (CFSE) staining were promoted after CARDS toxin stimulation (P<0.001, Figure 4B). Notably, F-DCs stimulated with CARDS toxin promoted the differentiation of $CD4^+$IFN-γ^+Th (Th1) cells(P<0.001), without affecting the differentiation of Th2 or Th17 cells (Figure 4C).

The IFN-γ-induced high expression of CXCL9 in M1-type Mφs can be blocked by transfection with STAT1-siRNA

IFN-γ promoted the level of CXCL9 expression in a dose-dependent manner at the same time (3 h, 6 h, and 9 h) by qPCR and ELISA (P<0.05); qPCR and ELISA also showed that the level of CXCL9 expression significantly increased after stimulation with IFN-γ (1 ng/ml, 10 ng/ml) (P<0.05). Upon stimulation with the same concentration of IFN-γ(1 ng/ml), the level of CXCL9 expression significantly decreased after Mφs had been transfected with STAT1-siRNA-1 (P<0.05) (Figures 5A, C, D).

Western Blot showed protein CXCL9 and p-STAT1 were expressed in a dose-dependent and time-dependent manner (P<0.05). The expression of protein CXCL9, STAT1 and p-STAT1 increased after stimulation with IFN-γ (1 ng/ml) (P<0.05); after Mφs had been transfected with STAT1-siRNA-1, the expression of these proteins significantly decreased (P<0.05) (Figures 5B, E).

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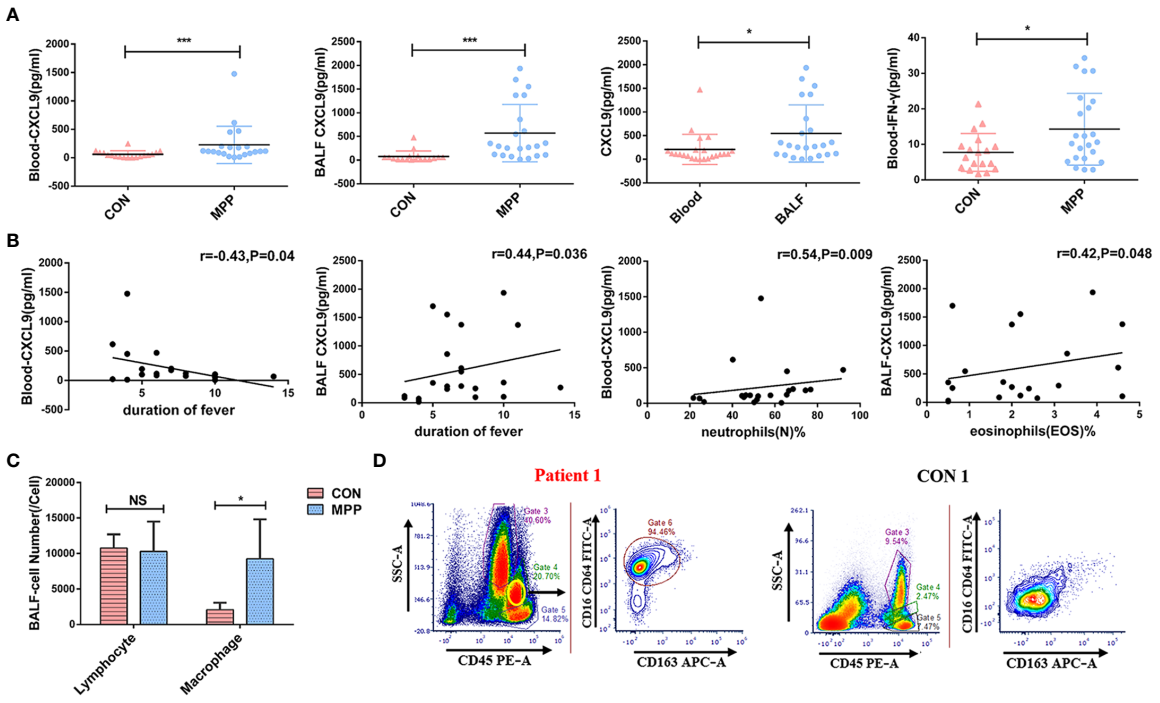

FIGURE 3
Clinical significance of IFN-γ/CXCL9 in peripheral blood and BALF from children with MPP in experimental group B and control group (MPP: n=23, CON=18). **(A)** Comparison of IFN-γ and CXCL9 expression between groups. **(B)** Correlations between CXCL9 and laboratory indexes. **(C)** Comparison of different types of cells from BALF between groups. **(D)** Flow cytometry result from the BALF between groups of representative cases (patient 1 and CON 1; M1-phenotype Mφs:$CD16^{+}CD64^{+}CD163^{-}$). Comparisons between groups were performed by the Mann–Whitney *U*-test; correlations between CXCL9 and laboratory indexes were performed using the *Spearman* correlation coefficient; comparison of different types of cells from BALF between groups were performed by *t*-tests. (*P<0.05; ***P<0.001; NS, not significant).

CXCL9 promotes the migration of Th1 cells

The expression of CXCL9 in the lower Transwell chamber increased after 24 h of Mφ (THP-1+PMA induction) stimulation with IFN-γ (1 ng/ml), and the number of migrating Th1 cells increased (P<0.001). Whereas, the number of migrating Th1 cells significantly decreased after Mφs had been transfected with STAT1-siRNA-1 (P<0.001) (Figure 5F).

Discussion

In recent years, the annual incidence of MPP has been increasing, and some children die because of severe *M. pneumoniae* infection. When it encounters an onslaught of proinflammatory cytokines and cellular elements (e.g., neutrophils, lymphocytes, Mφs, and mast cells) from the host, *M. pneumoniae* strongly adheres to the epithelial cell surface; it uses toxic molecules to damage host cells, thereby inducing ciliostasis and epithelial desquamation to acquire critical nutrients (26). Thus, there is a need to investigate the occurrence and specific regulatory mechanism involved in the immuno-inflammatory response to *M. pneumoniae*; the findings can facilitate early identification and targeted treatment of MPP in children.

In the early stages of this study, protein microarrays from clinical peripheral blood samples showed that CXCL9 was upregulated after *M. pneumoniae* infection. Therefore, the clinical significance of the chemokine CXCL9 in MPP was investigated. CXCL9 is induced by IFN-γ, which is usually secreted by peripheral blood mononuclear cells (24). We found the CXCL9 expression level was upregulated among patients with a higher fever peak, fever duration of greater than 7 days, an imaging manifestation of lobar or segmental, or combined pleural effusion, suggesting that the CXCL9 might have correlation with the disease severity. Our study also showed that the peripheral blood levels of IFN-γ and CXCL9, which were higher in patients than in the healthy control group, were positively correlated with each other. In patients, the CXCL9 expression level was significantly higher in the BALF than in the peripheral blood, and the BALF CXCL9 expression level was higher than that in the healthy control group. Consistent with the findings by Chung et al. (27), that CXCL9 was involved in the pathogenesis of acute respiratory infection involving *M. pneumoniae*. Cytokine

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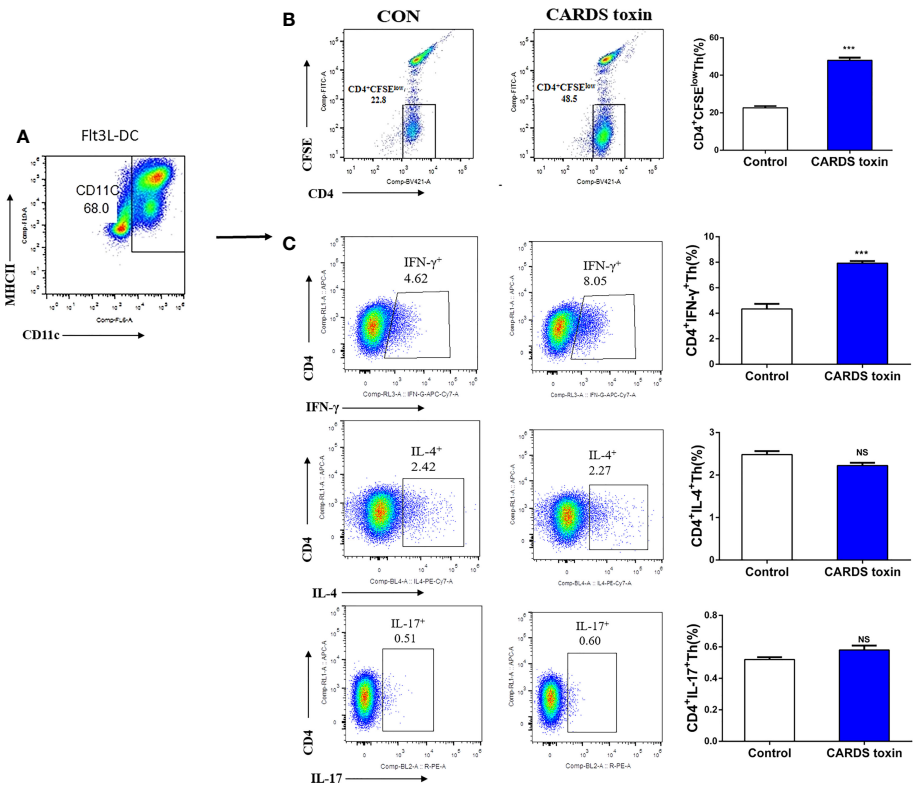

FIGURE 4
CARDS toxin stimulates F-DCs to regulate Th1 cell differentiation. **(A)** Screening and identification of F-DCs. **(B)** F-DCs were stimulated by CARDS toxin (10 ng/ml) and cell growth medium was used as the negative control; $CD4^+$T cells with low carboxyfluorescein diacetate succinimidyl ester (CFSE) staining were identified. **(C)** $CD4^+$IFN-γ^+Th, $CD4^+$IL-4^+Th and $CD4^+$IL-17^+Th cells were analyzed by flow cytometry and their proportion among all cells were analyzed between groups (CON vs. CARDS toxin). To detect statistically significant differences, *t*-tests was carried out (***P<0.001; NS, not significant).

release syndrome is regarded as the driver of coronavirus disease 2019 (COVID-2019) inflammation, and multiple studies have demonstrated that CXCL9 is associated with the severity of COVID-2019 (28, 29). Accordingly, CXCL9 may be useful as a clinical biomarker for disease diagnosis and treatment monitoring.

Previous reports (30) concerning experimental models of lower respiratory tract infection with *M. pneumoniae* have indicated that lung disease severity is directly associated with Th1-type cellular immunity; tigecycline treatment significantly reduced the levels of IFN-γ, tumor necrosis factor-α, and IL-1β, CXCL9, and other inflammatory mediators, thereby significantly reducing histological lung inflammation and disease severity. In another study, the ratios of IFN-γ/IL-4 and IFN-γ/IL-13 in BALF are significantly higher in children with MPP than in controls (31), suggesting that children with MPP exhibit a type 1 immune response dominated by Th1-type cellular immunity. Therefore, the type 1 immune response inflammatory factors IFN-γ and CXCL9 are closely associated with lung immuno-inflammatory injury. Furthermore, we found that the level of CXCL9 expression was significantly higher in BALF than in peripheral blood, indicating that lung tissue was the main site of the inflammatory response. IFN-γ stimulates M1-type Mφs to produce CXCL9, which guides Th1 cells along the CXCL9 concentration gradient (from low to high) toward the center of the inflammatory response; this results in a cascade expansion effect that comprises type 1 immune response positive feedback with IFN-γ/CXCL9 as the core circuit, along with a strong immuno-inflammatory response in the lungs.

CARDS toxin, an *M. pneumoniae*-related pathogenic factor, induces cytopathology *in vivo* and *in vitro*, replicates the infection process, and induces histopathological changes similar to *M. pneumoniae* infection; these changes include characteristic fibrillar arrest, cytoplasmic swelling and vacuolization, nuclear fragmentation, extensive inflammation, and histopathological damage (32). As reported (17), the expression level of CARDS toxin increase in the BALF of children with MPP, especially in MPP with mucus plugs and pleural effusion or RMPP. However, the mechanism by which CARDS toxin initiates the subsequent immuno-inflammatory response remains unknown.

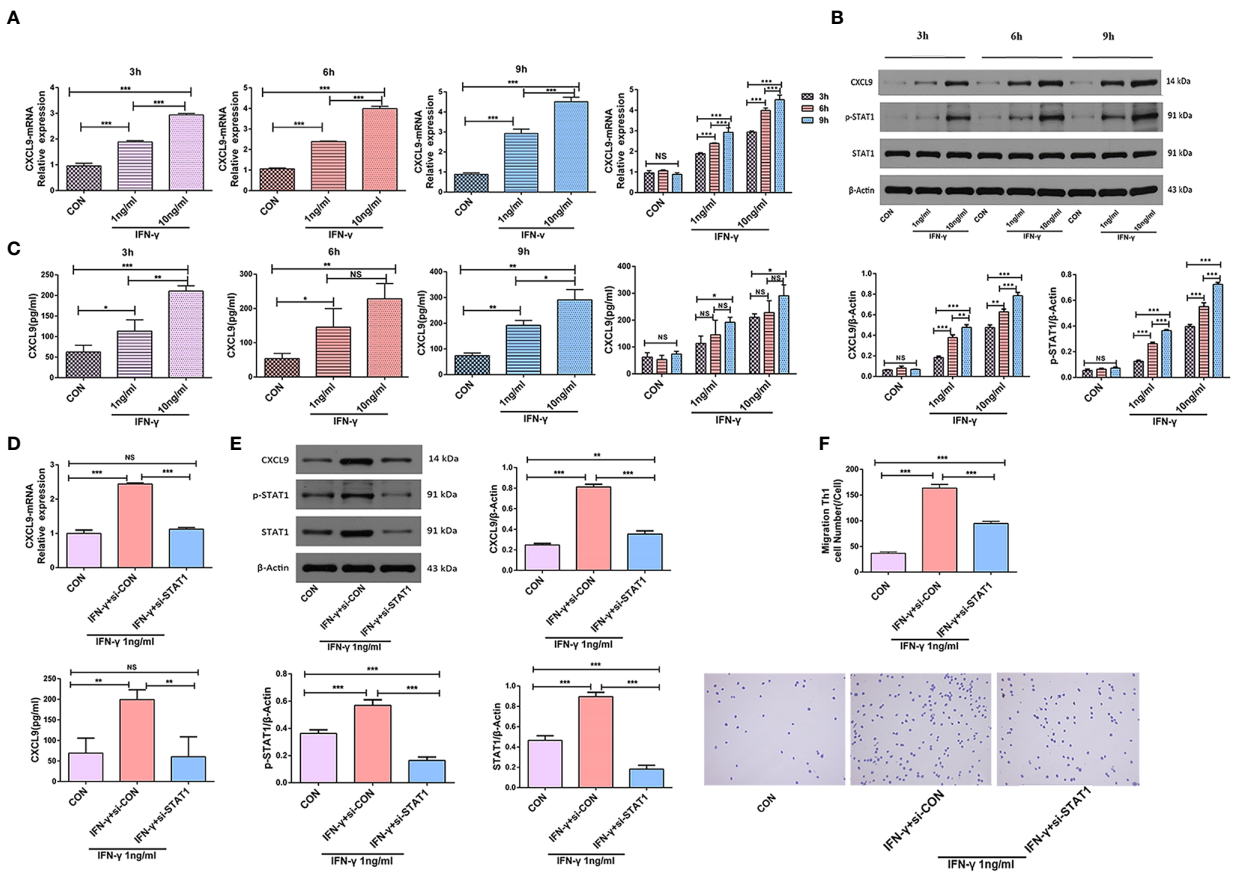

FIGURE 5
IFN-γ induced CXCL9 expression in a STAT1-dependent manner. **(A)** PMA was added to the THP-1 cells in suspension at a final concentration of 50 ng/ml and mononuclear Mφs were obtained after incubation for 24 h; then, mononuclear Mφs were stimulated with different concentrations of IFN-γ (CON, 1 ng/ml, or 10 ng/ml) and the expression level of CXCL9 was analyzed at different times (3 h, 6 h, 9 h) by qPCR. **(B)** Protein CXCL9, STAT1 and p-STAT1 were analyzed at different times and at different concentrations of IFN-γ by Western Blot. **(C)** The expression level of CXCL9 was analyzed at different times and at different concentrations of IFN-γ by ELISA. **(D)** The expression level of CXCL9 was analyzed by qPCR and ELISA before and after Mφs (IFN-γ 1 ng/ml stimulation) transfection with STAT1-siRNA-1. **(E)** Protein CXCL9, STAT1 and p-STAT1 was analyzed by Western Blot before and after Mφs (IFN-γ 1 ng/ml stimulation) transfection with STAT1-siRNA-1. **(F)** Number of migrating Th1 cells in Transwell experiment and observation of cell morphology by smear staining before and after Mφs (IFN-γ 1 ng/ml stimulation) transfection with STAT1-siRNA-1; Groups: different groups(bottom) + Th1 (top). To detect statistically significant differences, one-way analysis of variance was carried out (*P<0.05; **P<0.01; ***P<0.001; NS, not significant).

Then, through *in vitro* experiments, we explored whether CARDS toxin stimulated F-DCs (dendritic cells incubated with Flt3L) to promote Th-cell differentiation; we also investigated the IFN-γ-induced CXCL9 secretion pathway in macrophages and the role of CXCL9 in promoting Th1 cell migration.

DCs induced by the Flt3L-dependent bone marrow culture system (i.e., F-DCs) have diverse phenotypic characteristics, robust antigen processing, and a strong antigen presentation capacity (33). Therefore, we constructed an F-DC culture identification system to explore the ability of CARDS toxin to stimulate F-DCs to regulate Th cell differentiation; the results showed that F-DCs stimulated by CARDS toxin promoted the differentiation of $CD4^{+}IFN\text{-}\gamma^{+}$Th (Th1) cells without affecting the differentiation of Th2 and Th17 cells. Our findings suggest that CARDS toxin is closely associated with the Th1-type immuno-inflammatory response in the lungs after *M. pneumoniae* infection.

We also found that Mφs increased significantly in BALF after *M. pneumoniae* infection; M1-type Mφs were predominant in BALF from children with MPP. Mφs polarize into different activation states, play different roles, and participate in the progression of different diseases (34). Th1 cell differentiation is reportedly mediated by polarized M1-type Mφs through a mechanism that requires NLRP3 inflammasome activation; IL-1β, IL-6, tumor necrosis factor-α, CXCL9, and CXCL10 are the hallmark cytokines of M1-type Mφs (35, 36). PMA-induced differentiation of THP-1 cells into Mφs is a common *in vitro* model for the analysis of monocyte differentiation (37). Here, we

used PMA to induce THP-1 differentiation *in vitro*; we found that IFN-γ promoted the expression of CXCL9 in a dose-dependent manner at the same time. Upon stimulation with the same concentration of IFN-γ, the level of CXCL9 expression increased significantly. These findings showed that the activation of M1-type Mφs was initiated by IFN-γ stimulation; the activated M1-type Mφs then produced CXCL9, Lo et al. (15) reported similar results.

Inflammatory and growth factors often rely on the JAK/STAT signaling pathway to transmit signals that regulate biological effects such as cell growth, proliferation, survival, and inflammatory responses (38). JAK/STAT1 is activated by IFN-γ and plays a central role in Mφ differentiation, maturation, and host defense against pathogen infection (39–41). siRNAs degrade mRNAs with homologous complementary sequences; this loss-of-function mechanism is an important tool for analysis of the roles of genes in biomedical studies (42). To explore the mechanism underlying IFN-γ activation of the CXCL9 signaling pathway, we stimulated Mφs (THP-1+PMA induction) with IFN-γ (1 ng/ml) for 24 h; the level of CXCL9 expression significantly increased. After Mφs had been transfected with STAT1-siRNA-1, the level of CXCL9 expression significantly decreased. These results showed that STAT1-siRNA-1 mediated the reduction of CXCL9 expression in M1-type Mφs by downregulating the level of STAT1 expression. Therefore, the IFN-γ-JAK/STAT1-Mφ-CXCL9 pathway carefully regulates the host immune response in terms of gene and protein expression.

Our study also revealed that CXCL9 was produced in the lower Transwell chamber after Mφs had been stimulated with IFN-γ (1 ng/ml), thus promoting Th1 cell migration. After Mφs had been transfected with STAT1-siRNA-1, the level of CXCL9 expression decreased and the number of migrating Th1 cells significantly decreased. These results confirmed that CXCL9 promoted the migration of Th1 cells, and the migration of Th1 cells was correlated with the concentration of CXCL9.

To sum up, CARDS toxin promoted a Th1-type immuno-inflammatory response in the lungs after *M. pneumoniae* infection. Subsequently, IFN-γ activated the JAK/STAT1 signaling pathway and promoted the secretion of CXCL9 by M1-type Mφs; CXCL9 promoted the migration of more Th1 cells to the inflammatory response center, thereby forming a type 1 immune response positive feedback loop that amplified the inflammatory cascade and aggravated immunity-related damage in lung tissue (Figure 6).

Limitations

An important limitation in this study was that we did not characterize the specific mechanism by which CARDS toxin stimulates F-DCs and promotes Th1 cell differentiation. We will continue to focus on this mechanism in future studies.

Conclusions

Our findings suggest that the level of inflammatory factors IFN-γ/CXCL9 expression can provide new biological markers

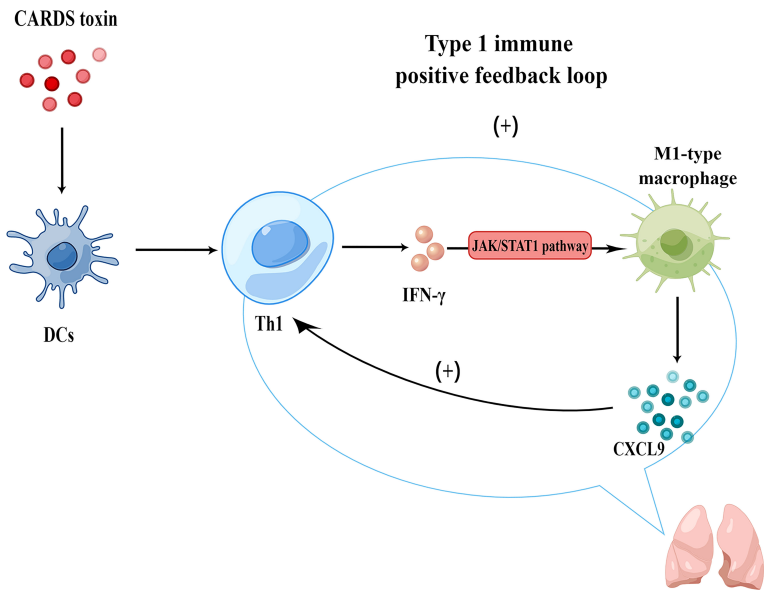

FIGURE 6
Mechanism of CARDS toxin-induced type 1 immune response positive feedback loop in MPP (generated using Figdraw).

for early identification, guidance of treatment and prognosis. CARDS toxin induces a type 1 immune response positive feedback loop during *M. pneumoniae* infection; this putative mechanism may be useful in future investigations of immune intervention approaches for *M. pneumoniae* pneumonia.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Children's Hospital of Soochow University (2019LW014). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by the Ethics Committee of Soochow University (SUDA20200510A02).

Author contributions

(I) Conception and design: YY and ZC; (II) Administrative support: YY and ZC; (III) Provision of study materials or patients: TW, HS, ZL, WJ, and GD; (IV) Collection and assembly of data: TW; (V) Data analysis and interpretation: TW, HS, and ZL; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: YY and ZC. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1054788/full#supplementary-material

SUPPLEMENTARY TABLE S1
List of primers used in experiments.

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Drastic transformation of visceral adipose tissue and peripheral CD4 T cells in obesity

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Obesity has a pronounced effect on the immune response in systemic organs that results in not only insulin resistance but also altered immune responses to infectious diseases and malignant tumors. Obesity-associated microenvironmental changes alter transcriptional expression and metabolism in T cells, leading to alterations in T-cell differentiation, proliferation, function, and survival. Adipokines, cytokines, and lipids derived from obese visceral adipose tissue (VAT) may also contribute to the systemic T-cell phenotype, resulting in obesity-specific pathogenesis. VAT T cells, which have multiple roles in regulating homeostasis and energy utilization and defending against pathogens, are most susceptible to obesity. In particular, many studies have shown that CD4 T cells are deeply involved in the homeostasis of VAT endocrine and metabolic functions and in obesity-related chronic inflammation. In obesity, macrophages and adipocytes in VAT function as antigen-presenting cells and contribute to the obesity-specific CD4 T-cell response by inducing CD4 T-cell proliferation and differentiation into inflammatory effectors *via* interactions between major histocompatibility complex class II and T-cell receptors. When obesity persists, prolonged stimulation by leptin and circulating free fatty acids, repetitive antigen stimulation, activating stress responses, and hypoxia induce exhaustion of CD4 T cells in VAT. T-cell exhaustion is characterized by restricted effector function, persistent expression of inhibitory receptors, and a transcriptional state distinct from functional effector and memory T cells. Moreover, obesity causes thymic regression, which may result in homeostatic proliferation of obesity-specific T-cell subsets due to changes in T-cell metabolism and gene expression in VAT. In addition to causing T-cell exhaustion, obesity also accelerates cellular senescence of CD4 T cells. Senescent CD4 T cells secrete osteopontin, which causes further VAT inflammation. The obesity-associated transformation of CD4 T cells remains a negative legacy even after weight loss, causing treatment resistance of obesity-related conditions. This review discusses the marked transformation of CD4 T cells in VAT and systemic organs as a consequence of obesity-related microenvironmental changes.

KEYWORDS

CD4 T cells, obesity, adipose tissue, osteopontin, immunosenescence

1 Introduction

The start of the obesity epidemic in the US in the early 1970s led to much research on adipose tissue, which regulates metabolic and nutritional homeostasis (1). Then, in the mid-1990s, Hotamisligil et al. proposed the intriguing hypothesis that immune cells are activated in response to a state of excess energy, which induces inflammation and, consequently, insulin resistance (2). Compared with subcutaneous adipose tissue, visceral adipose tissue (VAT) is composed of more diverse cell populations and is highly vascularized and contains numerous sympathetic and sensory nerves. Furthermore, during the development of obesity, a more complex diversity of immune cells arises in VAT than in subcutaneous adipose tissue. Since 2003, when Weisberg et al. and Xu et al. independently reported that macrophages accumulate in obese VAT, macrophages—which express high levels of inflammatory cytokines—have been considered the central players in VAT inflammation. However, subsequent studies revealed that VAT inflammation involves a greater variety of immune cells than previously thought and that not only the innate but also the acquired immune system is activated (3, 4). Obese VAT has a higher proportion of CD4 T cells, which have been recognized as central regulators of chronic VAT inflammation. Even in the lean state, VAT CD4 T cells continue to be weakly activated against an autoantigen specific to VAT. Interestingly, a much larger amount of regulatory T cells (Tregs) are present in VAT than in secondary lymphoid tissues, indicating that local immunometabolic homeostasis is maintained within VAT by enhanced immune tolerance (5).

During the development of obesity, strong activation of effector T cells initiates VAT inflammation (6). Furthermore, in chronic obesity, acute inflammation transitions to chronic inflammation and T cells become dysfunctional (because of T-cell exhaustion and senescence) (7–11). Once obesity has formed, the memory of obesity is imprinted on T cells and does not disappear with weight loss (12, 13); for this reason, weight rebound after weight loss induces more inflammation in the VAT than was present before weight loss (13).

VAT dynamically functions as not only a lipid storage organ but also an endocrine organ that produces a variety of soluble mediators, such as adipokines, cytokines, and lipids. The drastic changes in the VAT microenvironment in obesity affect the phenotype of not only VAT T cells but also systemic ones.

In this review, we summarize what is known about how T-cell activation, differentiation, and function change in response to obesity-associated factors.

2 Regulatory mechanisms of T-cell activity

In general, T-cell activation (survival, proliferation, differentiation, and functional enhancement) requires three positive signals: a T-cell receptor (TCR) signal, co-stimulation signal (CD28 signal), and cytokine signal. The TCR-mediated signal primarily activates the ZAP70-PLCγ pathway; the co-stimulation signal, the phosphatidylinositol-3-kinase-protein kinase B pathway; and the cytokine signal, the Janus family of kinases-signal transducer and activator of transcription (JAK-STAT) pathway (14). To suppress excessive responses, T cells are equipped with brakes on each of these three signals. TCR-mediated signaling is suppressed by programmed cell death 1 (PD-1) (15); PD-1 has an immunoreceptor tyrosine-based inhibitory motif, and upon stimulation by the ligands PD-L1 and PD-L2, it recruits Src homology 2 domain-containing protein tyrosine phosphatase 1, which dephosphorylates and inactivates ZAP7 (16, 17). Co-stimulation is suppressed by cytotoxic T-lymphocyte associated protein 4 (CTLA4), which has an extracellular domain similar to that of costimulatory receptor CD28 and competes with CD28 for binding to its ligand CD80/86; CTLA4 has higher affinity for CD80/86 than CD28 does, but it does not generate a signal (18) and thus physically blocks CD28-mediated signaling (19). And the cytokine signaling JAK-STAT pathway is suppressed by the suppressor of cytokine signaling family of molecules (20).

In addition to known T cell responses, obesity-associated insulin resistance and changes in adipokine secretion profiles all affect T-cell activation, function, and survival, not only in VAT but also in systemic organs (21). Obesity-associated adipocyte hypertrophy and hyperplasia cause VAT microenvironment remodeling, including impaired angiogenesis, deposition of extracellular matrix protein, and hypoxia-induced pyroptosis (22), which may result in changes in T-cell phenotype Obese VAT cells also regulate adipose T-cell activity through antigen presentation and co-stimulatory or co-inhibitory receptor signaling. Obesity-associated disruption of T-cell homeostasis may contribute to the development of an inflammatory state, which is followed by disruption of tissue homeostasis.

3 T-cell activation and suppression

The finding that T cells with a restricted TCR repertoire accumulate in the VAT of diet-induced obese (DIO) mice (23) suggests that in obesity, T cells recognize some kind of antigen. Antigen-presenting cells process an antigen and present it to T cells in the form of an antigen-peptide major histocompatibility complex (MHC). The MHC is recognized by the TCR on the T cell, and the T cell is activated. In addition, antigen-presenting cells highly express co-stimulatory molecules, which pair with co-stimulator receptors on the T-cell surface to modulate T-cell activation (24). Activation of T cells by antigen-presenting cells also plays an important role in triggering the VAT inflammation induced by a high-fat diet (HFD). In VAT T cells, MHC class II (MHCII)-T cell receptor interaction upregulates the expression of inflammatory Th1 marker genes, including *Tbx21* and *Ifng*.

Deletion of MHCII, which plays a role in presenting antigen-derived peptides to CD4 T cells, reduces VAT CD4 T helper type 1 (Th1) cell activity and macrophage accumulation within VAT (23, 25–27).

In VAT, besides the classical antigen-presenting cells, i.e., dendritic cells (DCs) (28), macrophages (27, 29), and B cells (30), adipocytes (26) also play an important role in the formation of T-cell responses as antigen-presenting cells (31). Adipocytes express MHCII molecules and the costimulatory molecules CD80/86 and thus act as antigen-presenting cells and promote CD4 T cell activation (26).Interestingly, in obesity, adipocytes have higher expression levels of MHCII molecules and costimulatory molecules (25).The above findings indicate that during the progression of obesity, antigens presented on MHCII molecules induce T-cell proliferation and differentiation into specific subclasses of inflammatory effectors and that this process is the basis for the initiation and persistence of inflammation in VAT. Knowledge about obesity-related antigens could potentially lead to the development of vaccines and treatments to prevent chronic inflammation of VAT, but unfortunately, such antigens have not yet been identified.

Immune checkpoint molecules maintain immune homeostasis by suppressing self-immune responses and excessive activation of T cells. Co-inhibitory receptors such as CTLA-4 and PD-1 are co-expressed on effector T cells and are involved in immune response homeostasis (19). T-cell exhaustion is characterized by restricted effector function, persistent expression of inhibitory receptors, and a transcriptional state distinct from functional effector and memory T cells. In general, PD-1 is expressed in T cells in response to most immune challenges; however, it is rapidly downregulated during the acute phase of the immune response, allowing for normal immune responses (15). On the other hand, PD-1 expression remains high in chronically stimulated antigen-specific T cells, so the immune response to additional stimulation is impaired (32–35).

T-cell exhaustion has also been identified in VAT in mice and humans with obesity (7, 9, 36).

In fact, in HFD-induced obese mice and patients with type 2 diabetes, adipose T cells are less able than lean adipose T cells to produce cytokines such as IL-2 and IFN-γ (9). HFD-induced obesity increases the PD-1 expression level in adipose T cells, and T cells with high PD-1 expression are a subset of T cells that acquire the exhaustion phenotype (7–9). Various obesity-associated environmental factors lead to heterogeneous T-cell exhaustion profiles, suggesting that obesity may not promote classical T-cell exhaustion.The transient elevation in the expression of immune checkpoint molecules during the priming process, in which native T cells are sensitized and activated by antigen-presenting cells, not only inhibits excessive activation but is also involved in determining the polarity of the effector T cells (37, 38). PD-L1 is expressed in large amounts on DCs in VAT of HFD-fed mice. DC-specific PD-L1 deficiency shifts the polarity of T cells in the VAT of HFD-fed mice toward Th1, exacerbating weight gain and abnormal glucose metabolism (39). PD-L1 expression on DCs is an important factor in suppression of Th1, Th17, and cytotoxic T cells in antitumor responses and autoimmune diseases (40, 41) and appears to play a similar role in the pathogenesis of obesity.

Upregulation of PD-L1 in human adipose tissue is positively correlated with body mass index, but not with type 2 diabetes (42). On the other hand, a negative correlation was reported between body mass index and PD-L1 expression (43). The pathology of human obesity is highly heterogeneous, with multifactorial contributions by dietary quantity and quality, physical inactivity, and genetic factors. Therefore, despite the common phenotype of accumulation of adipose tissue, the number and qualitative changes of immune cells involved in chronic inflammation of VAT are thought also to be diverse. These differences may stem from the different roles of immune cells: They are involved in the initiation, amplification, and/or suppression of chronic inflammation of VAT, and their roles differ greatly depending on the phase of obesity. The role of PD-1/PD-L1 signaling in human VAT T cells remains unclear and requires further elucidation.

4 T-cell metabolism

Differentiation of CD4 T cells into functional subsets is supported by complex metabolic programs (44). Th1, Th17, and Th2 effector cells generate energy by aerobic glycolysis rather than oxidative phosphorylation, whereas Tregs rely on fatty acid oxidation-fueled oxidative phosphorylation rather than glycolysis (45).

Obesity-related alterations in environmental signaling in VAT alter cellular metabolism and contribute to obesity-specific T-cell responses (46, 47). However, it is difficult to measure the metabolic state of VAT T cells in mice because of the complexity of the isolation process, so splenic T cell metabolism has mainly been studied.

Glucose uptake and oxygen consumption are increased in splenic CD4 T cells from obese mice compared with those from lean mice (46). Furthermore, β3-adrenergic receptor stimulation mimics T-cell metabolism in DIO mice and reduces expression of the mitochondrial-localized chaperone protein disulfide bond A oxidoreductase like protein (DsbA-L) in T cells. Although alterations in mitochondrial respiration are an important mechanism controlling cytokine production, loss of DsbA-L in T cells reduces mitochondrial oxidative phosphorylation capacity in both CD4 and CD8 T cells. Mice with T-cell–specific knockout of DsbA-L have reduced IFN-γ–producing Th1 cells in brown adipocytes, enhanced brown adipocyte thermogenic signaling, and less obesity and insulin resistance when fed a HFD (48). Thus, the obesity-related changes in T cell

metabolism greatly contribute to the pathogenesis of obesity, including insulin resistance; however, many aspects of the relationship between metabolism and function specific to VAT T cells remain unclear.

5 Substances that affect T cells in VAT, thymus and periphery

Adipocytes secrete a variety of bioactive substances, collectively referred to as "adipokines." Changes in adipokines associated with the progression of obesity affect T-cell proliferation, differentiation, and function (49). Adipocyte-derived lipids also affect T-cell phenotypes in obesity (Table 1).

5.1 Leptin

Leptin stimulates the satiety center in the hypothalamus. As body fat mass increases, adipocytes produce more leptin, and the serum leptin concentration rises. In normal-weight individuals, the higher serum leptin concentration stimulates the satiety center, which suppresses eating behavior, allowing body fat mass to return to its base level. In the periphery, leptin promotes fatty acid oxidation and glucose uptake in skeletal muscle (64).

Leptin has been reported to have various physiological activities in both normal weight and obese conditions. It also acts on the immune system by promoting T-cell formation by the thymus. Conversely, leptin deficiency results in thymic atrophy and decreased numbers of circulating T cells (50). Administration of leptin to young leptin mutant (ob/ob) and normal mice increases CD4 single-positive thymocytes in the thymus and CD4 T cells in the periphery (51). The long-chain leptin receptor ObRb is expressed on double-negative, double-positive, and CD4 single-positive thymocyte subsets, but not on CD8 single-positive thymocytes (52). Among other things, leptin may promote differentiation from double-positive into CD4 single-positive cells (52).

In obesity, thymus function is reduced despite hyperleptinemia (65). Individuals with obesity are known to have high blood levels of leptin, but their appetite is not suppressed and they are in a leptin-resistant state. The question whether the leptin resistance observed in the hypothalamus is also observed in the thymus or whether other factors, such as adipogenesis of thymic tissue, are involved in obesity-related thymic hypofunction needs further clarification.

Leptin is also involved in the differentiation and proliferation of CD4 T cells in the periphery: TCR stimulation upregulates the expression of leptin receptors on T cells (66), which require leptin for metabolic reprogramming in which activated effector T cells upregulate the glucose transporter Glut1 and enhance the glycolytic system (50). Leptin also promotes differentiation of T cells into Th17 cells *via* T-cell–like leptin receptors (53–57).

In VAT, leptin gene expression in adipocytes begins to increase within 1 week after HFD loading, indicating that leptin may be an initiator of the adipose inflammatory cascade. Leptin promotes differentiation of VAT T cells into Th1 cells and secretion of IFN-γ (26). On the other hand, ob/ob and db/db mice also develop VAT inflammation and severe insulin resistance, suggesting that humoral factors other than leptin may contribute to the initiation of VAT inflammation (67). In addition, in the pathogenesis of chronic obesity, leptin signaling contributes to T-cell exhaustion by activating homeostatic STAT3 signaling and thus inducing PD-1 expression (8).

TABLE 1 Effects of humoral factors on CD4 T cells in obesity.

Humoral factors	Effect on CD4 T cells	References
Leptin	T-cell differentiation in the thymus	(50–52)
	Induction of Glut1 on effector T cells	(50)
	Promotion of Th17-cell differentiation	(53–57)
	Promotion of differentiation of IFN-γ–producing VAT Th1 cells	(26)
	Induction of T-cell exhaustion *via* STAT3 signaling	(8)
Adiponectin	Suppression of T-cell proliferation and cytokine production and promotion of apoptosis	(58–60)
	Suppression of MHC2, CD80, and CD86 on DCs	(21)
	Upregulation of PD-L1 on DCs and suppression of Th1-cell differentiation	(61)
	Inhibition of glycolysis pathway in Th1 and Th17 cells	(62)
Fatty acids	Differentiation of IFN-γ–producing effector T cells	(63)

DCs, dendritic cells; IFN-γ, interferon gamma; PD-L1, programmed death-ligand 1; STAT3, Signal Transducer and Activator of Transcription 3; Th1, T helper 1; VAT, visceral adipose tissue.

In VAT, in which adipocytes are the major constituent cells, T cells may be exposed to high concentrations of leptin. In addition, the degree and duration of obesity, which determine the amount of exposure to leptin, greatly affect T-cell phenotypic changes. Leptin is one of the initiators of VAT inflammation that promotes Th1 differentiation of VAT T cells, and long-term exposure to leptin induces immune exhaustion. Thus, leptin is a key molecule in obesity-induced T cell phenotypic changes.

5.2 Adiponectin

Adiponectin would be expected to prevent obesity and obesity-related diseases by promoting insulin sensitivity and fatty acid oxidation and exerting anti-inflammatory effects (68). However, serum levels of adiponectin are negatively correlated with obesity, and obesity decreases adiponectin receptor expression (68).

In general, most T cells store the adiponectin receptors AdipoR1 and AdipoR2 in intracellular compartments. After antigen-specific stimulation, AdipoR1 and AdipoR2 are transported to the cell surface and expressed along with the receptor CTLA-A and other receptors. In various *in vivo* inflammatory models, adiponectin acts as a negative regulator of effector T cells by suppressing T-cell proliferation and cytokine production and promoting apoptosis (58–60).

Adiponectin also appears to affect the mechanism by which naïve T cells are activated by DCs, and adiponectin treatment reduces the expression of MHCII, CD80, and CD86 on DCs and suppresses production of IL-12p40 (21). Furthermore, PD-L1 expression is increased in adiponectin-treated DCs. Co-culture of adiponectin-treated DCs with allogeneic T cells *in vitro* decreases T-cell proliferation and IL-2 production, and this phenomenon can be partially reversed by blocking the PD-1/PD-L1 pathway (61).

Although the question whether the obesity-related decrease in adiponectin releases the brake on the inflammatory response of VAT T cells remains unanswered, *in vitro* research suggests that adiponectin may suppress the inflammatory response of obesity-activated T cells. In fact, obesity enhances the glycolysis pathway in splenic Th1 and Th17 cells, and adiponectin inhibits glycolysis in both an AMPK-dependent and -independent manner, resulting in the amelioration of inflammation (62). In obese patients, adiponectin expression is reduced not only in adipose tissue but also in serum (69). The reduction in VAT-derived adiponectin may affect systemic immune function and contribute to the development of obesity-specific inflammatory conditions.

5.3 Free fatty acids

During the progression of obesity, lipids released from adipocytes are also involved in the enrichment of IFN-γ–producing CD4 T cells in VAT (63). The differentiation of TCR-stimulated IFN-γ–producing effector T cells is enhanced by co-culture with adipocytes (62). Among the soluble factors, fatty acids were shown to be the strongest modulators of differentiation into Th1 (63). Patients with obesity have high serum fatty acid concentrations, showing that the bias for differentiation of naïve T cells into IFN-γ–producing Th1 cells is not limited to VAT. Indeed, upon antigen presentation in secondary lymphoid tissues, T cells primed with excess saturated fatty acids have been shown to undergo biased differentiation into pro-inflammatory effector memories that tend to cluster in pro-inflammatory nonlymphoid tissues such as obese adipose tissue and atherosclerotic lesions (70).

Because VAT CD4 T cells appear to acquire unique characteristics that differ from those of CD4 T cells in other organs, including blood and spleen, it is unclear whether fatty acids have the same effects on CD4 T cells in VAT as they do on those in blood. However, VAT CD4 T cells are exposed to higher concentrations of fatty acids, which may result in higher production of IFN-γ and development of VAT inflammation.

6 Homeostatic and pathogenic role of CD4 T cells in VAT

From an immunological perspective, VAT is a unique environment. In the physiological (non-obese) state, T cells are kept weakly activated against a self-antigen specific to VAT, and Tregs that are highly reactive to self-antigens are also assembled. In this way, peripheral immune tolerance is enhanced to suppress inflammation in VAT and maintain immunometabolic homeostasis. During the development of obesity, the storage of excess fat leads to further enhancement of T-cell activation signals. However, in the chronic phase of obesity, T cells in VAT become dysfunctional. In this section, we describe how the immune-tolerant state of VAT is breached in obesity, how T-cell activation affects adipocyte function, and how T-cell transformation is involved in chronic VAT inflammation.

6.1 T cells under homeostatic conditions in a lean state

Endogenous tissue Tregs differentiate and mature in response to tissue-specific environmental signals and play an important role in organ homeostasis (5). VAT Tregs are functionally specialized tissue-resident cells that prevent obesity-associated inflammation and maintain insulin sensitivity and glucose tolerance (71). Under homeostatic conditions in a lean state, VAT has a large population of Tregs, and research showed that as non-obese mice age, Tregs accumulate in the VAT (72). Indeed, studies in mice found that at 20 to 30 weeks of age, VAT Tregs represent a surprisingly high percentage (50%-80%) of the CD4

T-cell compartment (73, 74). For reference, the proportion of Tregs in the CD4 T-cell compartment in spleen and lymphoid tissues is about 5% to 15%.

VAT is thought have such a high proportion of CD4 T cells because it requires Tregs for immunological homeostasis. Within VAT, T cells continue to be loosely activated by autoantigens in adipose tissue. Tissue-resident Tregs play an important role in preventing the activation of effector T cells and the accompanying dysfunction of adipocytes and in maintaining systemic insulin sensitivity.

There are two types of Tregs: Tregs that are generated in the thymus (referred to as naturally occurring or thymic Tregs) and Tregs that differentiate from naïve CD4 T cells in the periphery (peripheral Tregs) (75).

The intestinal lamina propria has a large population of Tregs to prevent excessive immune responses to dietary components and intestinal bacteria; the population comprises peripheral Tregs, which increase locally depending on intestinal bacteria, and thymic Tregs (76). When all germs were experimentally removed from the intestinal mucosa, the population of Tregs in the intestinal mucosa decreased to the level seen in lymphoid tissues (61). Among intestinal bacteria, *Clostridium* species are known to be a strong inducer of peripheral Tregs (76). In VAT, most Tregs are thymic Tregs. CD4 T cells that undergo negative selection in the thymus and do not die express the lineage-determining transcription factor Foxp3 to become Tregs, which are self-recognizing and thus inherently more likely to invade self-tissue. Compared with Tregs in lymphoid tissue, VAT Tregs have a unique TCR repertoire that exhibits specific antigen recognition (77), meaning that VAT Tregs react to certain antigens specific to VAT.

Foxp3 and the signal-dependent transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ), as well as signaling by the cytokine IL-33 through the IL-33 receptor ST2, are important for the proliferation and functional maturation of Tregs in VAT (78). VAT Tregs express insulin receptors, which are rarely expressed on Tregs in lymphoid tissues, and highly express PPAR-γ and the ST2 receptor in an insulin signaling-dependent manner (79). VAT Tregs have transcripts driven by PPARγ that differ from lymphoid-organ and other nonlymphoid-tissue Treg populations (80). Mice lacking Treg-specific PPARγ have greatly reduced VAT Tregs.

In mice, the VAT Treg population was found to show sex differences and IL-33 was found to be particularly important for the maintenance of Tregs in adipose tissue of males (81). Under homeostatic conditions in a lean state, the Treg population is larger in male than in female mice. In female mice, adipose tissue inflammation is suppressed by estrogen, and the expression of inflammatory cytokines such as IL-6, C-C motif chemokine ligand 2, and IL-1b in VAT is more pronounced in males than in females (81). Consistent with this finding, male mice are more insulin resistant than females. In males, the environment of higher inflammation in the VAT causes more Tregs to be recruited from the spleen. Androgens regulate the differentiation of IL-33–producing stromal cell populations specific to VAT in males (81).

In male mice, VAT Tregs upregulate expression of the ST2 receptor in a manner dependent on transcription factor Blimp1, which is also highly expressed in males; the upregulated expression leads to local expansion of VAT Tregs in an IL-33 signaling-dependent manner (81).

VAT Tregs also enhance IL-10 production in a Blimp1-dependent manner. IL-10 produced by VAT Tregs suppresses not only effector T activity but also white adipose tissue browning (82). Because female mice have smaller VAT Treg populations, they have lower IL-10 levels and are thus more prone to white adipose tissue browning; lower IL-10 may also be one of the reasons why female mice are protected from white adipose tissue accumulation and glucose intolerance compared with age-matched male mice.

As obesity progresses, VAT Tregs suppress inflammation by regulating effector T cells, DCs, and macrophage activity directly or *via* IL-10 production (74). Tregs, which constantly express CD25 (also known as IL-2 receptor alpha chain), preferentially bind to IL-2 to inhibit proliferation of effector T cells (74). Tregs express the adhesion factors lymphocyte function-associated antigen-1 (LFA-1) and CTLA4, and the former enables them to strongly adhere to DCs (83). In addition, Tregs downregulate the expression of CD80/86 on DCs in both a CTLA-4- and LFA-1-dependent manner (83). IL-10 acts mainly on DCs and macrophages to suppress production of IL-12 and tumor necrosis factor alpha (TNFα) and the expression of CD86, MHCII, and CD40 by strongly activating STAT3 (84).

In severe obesity, the Treg cell population is reduced to 10% to 20% of the CD4 T-cell compartment (73, 74). One of the reported mechanisms for this decrease is the presence of ST2 soluble isoforms, which are secreted by adipocytes in obesity and function as decoy receptors for the ST2 receptor to weaken IL-33 signaling (85, 86).

The immune compartments of adipose tissue are markedly different in mice, humans, and primates: Lean male mice have a high proportion of Tregs in VAT, but the proportion is much lower in female mice, humans, and cynomolgus macaques (87). In healthy lean humans, the proportion of Tregs is significantly lower in VAT than in peripheral blood; however, OX40-expressing Tregs, which have potent suppressive activity and high proliferative capacity, are selectively distributed in VAT in all individuals and further increase with obesity. Upregulation of OX40 Tregs is thought to be a protective mechanism that suppresses excessive inflammation (88).

Although the actions of immune cells in the maintenance of VAT homeostasis appear to differ greatly between species, Tregs play an important role in regulating VAT inflammatory across species.

6.2 Early stage of obesity-induced inflammation in adipose tissues

The main players in VAT inflammation in obesity are macrophages. In the lean state, macrophages localize diffusely in the VAT, but in obesity, they explode in number and become intensively localized as crown-like structures, which comprise clusters of macrophages surrounding dead adipocytes (89). A wide variety of T cells modulate monocyte recruitment to the VAT and macrophage proliferation and differentiation in the crown-like structures (90).

CD8 T cells were first noticed as initiators of VAT inflammation. CD8 T-cell infiltration into VAT precedes accumulation of macrophages, and genetic deletion of CD8 T cells reduces VAT inflammation and ameliorates systemic insulin resistance. Adoptive transfer of CD8 T cells into CD8-deficient mice exacerbates VAT inflammation (91).

CD4 T cells also regulate VAT inflammation. Macrophage-driven inflammation is induced by the Th1 response. In VAT, this response is induced by a HFD. Th1 cells produce TNFα, which activates the vascular endothelium and promotes monocyte invasion, and IFN-γ, which induces polarization of proinflammatory M1 macrophages (92).

Thus, Th1 cells contribute to the recruitment of monocytes into the VAT and their differentiation into M1 macrophages (26). IFN-γ suppresses insulin signaling in mature adipocytes, which attenuates insulin-dependent glucose uptake and lipid storage, and also inhibits differentiation of pre-adipocytes to mature adipocytes (93). In sum, Th1 cells play a central role in the induction of early adipose inflammation and adipocyte dysfunction associated with a HFD. Th1 cells are elevated in VAT in patients with type 2 diabetes and correlate with obesity-induced inflammation and insulin resistance (94). In human adipocytes, IFN-γ sustains activation of the JAK/STAT1 pathway, attenuates lipid storage and insulin signaling, and suppresses differentiation (93). Indeed, obese IFN-γ knockout mice have smaller adipocytes and less accumulation of VAT inflammatory cells, resulting in improved insulin sensitivity (95).

In HFD-induced obese mice, the Th17 cell population in the spleen and circulating levels of IL-17 are increased in an IL-6–dependent manner, and IL-17 inhibits insulin signaling in hepatocytes and glucose uptake in skeletal muscle (96). IL-17 has a modulatory effect on adipocytes, inhibiting lipid uptake and glucose uptake by insulin and suppressing adipogenesis (97). Obese insulin-resistant patients have more Th17 cells in their abdominal subcutaneous visceral fat than insulin-sensitive obese patients (98). Furthermore, in obese and metabolically unhealthy individuals, Th17 cells infiltrate obese adipose tissue and Th17 cytokines promote TNF-α production and induce inflammation (99).

Th2 cytokines, such as IL-4 and IL-13, have complex effects in VAT, i.e., they do more than antagonize Th1 cytokines and exert anti-inflammatory effects. In healthy adipose tissues, metabolic homeostasis is maintained by local IL-4 secretion by VAT eosinophils and maintenance of alternatively activated macrophages. In the absence of eosinophils, the numbers of alternatively activated macrophages in VAT are greatly reduced (100). In obese humans, the number of Th2 cells is decreased in subcutaneous and visceral fat and peripheral blood and Th2 frequency is inversely correlated with insulin resistance and serum levels of C-reactive protein, a marker of systemic inflammation (101). Th2 cytokines are abundant in the crown-like structures of obese VAT, where macrophages actively proliferate in a manner dependent on the IL-4 receptor α-chain (IL-4Rα), a molecule essential for Th2 signaling. In these structures, IL-6 functions as a major driver of proliferation of VAT macrophages in obesity by upregulating IL-4Rα (102). The role of the Th2 cytokines IL-4 and IL-13 in VAT inflammation caused by a HFD load is supported by the finding that inflammation is reduced in mice with myeloid cell-specific knockout of IL-4Rα (103). Thus, in the obese environment, Th2 cytokines play an important role in the maintenance of fast-proliferating macrophages within the crown-like structures (102). In the physiological state, innate lymphoid cells (ILC2s) are important producers of type 2 cytokines, which are critical for maintenance of alternatively activated or M2-like adipose tissue macrophages and glucose homeostasis (104). In obese VAT, ILC2s regulates saturated fatty acid absorption, resulting in the amelioration of chronic inflammation (105); however, in obesity the number of ILC2s decreases in both mouse and humans (104). The sources and roles of type 2 cytokines appear to be significantly different between VAT homeostasis and obesity-induced exacerbation of chronic inflammation, and further research is needed.

6.3 Obesity-induced chronic inflammation in adipose tissues

Overeating activates the effector function of T cells in VAT and initiates inflammation of the tissue. However, the mechanism by which this inflammation becomes chronic is still being elucidated. Recent studies have revealed that T-cell dysfunction due to both T-cell exhaustion and aging is involved in the chronicity of VAT inflammation, and these two aspects are discussed below.

6.3.1 T-cell exhaustion

As a result of chronic inflammation, obese T cells exhibit an exhausted phenotype that includes long-term antigen-stimulation, stress responses, and hypoxia (106). Given that a significant proportion of VAT CD4 T cells are not recruited from the circulation and proliferate *in situ* (107), T-cell exhaustion can be expected to significantly interfere with

antigen-specific and memory responses. Soluble factors from the obese stromal vascular fraction inhibit activation of VAT T cells, suggesting that the microenvironment of obese VAT may trigger T-cell exhaustion (9). In mice, chronic HFD intake attenuates the inflammatory capacity of effector T cells in VAT such that they lose their ability to respond to TCR-specific stimulation (9). Strikingly, VAT T-cell dysfunction has been suggested to occur early, i.e., before macrophage infiltration into the VAT (9).

VAT T cells from DIO mice fed a HFD for 18 weeks fail to upregulate CD25 or secrete T cell effector cytokines such as IFN-γ and IL-2 (9). CD25 is required for formation of TCR-stimulated, high-affinity IL-2 receptors. The same is true for VAT T cells in humans with obesity. On the other hand, the ability of Tregs to suppress effector T cells is reduced by downregulation of CD25.

The dysfunction of VAT T cells in DIO mice involves persistent antigen presentation from antigen-presenting cells. Although many T cells in the VAT of DIO mice express high levels of PD-1, blocking PD-1 in the VAT is not able to restore the ability to produce cytokines upon TCR stimulation (9).

In addition to persistent antigen presentation by antigen-presenting cells, some soluble factors in obese VAT are involved in T-cell dysfunction. One candidate for soluble factors is leptin (see also section 5.2). The high levels of leptin associated with obesity lead to T-cell exhaustion by inducing PD-1 expression through strong activation of STAT3 (8, 108). However, adipose tissue T cells from db/db mice also cause T-cell exhaustion (9), suggesting that it involves other humoral factors besides leptin.

Obese VAT is in a state of insulin resistance, in which glucose uptake and fatty acid synthesis are suppressed in the presence of high insulin levels and, conversely, triglycerides are broken down and fatty acids and glycerol are released (109). Impairments in angiogenic capacity also occur in obese VAT. Inhibition of angiogenesis leads to rarefaction of capillaries, impaired proliferation of multipotent progenitor cells, and adipocyte hypertrophy (110). These changes in the environment surrounding T cells may affect the intracellular metabolic system and induce T-cell exhaustion.

Aerobic glycolysis is activated when TCRs are stimulated and suppression of the glycolytic system induces elevated PD-1 expression and T-cell exhaustion, which decreases IFN-γ production. One group proposed a mechanism by which, when the glycolytic system is suppressed, glyceraldehyde 3-phosphate dehydrogenase binds to the 3'UTR of IFN-γ mRNA and inhibits its translation, resulting in decreased IFN-γ production (111).

In the cancer microenvironment, glucose deprivation by cancer cells results in reduced T-cell glycolytic flux, decreased IFN-γ production, and increased PD-1 expression (111). *In vitro* co-culture experiments have shown that in the cancer microenvironment, metabolic constraints, rather than chronic antigen stimulation per se, may be responsible for T-cell exhaustion (112). In the obese environment, a reduction in the glycolytic flux of VAT T cells may also accelerate T-cell exhaustion.

PD-1 is one of the indicators of T-cell exhaustion, and inhibitors of the PD-1/PD-L1 axis, which restores T cells from exhaustion, have provided major clinical breakthroughs in cancer (16, 113–115). PD-1 expression on T cells is elevated in people with obesity (8). Highly exhausted T cells are susceptible to PD-1 blockade, and obesity is positively correlated with the efficacy of PD-1/PD-L1 inhibitors in patients and mice with cancer (8, 116, 117), together suggesting that obesity induces T-cell exhaustion.

Obesity also induces CD4 T-cell exhaustion in obese VAT, and exhausted CD4 T cells highly express PD-1 (7). However, the finding that PD-1 blockade does not improve VAT CD4 T-cell exhaustion indicates that VAT CD4 T cells may have acquired a unique exhaustion phenotype independent of PD-1/PD-L1 signaling (9). CD8 T cells also appear to acquire diverse exhaustion traits in obese VAT. VAT CD8 T cells from obese mice highly express T-cell exhaustion markers such as *Pdcd1*, *Tox*, *Entpd1*, *Tigit*, and *Lag3* and have an exhaustion profile similar to T cells chronically infected with Lymphocytic choriomeningitis virus (36). On the other hand, obese VAT CD8 T cells do not have increased immune checkpoints such as *TIM-3*, *LAG-3*, *TIGIT*, and *EB4* or exhaustion markers such as *TOX*, *TCF-7*, and *Eomes*; instead, VAT CD8 T cells upregulate the co-inhibitory receptor B and T Lymphocyte Attenuator (*Btla*), *Nlrc3*, and *Dicer1* genes, which suppress TCR signaling by a mechanism different from that of PD1 signaling (118).

Differences in T-cell exhaustion profiles in VAT may result from the intensity and duration of exposure to specific antigens associated with HFDs and obesity. Immunosuppressive signals of VAT are also considered to be a defense mechanism that suppresses excessive obesity-related inflammation of VAT. However, VAT is an important reservoir of immune cells in obesity, and exhaustion of VAT T cells may be disadvantageous in case of various pathologies, such as infectious diseases and cancer.

To sum up, in developing obesity, a HFD load causes acute inflammation by inducing excessive activation of VAT T cells. On the other hand, in the chronic phase of obesity, inhibitory signals suppress the normal VAT T cell response. The fact that chronic inflammation in VAT persists suggests that the presence of not only T-cell exhaustion but also cell-intrinsic functional changes in VAT T cells, which more actively sustain inflammation.

6.3.2 Regression of the thymus and maintenance of T cells by homeostatic proliferation

TCR spectratyping analyses show that diet-induced obesity causes thymic regression and limits TCR diversity. Obesity due to melanocortin-4 receptor deficiency, the most common genetic cause of human obesity, also reduces T-cell repertoire diversity (65). A key factor in obesity-induced thymic regression is the conversion of thymic fibroblasts to adipocytes as a result of lipid

accumulation (119). Adipocyte proliferation leads to an increase in leukemia inhibitory factor, oncostatin M, and IL-6, which inhibits thymic function and induces thymocyte apoptosis, resulting in a compromised pool of T-cell progenitor cells (120). After thymic degeneration, peripheral T cells are maintained by homeostatic proliferation. In humans, homeostatic proliferation of circulating CD4 T cells is accelerated in individuals with obesity (121).

6.3.3 Mechanism of the repertoire restriction of T cells

VAT T cells have significantly less TCR diversity than splenic T cells, and obesity exacerbates this difference (23). Repeated antigen stimulation, the effects of antigen-presenting cells themselves, and homeostatic proliferation may contribute to the repertoire restriction of T cells.

After thymic degeneration, peripheral T cells are maintained by homeostatic proliferation, but a significant proportion of VAT CD4 T cells are not recruited from the circulation (107). Furthermore, VAT contains a huge pool of T cells in obesity (122). These findings suggest that to compensate for the decrease in VAT T cells, VAT T cells are activated and maintained by repeated antigen stimulation rather than by homeostatic proliferation. Studies showed HLA- and MHC2-mediated activation of T cells by antigen-presenting cells in obese humans and mice (25–27, 123, 124), suggesting that repeated stimulation with some kind of antigen may result in TCR repertoire restriction. Differences in the expression of various co-stimulatory and inhibitory receptors expressed on antigen-presenting cells also influence T-cell phenotypic changes (24–27, 29, 31), so antigen-presenting cells may also contribute to T-cell repertoire restriction.

On the other hand, a specific subset of T cells that emerges with obesity may be maintained through homeostatic proliferation because studies showed that during the physiological aging process, T cells are maintained through homeostatic proliferation triggered by homeostatic cytokines and MHC associated with self-peptides (123, 124) and we previously found that obesity accelerates T-cell senescence and that senescent T cells have similar characteristics to T cells created by homeostatic proliferation during aging (7). Although no studies have directly demonstrated homeostatic proliferation of T cells in VAT, we hypothesize that obesity-associated senescent T cells may be maintained by homeostatic proliferation in the same way as aging-associated senescent T cells.

6.3.4 Obesity-induced T cell senescence

Cellular senescence is triggered by DNA damage, telomere dysfunction, inflammation, and metabolic dysfunction and is accompanied by irreversible cell cycle arrest and the acquisition of the senescence-associated secretory phenotype (125). Oxidative stress, inflammation, and repeated antigenic stimulation associated with obesity may induce shortening of telomere length and accelerate cellular aging (111, 126, 127). Obesity is also associated with leukocyte DNA methylation changes that can lead to immune dysfunction (128). However, the effects of long-term exposure to obesity on gene expression and metabolic status in T cells remain to be elucidated.

In mice fed a HFD, the absolute number of CD4 T cells per gram of VAT continues to increase as obesity progresses. One study attributed this increase in the absolute number of VAT CD4 T cells to an increase in antigen-stimulated activated $CD44^{hi}CD62L^{lo}$ cells (7). In this study, a unique population of $CD44^{hi}$ $CD62L^{lo}$ CD4 T cells that constitutively express CD153 and PD-1 was found to exhibit cellular senescence properties. A $CD153^{+}$ $PD\text{-}1^{hi}$ subset expressing T-bet, the master transcription factor of Th1, had high senescence-associated beta-galactosidase activity and were positive for the DNA damage marker γH2AX, indicating characteristics of cellular senescence. The authors concluded that $CD153^{+}$ $PD\text{-}1^{hi}$ subset continues to secrete abundant osteopontin (OPN) without PD-1–mediated negative signaling inhibition at the cost of normal function and causes VAT inflammation (7). In fact, OPN is elevated in the circulation of patients with obesity and enhances VAT inflammation, leading to the development of diabetes (129, 130).

OPN functions as a potent chemoattractant for macrophages. Chronic inflammation is characterized by macrophage retention, and OPN is a particularly important molecule in promoting macrophage migration and retention. OPN also regulates cytokine production by macrophages and acts on T cells to promote IL-12 production while inhibiting IL-10 production and promoting Th1 cell-mediated responses (131).

A portion of Th1 effector cells terminally differentiates into OPN-producing senescent T cells, and increased numbers of senescent T cells produce large amounts of OPN and maintain high levels of OPN in VAT (7). In turn, high OPN levels cause persistent accumulation and activation of macrophages and induce a Th1 response. These experimental results suggest that, at least in mice, VAT CD4 T cells that have deviated from immune checkpoint mechanisms and acquired a senescent phenotype continue to produce OPN independently of classical TCR stimulation and that this process is closely related to the maintenance of chronic inflammation in obesity. Recently, vaccination targeting CD153 was shown to reduce senescent cells in visceral fat of obese mice fed a HFD for 10 to 11 weeks and to improve VAT inflammation and insulin resistance (132). Senescent T cells were suggested as a potential therapeutic target for obesity-associated immunometabolic disorders.

$CD153^{+}$ $PD\text{-}1^{hi}$ CD4 T cells were originally identified as a characteristic T-cell subset that emerges with aging and were named senescence-associated T cells (124, 133). Noteworthy is that the same cells also appear in the VAT microenvironment in obesity and are involved in chronic local inflammation, making T cell senescence a common underlying pathology for both obesity-related and aging-related diseases.

VAT CD4 T cells play an important role in regulating inflammation and metabolism in obesity, but the underlying mechanisms are largely unknown. Recently, DIO was shown to

increase p38 activity in VAT T cells and promote obesity-associated adipose tissue senescence. p38α, an essential subunit of p38, promotes T-cell glycolysis through a mechanistic target of rapamycin signaling, resulting in enhanced Th1 differentiation. T cell-specific deletion of p38α protected mice from HFD-induced obesity, fatty liver, adipose tissue inflammation, and insulin resistance (134).

To sum up, the negative linkage between obesity-accelerating T-cell senescence and T-cell activation-accelerating VAT senescence plays an important role in the development of chronic VAT inflammation (Figure 1).

7 Negative legacy of obesity

Obesity can be corrected by weight loss. However, weight loss through caloric restriction and increased physical activity is not as easy as it sounds, and even if weight is lost, it may be regained quickly. Traditionally, bariatric surgery has been performed in severely obese patients who are refractory to medical therapy because of a combination of factors such as dietary environment and unique personality traits. With the recent launch of a glucagon-like peptide 1 receptor agonist and dual glucose-dependent insulinotropic polypeptide/glucagon-like peptide 1 receptor agonist, long-term maintenance of successful lost weight by medication has finally become feasible (135, 136). However, studies on the long-term effects of weight reduction on cardiovascular events and life expectancy in patients with obesity and type 2 diabetes have yielded conflicting results. A meta-analysis showed that weight reduction is associated with reduced mortality in unhealthy individuals with obesity (137), and bariatric surgery reduced the incidence of myocardial infarction in patients with obesity and type 2 diabetes over a mean follow-up period of 13.3 years (138). However, a prospective randomized study found that an intensive lifestyle intervention focused on weight reduction was not associated with reduced cardiovascular disease and mortality in adults with overweight or obesity and type 2 diabetes at almost 10 years of follow-up (139).

The phenotypic changes in T cells upon weight reduction in obesity appear to be very complex. In obese mice, weight reduction induces the accumulation of CD4 T cells in VAT, resulting in the recruitment and retainment of pro-inflammatory

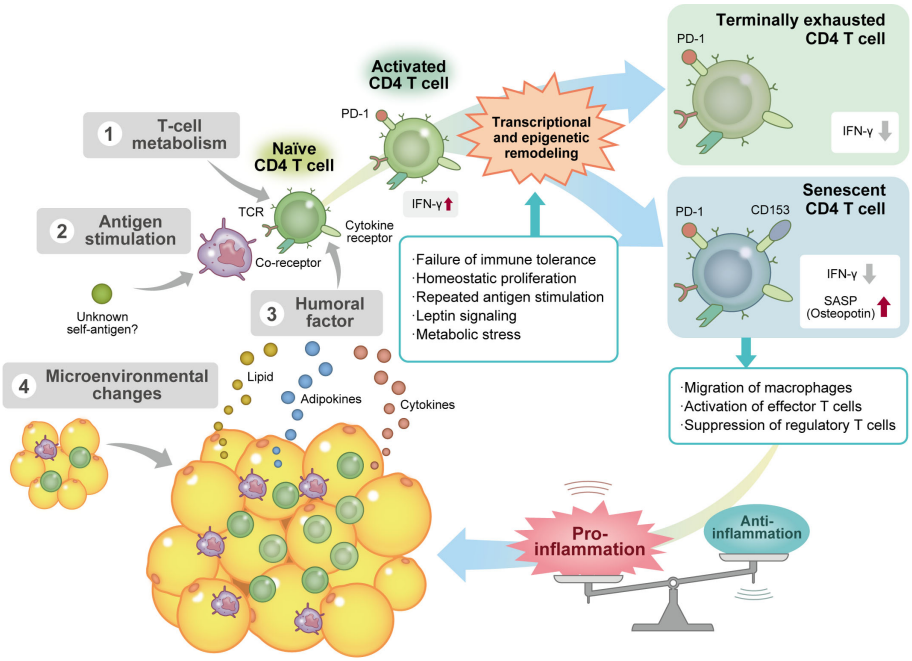

FIGURE 1
Obesity induces CD4 T-cell senescence *via* several mechanisms. Factors affecting the differentiation and maturation process of visceral adipose tissue (VAT) CD4 T cells in obesity include 1) alteration of T-cell metabolism; 2) repeated antigen stimulation by antigen-presenting cells, such as macrophages and dendritic cells; 3) various humoral factors, such as cytokines, adipokines, and lipids; and 4) changes in the VAT microenvironment. These stimuli activate T helper 1 (Th1) CD4 T cells and induce various types of transcriptional and epigenetic remodeling. Chronic activation of T cells induces immune exhaustion and reduces the secretion of proinflammatory cytokines, including interferon γ. On the other hand, some Th1 effector T cells deviate from the immune checkpoint mechanism and acquire a cellular senescence phenotype without anergy because of repeated antigenic stimulation. Senescent CD4 T cells that produce a large amount of osteopontin accelerate macrophage migration and effector T-cell activation and suppress regulatory T cell function, resulting in the maintenance of chronic VAT inflammation. Ifn-γ, interferon γ; PD-1, programmed cell death protein 1; SASP, senescence-associated secretory phenotype; TCR, T-cell receptor.

macrophages in VAT despite normalization of body weight (13, 140). In mice with weight reduction after being switched from a HFD to a control diet, body weight and visceral fat decreased to the same level as lean mice fed the control diet; however, the VAT of the weight-loss mice showed dense infiltration of macrophages, which formed more crown-like structures than those in HFD-fed obese mice. Mechanistically, $CD153^{+}$ $PD\text{-}1^{hi}$ CD4 T cells are long-lived and not easily eliminated after weight loss, and the continued presence of senescent T cells is associated with the production of large amounts of OPN, creating a chronic inflammatory loop (Figure 2). Thus, senescent CD4 T cells are suggested to be a negative legacy effect of obesity (13).

Interestingly, in a weight gain-loss-regain model, mice with a history of obesity regain weight very quickly. Although the molecular mechanism for this obesity memory is unknown, CD4 effector T cells were shown to be involved (12). Furthermore, one study reported that glucose tolerance was worse in DIO weight-loss-regain mice than in normal DIO mice and that this metabolic dysfunction was associated with increased T-cell accumulation in VAT (36).

The above experimental results explain the body's propensity to repeatedly gain and lose weight, which makes it difficult to maintain weight loss and increases the risk of developing diabetes. Noteworthy is that the background imprinting of an obesity-related immune phenotype is present in VAT.

8 Conclusions

VAT is not only a storage organ for lipids, but also an endocrine organ that regulates energy balance in the body by secreting various bioactive substances called adipokines, which are involved in food intake and insulin resistance. Dysregulation of adipokine function and production in obese VAT plays a major role in the development and progression of metabolic syndrome. Furthermore, it is becoming clear that various immunocompetent cells, including macrophages, increasingly infiltrate obese VAT and that the accompanying mild systemic chronic inflammatory response is the underlying pathogenesis of lifestyle-related diseases such as metabolic syndrome.

In this review, we sought to summarize the role of CD4 T cells in maintaining VAT homeostasis, inflammation caused by excessive fat accumulation in VAT, and the chronicity of this inflammation. In addition to TCR signals, co-stimulation

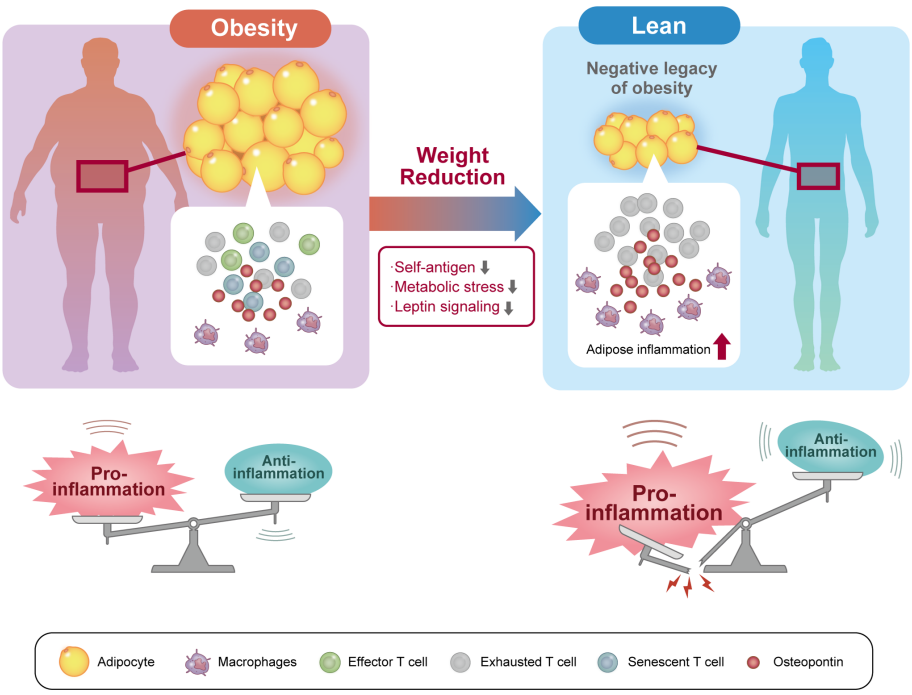

FIGURE 2
Senescent CD4 T cells are a negative legacy of obesity. In the pathophysiology of obesity, not only effector and exhausted T cells but also senescent T cells accumulate in visceral adipose tissue (VAT). VAT inflammation induced by these VAT T cells induces systemic insulin resistance and contributes to the pathogenesis of diabetes. Weight reduction reduces exposure to self-antigens, metabolic stress, and humoral factors in the VAT microenvironment. However, long-lived senescent CD4 T cells concentrate in VAT after weight loss and continue to secrete proinflammatory osteopontin. As a result, VAT inflammation persists after weight reduction and may represent a residual risk factor for cardiovascular disease.

signals, and cytokine signals from antigen-presenting cells, T cells undergo tissue-specific differentiation in response to organ- and disease-specific environmental signals.

To date, most of the findings on chronic inflammation of VAT associated with obesity are from analyses of DIO mice fed a HFD rich in saturated fatty acids. Therefore, future research needs to carefully examine the extent to which findings from DIO mice can be extrapolated to humans. Epidemiological evidence indicates that obese VAT increases the risk of developing cardiovascular disease. However, the mediators that affect remote organs *via* immunological changes in VAT remain unclear. In obesity, ectopic fat accumulates around the heart and blood vessels, and development of cardiovascular disease may be more strongly influenced by this neighboring ectopic fat. Obesity also affects systemic immune function. When considering the relationships between obesity and infectious diseases, cancer, and autoimmune diseases, the hypothesis is attractive that immunological abnormalities in obese VAT can also affect systemic immune function. In fact, some research suggests such an association, but proving a causal relationship remains a challenge for the future. Nevertheless, an understanding of the qualitative and quantitative changes of immune cells within the microenvironment in VAT will undoubtedly help to elucidate the mechanisms by which obesity causes various diseases, including lifestyle-related ones, *via* excessive local or systemic activation of the immune system. In the future, tailored therapeutic strategies based on an understanding of immune cell trait changes may contribute to improving the prognosis of obesity-related diseases.

Author contributions

Both KS and MS contributed to conceptualization, writing (original draft preparation, review, and editing), and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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T cells and their products in diabetic kidney disease

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Diabetic kidney disease (DKD) is the most common cause of end-stage renal disease and has gradually become a public health problem worldwide. DKD is increasingly recognized as a comprehensive inflammatory disease that is largely regulated by T cells. Given the pivotal role of T cells and T cells-producing cytokines in DKD, we summarized recent advances concerning T cells in the progression of type 2 diabetic nephropathy and provided a novel perspective of immune-related factors in diabetes. Specific emphasis is placed on the classification of T cells, process of T cell recruitment, function of T cells in the development of diabetic kidney damage, and potential treatments and therapeutic strategies involving T cells.

KEYWORDS

diabetic kidney disease, T cells, cytokines, immunologic function, differentiation, recruitment, therapeutic methods

Abbreviations: DKD, Diabetic kidney disease; MHC, Major histocompatibility complex; MAIT, Mucosal-associated invariant T cells; NKT, Natural killer T cells; Trm, Tissue-resident memory T-cells; TFH, T follicular helper cells; IFN-γ, Interferon-γ; TNF-α, Tumor necrosis factor-α; STAT, Signal transducer and activator of transcription; TIM3, Immunoglobulin domain and mucin domain 3; Treg, Regulatory T cell; FasL, NF-α/Fas ligand; S1PR1, Sphingosine 1-phosphate receptor 1; HbAlc, Glycated hemoglobin; CXCL, C-X-C motif chemokine ligand; CXCR, C-X-C motif chemokine receptor; CCL2, Chemokine ligand 2; CCR, Receptor C-C chemokine receptor; AGE, Advanced glycosylation end; TIM-1, T-cell immunoglobulin and mucin-containing molecule-1; KIM-1, kidney injury molecule-1; tolAPCs, Tolerogenic antigen presenting cells; IDDM, Insulin-dependent diabetic; TP, Triptolide; TCR, T-cell-specific antibody anti-T-cell receptor; GRK2, G protein coupled receptor kinase 2; DT, Diabetea teame.

1 Introduction

Diabetic kidney disease (DKD) is a highly prevalent microvascular complication of diabetes that affects >50% of incident cases of diabetes mellitus (DM) and profoundly contributes to patient morbidity and mortality (1). Clinically, DKD is characterized by the presence of albuminuria and decreased estimated glomerular filtration. DKD is diagnosed based on glomerular basement membrane thickening, mesangial expansion, diffuse or nodular glomerulosclerosis, podocyte loss, and interstitial fibrosis on pathology and histology (2). Multiple mechanisms contribute to the outcome of DKD. Among them, nonimmune factors, metabolism, and hemodynamics are considered the most crucial causes of renal damage in patients with type 2 DM (T2DM) and DKD in traditional perceptions (3–5). Therefore, optimal control of hyperglycemia and intensive treatments for elevated blood pressure remain the current management strategies for patients with diabetes. However, current treatments are insufficient to prevent its progression in a large proportion of patients, and the prevalence of DKD is still increasing every year. The mechanisms leading to the development of renal dysfunction in diabetes are not fully understood; therefore, there is an urgent need to identify the pathogenesis and therapeutic approaches to DKD.

In comparison to merely considering DKD a non-immune metabolic disease induced by hyperglycemia, current studies emphasize that DKD is also an inflammatory disease (6, 7). The infiltration of immune cells, which is related to innate and adaptive immunity, may be involved in hyperglycemia-induced renal injury (8). In particular, the role of T-cells in the development of DKD has been confirmed (9). On the one hand, high glucose has been verified to induce T cells recruitment, activation, differentiation, and maturation, even the cytokine factor expression profiles of T cell (10). On the other hand, serum concentrations of chemokines and cytokines produced by T cells have been assessed in patients with diabetes, which are supposedly to predict the onset of diabetic complications.

Hence, we summarized the updated progress in the aspects of differentiation, recruitment, function of T cells, and their products in the DKD as well as the potential strategies for the treatment of DKD, hoping to provide insights for future research.

2 Classification and differentiation of T cells in diabetic kidney disease

T cells are involved in host defense and clearance of pathogens. In general, T cells are divided into two species according to their constitutive chains, called “conventional T cells” and “unconventional T cells,” which operate in utterly different ways to regulate and coordinate immune responses in the kidney.

Classically, T cells that express T-cell receptors (TCRs) with α- and β-chains are classified as conventional T cells; specifically, conventional T cells can be separated into $CD8^+$ T cells and $CD4^+$ T cells, and these cells recognize peptides presented by the major histocompatibility complex class II and I. Based on the specific function, the differentiated $CD4^+$ T cell subsets were further distinguished into T-helper (Th) cells and regulatory T cells (Tregs).

According to previous research, unconventional T cells recognize antigens in the absence of classical restriction *via* the major histocompatibility complex and respond rapidly upon antigen encounters (11). In the kidneys, unconventional T cells include mucosal-associated invariant T (MAIT), natural killer T (NKT), and γδT cells (12).

In addition, tissue-resident memory (TRM) T-cells, the most abundant memory T-cell subset, have been identified as a class of T cells that reside in the kidney (13, 14). The different phenotypes and functions of TRM are derived from its position in various tissues (15). Due to the synergistic effect of the anatomical localization effector and memory phenotype, TRM T-cells located in the kidney are critically involved in DKDs.

2.1 Overview of T helper cells

Th cells are a cluster of highly plastic $CD4^+$ T cells and are simultaneously important contributors to the autoimmunity and inflammation induced by DKD. Many modulatory mechanisms employed by Th cells contribute to the adjustment of renal tissue damage, such as by mediating the production of local cytokines. According to their cytokine and transcription factor expression profiles, Th cells are primarily grouped into Th1, Th2, Th3, Th9, Th17, Th22, T follicular helper (Tfh), and Tregs.

As a flock of plastic cells, Th cell subsets can acquire regulatory functions upon chronic stimulation in diabetes, opening a new perspective for the exploration of immunomodulatory mechanisms for diabetes (8, 16). Hence, the classification and differentiation of Th cells in diabetes and its renal complications are associated with their unique subsets, which are described in the following sections.

2.1.1 Th1

Since 1986, a groundbreaking study has elaborated the patterns of lymphokine activity production of Th1 and Th2 cells; Th1 expresses its signature cytokines such as interleukin (IL)-2, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and transcription factor T-box (T-bet), among others (17–19), owing to which it participates in the activation of macrophage cell-mediated immunity and systematically regulates cellular function.

Notably, the level of Th1 can be mediated by multiple factors. For example, STAT4 and STAT1, members of the signal transducer and activator of transcription (STAT) family, are crucial for inducing differentiation and maintaining the Th1 cell phenotype (20, 21). T-bet also modifies the level of Th1 by activating STAT1 (22). In contrast, the cell immunoglobulin domain and mucin domain 3 are extensively considered suppressants of IFN-γ-producing T-cells (23).

Meanwhile, Th1 has been shown to respond to preceding and accompanying immunoreaction in DM (24). In clinical settings, Th1 cells dramatically increase in patients with type 2 diabetic nephropathy (T2DN), and the degree of proteinuria is positively correlated with aberrant cytokine production, such as IFN-γ and IL-2R (25). Creatinine clearance is also negatively correlated with plasma TNF-α and urinary MCP-1 levels.

2.1.2 Th2

Th2 and its produced IL-4, IL-5, IL-9, IL-10 and IL-13 are related to the pathogenesis of DKD (26, 27). Furthermore, the inherent link between Th1 and Th2 has been discussed in the immunopathogenesis of diabetes. In contrast, IL-10 and IL-4 produced by Th2 can dampen IFN-γ secretion and suppress Th1 cell activation (in the regulation of humoral immunity, among other processes (27, 28).

Contrastingly, the decrease in T-bet produced by Th1 corresponds with the increase in plasma IL-4 secreted by Th2, implying an imbalance between Th1 and Th2 (29). Hence, upregulating GATA-3 and IL-4 expression and downregulating T-bet and IFN-γ levels may provide a novel therapeutic method for type 1 diabetes (T1D) treatment in non-obese diabetic (NOD) mice (30). Intriguingly, GATA-3, a promoter of Th2 responses, was increased in diabetes (31, 32), while peritoneal dialysis may increase the frequency of Th2 cells during the treatment of DKD (33).

2.1.3 Th17

As discovered in 2005, Th17 secrete IL-17 as its signature cytokine (34). After Th17 cells receive active signaling, the JAK/STAT pathway directly culminates in activation of STAT3 of RORγT, resulting in the production of IL-17 (35). In contrast, IL-2-induced activation of STAT5 causes a decrease in ROR-γt and a transient downregulation of IL-17 (36). In addition to ROR-γt, the differentiation of Th17 cells can be directed by transforming growth factor (TGF)-β, IL-6, IL-1β, and IL-23 (37, 38). Interestingly, unique cytokines can induce different types of Th17 cells. For example, the proinflammatory subtype of Th17 cells is induced by TGF-β, whereas the less pathogenic subtype is promoted by IL-1β (36, 39). In peripheral blood lymphocytes from patients with diabetes, promoter activation was verified as the core principle of the change in IL-17 and its downstream signaling (40).

On the immune-mediated kidney disease, Th17 cells are likely to get upregulated in DKD, resulting in a general increase of IFN-γ and IL-17A in streptozotocin (STZ)-induced diabetes (41). A clinical study based on blood samples collected from 56 patients with nephropathy and 57 patients with diabetes revealed that patients carrying at least one allele of the IL-17A *(rs2275913)* gene polymorphism were vulnerable to DKDs (42). In a cross-sectional study, the level of serum IL-17 was also found to be lower in individuals with diabetes or renal lesions in Asian and Indian populations (43).

Furthermore, in terms of DKD treatment, IL-17A gradually demonstrates dose-dependent properties. As mentioned above, presence of IL-17A in individuals with diabetes and diabetic mouse models is an obvious characteristic, and serum and urinary levels of IL-17A in the former with advanced DKD confirms this finding; additionally, low doses of IL-17A have a noteworthy therapeutic effect on podocytes and tubular cells (44). The protective effect of IL-17 may also be dependent on its subsets, as low doses of IL-17A and IL-17F can prevent severe impairment of renal function at the beginning of the course of DKD; however, IL-17C or IL-17E do not show a similar effect (40).

With respect to the relative ratio of Th17 cells, interesting studies have demonstrated that the Th17/Treg ratio promotes inflammation and may hasten the development of diabetic complications. The increase in Th17 or decrease in Tregs may be a contributing factor to the deterioration of kidney function (45, 46). The Th17/Th1 response ratio is a potential contributor to β cell destruction and provides a novel biomarker for the rapid diagnosis of T1D preceding the clinical end. Moreover, similar investigations have been performed on the serum levels of relevant cytokines in patients with T2DM, and the Th1/Th2/Th17/Treg paradigm has been demonstrated to skew toward Th1 and Th17 (26).

2.1.4 Th3, Th9, and Th22

As the research has progressed, various types of Th cells have been discovered to be involved in diabetic complications. Characterized by high expression of TGF-β, Th3 has a negative correlation with DKD at the onset of the disease rather than in the prediabetic phase (47). Another subset, named Th9 cells, is designated as IL-9 producers. With the technical support of nanoscale flow cytometry, Semenchuk et al. found that IL-9 is inversely related to the quantification of urinary podocyte-derived extracellular vesicles (48). Additionally, Th22 participates in the regulation of DKD by producing IL-22 (49).

2.2 Tfh cells

Tfh cells are another distinctive Th subset of cells that require the synergistic action of IL-6 and IL-21 to drive differentiation (50, 51). Tfh cells are involved in diabetic syndrome, leading to elevated levels of CXCR5, ICOS, PDCD1, BCL6, and IL21 (52). Many subsets of Tfh cells, such as $CXCR5^+$ $PD1^+$ $ICOS^+$ and $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$, are increased in children and adults with diabetes (53, 54). In particular, $CD4^+$ $CXCR5^+$ Tfh cells have been confirmed to manipulate the levels of estimated glomerular filtration rate (GFR), creatinine, urea, urinary protein, fasting and postprandial blood glucose, and hemoglobin A1c in patients with DKD (55).

2.3 Regulatory T cells

Analysis of gene polymorphisms revealed that $FOXP3^+$ Tregs were reduced in patients at the onset of diabetes (56). The apoptosis of Tregs is affected by aberrant IL-2R signaling, leading to a decrease in FOXP3 persistence and impacting the establishment of tolerance (57). Therefore, a single infusion of autologous polyclonal Tregs and recombinant human low-dose IL-2 may be a novel treatment for diabetes (58).

In addition to suppressing T cells, NK cells, NKT cells, B cells, and dendritic cells in the adaptive immune responses, Treg cells play a fundamental role in the pathological development of DN, maintaining a dynamic equilibrium between inflammatory cytokines and anti-inflammatory cytokines (59–61). Treg cells can control phenotypic changes by increasing (IFN-γ, IL-2, and IL-17) and decreasing (IL-10, IL-35, and TGF-β) the levels of anti-inflammatory cytokines (62, 63). Generally, the population and function of Tregs has a peculiar effect on immunoregulation in patients with diabetes.

2.4 $CD8^+$ T cells

Recent findings have reported that $CD8^+$ T cells were increased in patients with diabetes and that suppressing $CD8^+$ T cells may alleviate the pathological reaction of DKD (64). Furthermore, infiltration of $CD8^+$ effector T cells is important for recruiting macrophages to ameliorate systemic insulin resistance in mice fed a high-fat diet (65). Interestingly, the proportion of $CD8^+$ TRM cells was increased in DKD and further promoted podocyte injury and glomerulosclerosis,

suggesting a pivotal role of $CD8^+$ T cells in podocyte damage in insulin-resistant patients with DN (66).

2.5 NKT cells and γδ T Cells

NKT cells are another characteristic T-cell subset that links the innate and adaptive immune systems, and abnormalities in the frequency and activity of NKT cells may be attributed to the exacerbation of T1D (67). NKT cells play a fundamental role in various renal diseases involving abnormal metabolism. For instance, inappropriate overactivation of NKT cells can cause kidney damage *via* the TNF-α/Fas ligand pathway (68). In progressive non-alcoholic fatty liver disease, NKT cells also cause glomerular function and renal immunotoxicity (69). Furthermore, during chronic kidney disease (CKD) progression, the raise of $CD3^-$ $CD56^+$ NK cells were observed in tubulointerstitial, and the frequency of $CD3^-$ $CD56^+$ NK cells and $CD3^+$ $CD56^+$ NKT cells were also remarkably elevated in the peripheral blood of diabetic patients (70, 71). Simultaneously, NKT cells express IL-4, IFN-γ, natural-killer group 2 member D, and IL-17, thus inducing vascular injuries (72).

The subsets of γδ TCR^+ cells, such as $CD27^-$ CD44hi and $CD27^+$ CD44lo, have also been increased in prediabetic NOD mice; however, the knowledge of concrete mechanism of γδ T in DKDs has been limited until now (73).

2.6 Mucosal-associated invariant T cells

MAIT cells not only belong to a specialized subset of unconventional (non-major histocompatibility complex-restricted) T cells but have also emerged as key players in immunity and pathological inflammation. First, human MAIT cells express a semi-invariant TCRa chain (Va7.2, coupled with restricted Ja segments), coexpressed with high levels of the C-type lectin receptor CD161, which is beneficial to its presentation in human barrier sites such as the kidneys (74, 75). Moreover, MAIT cells were reported to sharply increase with a cytokine cocktail comprising IL-12, IL-15, and IL-18, which participates in the progression of chronic inflammation (76).

Furthermore, researchers have found that dysregulation of MAIT cells may influence the severity of insulin resistance. The frequency of MAIT has been shown to be influenced by BMI, and there is a positive correlation between MAIT and HbA1c levels, accompanied by an increase in CD25 and CD69 (77, 78). Another study by Harms et al. observed a significant increase in the CD27- MAIT cell subset and IL-17A in patients with T1DM, particularly in younger patients (77).

2.7 Tissue-resident memory T-cells

$CD69^+$ $CD103^+$ and $CD69^+$ $CD103^-$ TRM cells have been identified as two primary subsets of TRM cells (15). CD69 binds to S1PR1 on the T cell membrane, restraining the migration of memory T cells from the blood to peripheral tissues (79). Therefore, a mass of TRM-T cells exists in the kidney rather than in the circulation. After encountering antigens *in vivo* and *in vitro*, native T cells rapidly produce effector T cells and swiftly migrate to lymphoid and non-lymphoid tissues, persisting through barrier tissues, such as the kidney (80, 81). Following inflammation resolution, antigen-specific effector T cells differentiate into diverse memory T cell subsets with distinct trafficking properties.

In the tubulointerstitium of DKD, a recent xCell analysis has identified immune cells, thus revealing significant changes, including activated Th2 cells, $CD4^+$ T cells, $CD8^+$ T cells, dendritic cells, conventional dendritic cells, M1 macrophages, and restrained Tregs (82). However, knowledge of T cell differentiation in DKD remains limited.

3 Recruitment and activation of T cells in diabetic kidney disease

As early as 2012, the aberrant recruitment and activation of T cells in DKD had been discussed (83). The results showed an increase in $CD4^+$, $CD8^+$, and $CD20^+$ cells in the interstitium, indicating that aberrant intrarenal infiltration and recruitment of T cells are potential immunopathological mechanisms of diabetic kidney lesions. Immunohistochemical analysis also showed that a substantial proportion of juxtaglomerular apparatuses in patients with T1DM contained abundant T cells (84).

To exert their local effects on renal injury, circulating T cells must reach the site of inflammation. Typically, some T cells, such as Th cells, do not possess a residency status similar to that of other immune cells, such as kidney TRM-T cells.

A series of tissue-specific markers has been reported to activate T cells in the kidney. Once activated, T cells can expand their immunoreaction, inducing chemokine release and more widespread recruitment of T cells (85). However, little is known about the trafficking of T cells into the kidney under hyperglycemic conditions, and their migration patterns have been the subject of extensive studies (17). Hence, the methods for circulating T cell migration into kidney should be assessed in the next step of research.

3.1 Chemokines and its receptor

There is a positive feedback between chemokines and T cells in the inflammatory response and immune adjustment. In other words, chemokines facilitate the attraction of circulating T-cells and stimulate their infiltration into tissues. T cells also participate in the regulation of the pathophysiological progression of renal insufficiency by producing chemokines. In this section, we focus on the chemokines involved in the recruitment of T cells in DKD.

3.1.1 CXCL9-CXCR3

Multiple studies have shown that the urinary level of C-X-C motif chemokine ligand 9 (CXCL9) mRNA is significantly elevated and correlated with eGFR decline, which can be utilized to measure and stratify the risk of DKD (86, 87).

On the other side of the CXCL9-CXCR3 axis, C-X-C motif chemokine receptor 3 (CXCR3) is a well-known chemokine receptor predominantly expressed on the surface of Th1 polarized T cells and regulates the recruitment of Th1 cells (88). Moreover,

CXCL9 and CXCR3 have been found to be influenced by advanced glycosylation end products (AGEs), implying that Th1 can be recruited under diabetic conditions (89).

3.1.2 CXCL10/CXCR3

In an exploratory study, the CXCL10/CXCR3 axis was observed in the autoimmune process in T1D. Serum levels of CXCL10, a well-known Th1 chemokine, are elevated in patients with T1D, suggesting that CXCL10 plays a critical role in predicting T1D (90). Most importantly, CXCL10 may be induced by IFN-γ, promoting T cell infiltration and accelerating beta cell destruction (91).

3.1.3 CXCR5

In individuals with DN, the increase in $CD4^+$ $CXCR5^+$ Tfh cells may significantly increase creatinine, urea, urinary protein levels, fasting blood glucose, postprandial blood glucose, and HbA1c and decrease estimated GFR (55). In the future, the increased number of $CD4^+$ $CXCR5^+$ $PD\text{-}1^+$ Tfh cells in patients with DN may be a new target for intervention in DKD (47).

3.1.4 CX3CL1-CX3CR1

At an early stage of nephropathy, $CX3CR1^+$ T cells are elevated and induce IL-17A production in renal impairment (92–94). In addition, the polarization of TH17 or Treg cells may be associated with an increase in CX3CR1 reporter gene expression in T cells (92). Several studies have shown that CX3CR1 and CX3CL1 are upregulated in the kidneys of patients with diabetes, accompanied by an increase in urea, creatinine, A/C ratio, HbA1C, and IgG; however, the concrete mechanism of CX3CL1-CX3CR1 recruiting T cells requires further exploration in DKD (93).

3.1.5 CCL5 (RANTES)- CCR5

CCL5 is a β-chemokine, which is also known as RANTES (regulated on activation, normal T cell expressed and secreted), and can function as a chemotactic factor for T cells and induce cellular activation of normal T cells (95) In inflammatory kidney diseases, constitutive RANTES expression facilitates the accumulation of CD4+ T cells in the kidney, while the administration of RANTES-neutralizing antibody is helpful in reducing the accumulation of T cells in the kidneys to a large degree. Moreover, RANTES-neutralizing antibodies can reduce the deposition of collagen in obstructed kidneys (96).

There is no doubt that CCR5 is a characteristic of Th1 lymphocytes and a critical chemokine receptor for trafficking of TH1 cells to the kidney (88, 97); however, the status of CCR5 in T2DM and microvascular complications remains controversial. The problems are mainly focused on the significant discrepancy in the allelic frequency of CCR5 between different ethnic groups. In Asian populations and people with T2DM, the CCR5 *59029G/A* polymorphism is significantly associated with an enhanced susceptibility to DN (98). Nevertheless, the CCR5 *59029 A* allele only has a convincing association with nephropathy in T2DM Malaysian Chinese population but is weakly associated with nephropathy in Malaysian Indian population (99). Additionally, in native Estonian patients with T2D, there was a lack of association between the *CCR5-Δ32* mutation and DKDs (100). Hence, further research is needed to determine whether CCR5 is associated with DKD worldwide.

3.1.6 CCL2 (MCP-1)- CCR2

Chemokine ligand 2 (CCL2) binds to its receptor, C-C chemokine receptor 2 (CCR2), initiating the migration and infiltration of T cells and regulating tissue inflammation (101). A longitudinal analysis followed the fate of $CCR2^{-/-}$ T cells and observed that CCR2 regulates the immune response by modulating the effector/regulatory T ratio. Additionally, CCR2 deficiency in T cells decreases the levels of Th17 cells while promoting a program that induces the accumulation of Foxp3+ Tregs *in vivo* (102).

Recent studies have suggested that CCL2 (MCP-1) is a key chemokine involved in DN. In a blood sample analysis conducted in Iran, CCL2 was gradually elevated in patients with T1D with disease duration (103). Furthermore, the blockade of this pathway plays a protective role in insulin resistance, modulation of adipose tissue, restoration of renal function, and restraint of progressive fibrosis in hyperglycemic kidneys (104, 105). A phase Ia study targeting emapticap impeded the CCL2/CCR2 receptor axis and exerted beneficial effects on ACR and HbAlc in albuminuric T2D (106). Overall, the CCL2/CCR2 receptor axis is thought to be crucial for the progression of DKD.

3.1.7 Interleukins

T cells not only produce several members of the IL family but are also recruited by other immunocytes produced ILs, such as IL-18, IL-19, among others.

IL-18 is not mainly produced by T cells but plays an underlying pathophysiological role in the progression of T cell differentiation in DKD. IL-18 induces plasticity in established Th1 and Th2 cells (107–109). It also acts synergistically with IL-12 to increase the level of IFN-γ, a Th1 cytokine (110). In recent years, a cross-sectional study of patients with T2D showed that IL-18 levels were significantly boosted at a low eGFR and positively correlated with the development of DN and urinary albumin excretion (UAE) rate (111, 112).

Similarly, IL-19 were markedly positively correlated with Hs-CRP, cystatin C, and UAE in patients with DN (113). The reduction in IL-19 levels contributes to the suppression of T-cell responses and inhibition of the regulatory activity of $CD4^+$ T cells, causing cell-mediated immunosuppression (114). Therefore, IL-19 may be another target for regulating T cell differentiation in DKD.

3.1.8 TGF-β

In renal inflammatory diseases, TGF-β has been demonstrated to orchestrate the differentiation of T cells, including Th17 and $Foxp3^+$ Treg cells (96) Additionally, rats with hyperglycemia-induced microalbuminuria possess upregulated TGF-β and serum creatinine levels (115). Recently, the role of TGF-β in promoting the characterized T cell cytokines, IL-9 and IL-17, has become more widely accepted. TGF-β controls the secretion of both these cytokines, subsequently mediating fasting and postprandial glucose and HbAlc levels in patients with DN (116). Taken together, restraining TGF-β may be considered as an approach aimed at attenuating T1D in the immediate future.

3.2 Other factors that regulate T cells

Similar to chemokines, there are many other factors that facilitate the assembly and infiltration of T cells, such as C3a and its receptor, AGE, KIM-1, Chromogranin A, among others.

3.2.1 Complement C3a and its receptor

Emerging evidence suggests that the expression of C3a and C3aR is involved in DN pathogenesis (117). Compared with normal controls, C3aR was significantly increased in the renal specimens of patients with diabetes and wild-type (WT) diabetic mice. *In vitro* microarray profiling revealed the underlying mechanism that C3a plays a role in suppressing T-cell adaptive immunity by interfering with $CD4^+$ and $CD8^+$ T cell infiltration, and in an *in vitro* study, C3a was able to enhance differentiation of the T-cell lineage in inflammatory responses (118). Thus, C3aR may be a promising target for T cell recruitment and activation.

3.2.2 Advanced glycosylation end products

In peripheral blood T lymphocytes, the expression of AGE binding sites serves to target T cells to the AGE-rich renal tissues. With the increase and accumulation of AGE products and AGE-modified proteins, their binding to the AGE receptor on T cells is remarkably increased, promoting the synthesis and release of proinflammatory cytokines in diabetes (119).

3.2.3 KIM-1

KIM-1 is also known as T-cell lg mucin 1 (TIM-1) or hepatitis A virus cellular receptor 1 and has been reported as a transmembrane glycoprotein receptor on T cells (120). Recent studies have revealed elevations in KIM-1, suggesting that glycemic variations may increase the production of KIM-1 in $CD8^+$ T cells in individuals with DKD, thereby increasing the risk of DKD (121). The elevations in circulating KIM-1 also increases the urinary KIM-1 in DN, verifying that KIM-1 can be a biomarker and a reliable predictor of diabetic kidney injury (122).

3.2.4 Chromogranin A

The β-cell secretory granule protein, also known as chromogranin A, is a new autoantigen in T1D. A recent study identified chromogranin A as a forceful inducer of the reacting $CD4^+$ T cells in the pathogenic process of T1D in NOD mice (123). However, studies on the function of chromogranin A in diabetic vascular complications and DKD are still insufficient.

4 T cells regulate inflammation in diabetic kidney disease through inflammatory cytokines

In DKD, the inflammatory cytokines secreted by T cells can cause the epithelial-to-mesenchymal transition and the extracellular matrix accumulation (124). In this section, we have elaborated on the mechanisms by introducing, summarizing, and comparing the inflammatory mediators in DKD, which may prove useful in future researches.

4.1 IL-1β

Based on the different encoding genes, IL-1, a classical chemokine, is divided into IL-1α and IL-1β. Both can bind to the primary receptor point of distinction (IL-1RI), while only IL-1β is secreted by T cells and macrophages (125). In diabetic metabolic syndrome, high glucose and oxidative stress can induce IL-1 activation, which occurs earlier than the pathophysiological manifestations. IL-1β production may be related to TNFR-Fas-caspase-8-dependent pathway in $CD4^+$ T cell-driven autoimmune pathology (126). Moreover, IL-1β was also identified to cause endothelial cell damage in resistance arteries and affect the NADPH oxidase activation (127, 128). In addition, studies have shown that repressing IL-1β and its receptor can reduce systemic inflammation in patients with T2DM (128, 129).

4.2 IL-2

IL-2 can be produced by Th and kidney-derived MAIT cells. The function of the IL-2/IL-2R in renal dysfunction has been discussed in early studies, which has indicated that serum soluble IL-2R (sIL-2R) levels increase with a decrease in creatinine clearance (130). In the autoimmune diabetic NOD mice, two separate research groups have revealed that deficiency in IL-2 production or the responsiveness of Tregs to IL-2 may be associated with the development of the immune response (131, 132). Given its crucial role in the expansion and function of Tregs, IL-2 has been used to regulate tissue damage and limit the immune response following infection (133). Low-dose IL-2 selectively induces $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in patients with CKD, and these Tregs limit the levels of proinflammatory Th1 and Th17 cells (133). In other mouse models of autoimmune diseases, such as C57BL/6 mice, $CD4^+$ $CD25^+$ Tregs are also induced by recombinant IL-2, thus preventing the progression of diabetes (134). Hence, it would be interesting to explore the effect of IL-2 on new therapeutic schedules for patients with DKD.

4.3 IL-4

IL-4, partly produced by Th2 and NKT cells, can expand the proliferation of activated T and B cells and regulate the differentiation of Th1 and Th2 cells (135). The role of IL-4 in DM remains controversial. Data investigation of Filipino patients suggested that the risk of T1D was partly determined by specific polymorphisms. The variability in promoters, coding sequences, and specific combinations of genotypes indicated that IL-AR of IL-4 and IL-13 were significantly associated with susceptibility to T1DM (136). In contrast, no significant change in IL-4 plasma levels between patients with T2DN and those without nephropathy was observed in a study (25). The IL-4 *rs2243250* polymorphism is irrelevant to DN in Slovenian patients with T2DM (137). As a result, the relationship between IL-4 and DN may depend on race, ancestry, geographical conditions, and national customs to some extent, which needs to be proven by more prospective evidence.

4.4 IL-6

IL-6 levels are significantly increased in the plasma of patients with DN patients than those with diabetes but without nephropathy (138). Furthermore, the *IL-6-174 G* allele was found to increase the occurrence rate of DN, confirming the correlation between IL-6 and DN (139). Similarly, a recent meta-analysis revealed the significance of different IL-6 polymorphisms in DN progression. The results showed that IL-6 *rs1800795, rs1800796*, and *rs1800797* were associated with DN, whereas IL-6 *rs2069837* and rs2069840 may be indifferent to the risk of renal complications in patients with T2DM (139).

Classically, IL-6 participates in the pathogenesis of DKD by various methods, including binding to the receptor IL-6R, sIL-6R trans-signaling pathway, and IL-6 autocrine signaling (140). IL-6 influences renal cells by relying on diverse signaling pathways. For instance, IL-6 facilitated mesangial expansion by infiltrating the mesangium, interstitium, and tubules, which has been observed in human renal biopsies (141). Second, the determination of samples from patients indicated that the width of the glioblastoma (GBM) was directly associated with fibrinogen and IL-6 levels in diabetic glomerulopathy (142). Moreover, the effects of IL-6 on diabetic renal injury may be due to increased insulin resistance and promotion of the inflammasome (143).

4.5 IL-9

IL-9 is mainly produced by a flock of T cells, such as Tregs and Th2 cells, and manipulates signaling pathways in renal immune diseases. For example, IL-9 protects against progressive glomerulosclerosis and tubulointerstitial fibrosis and regulates T cell-induced immune suppression in adriamycin-induced nephropathy and acute kidney injury (144, 145). Meanwhile, as characterized by T cell cytokines, IL-9 levels were evidently reduced in the diabetic group and positively correlated with the level of urea and microalbuminuria, which may be considered as an approach of T cells to address hyperglycemia damage (43).

4.6 IL-17

IL-17A can be produced by many types of $CD4^+\alpha\beta$ and $\gamma\delta$ T cells, particularly Th17 cells (146). The IL-17 family is essential for the inflammatory response and includes six structurally related isoforms: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. However, only IL-17A and IL-17F have unique functions in DN, whereas IL-17C and IL-17E are indifferent to DN (44).

Clinically, the decline in IL-17 levels is synchronous with the progression of DKD and is correlated with declining GFR (43, 147). IL-17A has been proven to not only trigger inflammatory signaling pathways associated with NF-κB downstream but also regulate the viability of T cells (147). However, another study reported the opposite result, indicating that IL-17A may increase the infiltration of inflammatory cells in renal tissue and blood pressure in mice (148)

As a potential immunologic therapeutic target for DKD, studies have suggested that intrarenal IL-17A1 CD41 T cells can be suppressed by mycophenolate mofetil, which is beneficial for treating albuminuria and tubulointerstitial fibrosis (41). All-trans retinoic acid was used to retain the capacity of Tregs to secrete IL-17 during hyperglycemia, implying an important role of IL-17 in DKD (149).

4.7 IL-22

In addition to studies on DKD, IL-22, mainly produced by Th22, was found to be downregulated in patients with DKD. Further observations indicated that the mechanism of IL-22 participating in inflammatory processes of DKD is intricate and comprehensive. Chen et al. demonstrated that IL-22 induced AMPK/AKT signaling and PFBFK3 activity, alleviating the level of dysfunctional mitochondria and the accumulation of reactive oxygen species (150). In addition, IL-22 can ameliorate renal fibrosis and attenuate microalbuminuria in DKDs (150, 151).

4.8 IL-35

Anti-inflammatory cytokine IL-35 is expelled by Tregs, regulatory B cells, and tolerogenic antigen presenting cells. Tregs were reported to infiltrate renal tissues to maintain homeostasis of the immune system in patients with diabetes and use IL-35 to intervene in the development of DKD (63).

4.9 INF-γ

Several studies have reported that T cells can be stimulated by high glucose concentrations and expedite IFN-γ production (83, 130, 152). Under conditions of high glucose concentrations, IL-12 can stimulate CD4 cells to produce IFN-γ. AGE-modified proteins bind to the receptor for AGE and T cells, inducing the synthesis and release of IFN-γ and accelerating inflammation of renal tissues (130).

4.10 TNF-α

As a synthetic product of T cells, TNF-α may be used as an indicator for evaluating DKDs (153). Many clinical studies have found that TNF-α is increased in the plasma and urine of patients with diabetes, leading to a higher risk of mortality, more serious macroalbuminuria, sodium retention, and renal hypertrophy (154–157). Specifically, TNF-α participates in the pathophysiological reaction in DN *via* diverse pathways, including altering intraglomerular blood flow, reducing glomerular filtration, inducing cytotoxicity to renal cells, and producing local reactive oxygen species (158–160).

5 Summary of other functions of T cells in diabetic kidney disease

Pathologically, hyperglycemia stimulates T cells to produce chemokines and cytokines that not only participate in the

promotion of inflammation and activation of macrophages and endothelial cells but also damage renal function through different mechanisms. First, these proinflammatory molecules highlight the role of T cells in the process of insulin resistance. Second, T cells mediate the glomerular filtration barrier through podocytes. Third, T cells contribute to extracellular matrix deposition and the differentiation and proliferation of myofibroblasts. Ultimately, T cells lead to proteinuria and the development of DKD (Figure 1).

5.1 Function of T cells in insulin resistance

Insulin resistance is often regarded as a strong marker of DKD and is characterized by hyperinsulinemia and reduced insulin action, affecting many classical insulin-regulated pathways in the kidney and vasculature (161). For instance, the lack of insulin resistance in the kidney has been verified as an inducer of sodium retention, resulting in salt-sensitive hypertension. Podocyte insulin sensitivity is critical for glomerular alterations and disorders in DKDs (162, 163). In recent years, T-cells have been reported to improve glucose tolerance, enhance insulin sensitivity, and reduce weight gain in mouse models (164). However, a summary of T cells in renal insulin resistance is currently insufficient.

The relationship between insulin resistance and T cells has been described, involving Tregs, $CD8^+$ T cells, Th cells, and MAIT cells. The depletion of Tregs leads to enhanced insulin resistance and impaired insulin sensitivity accompanied by albuminuria and glomerular hyperfiltration (165). In contrast, insulin sensitivity in DKD can be significantly rescued by adoptive transfer of $CD4^+$ $FoxP3^+$ Tregs in a murine model, resulting in less diabetic kidney damage (166). In addition, $CD4^+$T cells in visceral adipose tissue have also been demonstrated to regulate insulin resistance and control glucose homeostasis in diet-induced obesity progression. When Th1 statically overwhelms $CD4^+$ $FoxP3^-$ Tregs, weight gain and insulin resistance are reversed (166).

Moreover, the depletion of CD8+ T cells has been reported to alleviate macrophage infiltration of CD8+ T cells. CD8 + T cells recruit macrophages to mediate insulin resistance and adipose tissue inflammation (65). Conversely, systemic insulin resistance is aggravated by adoptive transfer of CD8+ cells (167). In addition, Th1 and MAIT cells can regulate insulin resistance (77, 168).

As mentioned above, secretion and release of T cells produce proinflammatory cytokines that not only induce insulin resistance but also impair kidney function. IL-1, IL-6, IL-17, IL-33, GATA-3, and other proinflammatory cytokines also play important roles in renal insulin resistance. First, the blockade of IL-1 improves glycemia and β-cell secretory function; repression of the IL-6 receptor relieves diabetic renal injury and insulin resistance, and suppression of GATA-3 restores insulin sensitivity (129, 143, 169). In clinical investigations, TGF-β is positively correlated with insulin resistance markers, including fasting and postprandial glucose levels and HbA1c, whereas IL-17 is negatively associated with them (43). Additionally, studies have shown a serial decline in IL-33 levels in DN, resulting in an increased severity of insulin resistance and microalbuminuria (170). Overall, insulin resistance in DKD is closely associated with proinflammatory cytokines produced by T cells.

5.2 T cells and podocyte damage in DKD

Normal function and structural integrity of podocytes are essential for the occurrence of albuminuria and progression of diabetes (171). T cells and their production have been described as novel factors influencing podocytes in patients with diabetes (172). Therefore, podocytes may be regarded as an essential part of T cells that mediate pathological effects in DKD.

Firstly, CD28/B7 and cytotoxic T lymphocyte-associated antigen-4 (CTLA4) are critical for Th cells and podocytes. With regard to T-cell proliferation, differentiation, and survival, costimulatory molecules composed of CD28, B7-1 (CD80), and B7-2 (CD86) have been reported to play crucial roles (173). As a novel biomarker for podocyte damage, B7-1 is upregulated in podocytes under high glucose conditions. After activation, the CD28/B7-1 pathway mediates circulating T cells to aggravate podocyte damage (174, 175). Moreover, CTLA4 is a negative regulator of T cell activation,

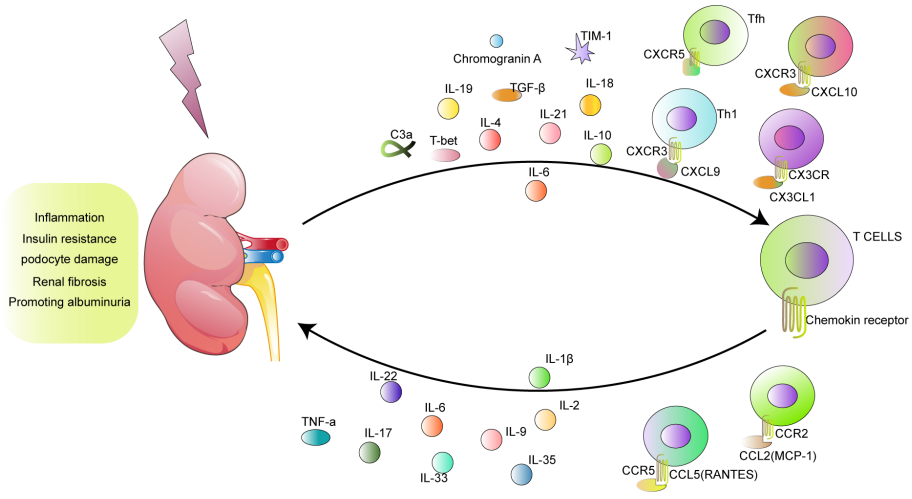

FIGURE 1
The model of T cells and their products participating in DKD.

and genetic polymorphisms in CD28/B7/CTLA4 are related to susceptibility to T2DM (176). However, the fact that B7-1 was not inducible in podocytes in patients with DKD is contradictory; therefore, further investigation is required (177).

Researchers have also discovered that IL-17A is a characteristic proinflammatory cytokine in the serum and urine of patients with diabetes, and CD40 expression was observed to be increased in podocytes with DN (38, 147). The synergistic action of IL-17 and CD40L regulates the inflammatory response and mediates remodeling of glomerular sclerosis in DN.

Furthermore, podocyte damage is affected by TNF. Albuminuria is partly attributed to TNF-induced ABCA1 deficiency in podocytes. Studies have indicated that TNF is sufficient to cause free cholesterol-dependent podocyte injury through an NFATC1/ABCA1 dependent mechanism (155).

Podocyte apoptosis is triggered by $CD8^+$ TRM cells. In db/db mice, the relative proportion of $CD8^+$ TRM cells is remarkably increased under pathological conditions, and renal $CD8^+$ TRM cells have cytotoxic effects on podocytes and enhance podocyte apoptosis (66).

5.3 T cells and renal fibrosis in DKD

Pathologically, fibrosis is one of the most fundamental characteristic mechanisms in the onset and progression of DKD, and renal T-cell infiltration is helpful for fibrosis. Therefore, hyperglycemia stimulates T cells and T cell-derived products, including IL-1, IL-6, IL-17, and IL-22, which are of central importance in progressive fibrosis in DKD (66).

Primarily, IL-1β induces proximal tubule damage and fibrosis in renal tubule interstitials (178). One study showed that IL-1β participates in the dysregulation of glycolysis and matrix activation, leading to tubulointerstitial fibrosis (147). In contrast, another report on CKD described the relationship between IL-1β and fibrosis initiation and progression (178).

Second, IL-6 trans-signaling may be a crucial factor in the development of renal fibrosis, thus influencing the width of the GBM in the pathogenesis of diabetic glomerulopathy (142, 179). Simultaneously, targeting IL-6 trans-signaling, Fc-gp130, could be a novel therapeutic strategy for renal fibrosis.

Moreover, IL-17 suppresses fibrosis *via* the STAT-3 and WAP domain protein pathways in models of T1D and T2D, and tubulointerstitial fibrosis can be rescued by suppressing intrarenal IL-17A1 CD41 T cells (41, 44). Furthermore, through the NLRP3/caspase-1/IL-1β pathway, IL-22 can reverse the overexpression of fibronectin, collagen IV, and extracellular matrix in mouse renal glomerular mesangial cells, thereby ameliorating renal fibrosis and proteinuria excretion in DN (150, 180).

Additionally, sphingosine 1-phosphate receptor 1 activation in T cells leads to fibrosis in normoglycemic conditions but exacerbates fibrosis in a model of STZ-induced diabetic cardiomyopathy (181).

5.4 T cells and albuminuria in DKD

5.4.1 The quantity of T cells and albuminuria

To elaborate the internal relationship between T cells and albuminuria in DN, preliminary exploration was performed. Under STZ stimulation, only $Rag1^{(+/+)}$ mice, which have mature T lymphocytes, had glomerular immunoglobulin deposition. However, $Rag1^{(-/-)}$ mice, which lack kidney infiltration with T cells, were protected from albuminuria (172). Additionally, the degree of albuminuria is regulated by the number of T cells infiltrating the kidneys of DKD animals, and abatacept ameliorates DKD by blocking systemic T-cell activation (182).

However, there are also a series of contradictory reports. Absolute and percent T-lymphocytes were found to be relatively lower in patients with nephrotic proteinuria and long-standing insulin-dependent diabetes (183). In contrast, Another study showed that T cell-positive patients had a shorter duration of diabetes and lower albumin excretion rates than T cell-negative patients (84). Hence, the exploration and summary of the relationship between T cells and proteinuria in DN are significant.

5.4.2 Types of T cells associated with proteinuria

In general, circulating $CD8^+$ T cells and Tregs are considered the main types of T cells that are associated with albuminuria in DN. A cross-sectional study showed that the percentage of circulating $CD8^+$ T cells was correlated with albuminuria in T2DM, indicating that systemic inhibition of T lymphocytes provides a new therapeutic direction for albuminuria in DKD (184). In addition, $FoxP3^+$ Tregs exert a protective effect in the kidneys of diabetic mice, although it reduces glomerular hyperfiltration and albuminuria. Moreover, depletion of Tregs with anti-CD25 antibodies can accelerate the progression of albuminuria (165).

5.4.3 Product of T cells and albuminuria

T cells regulate albuminuria through cytokines including IL-6, IL-9, TNF-α, IL-22, IL-33, and IL-233.

IL-6, associated with higher albuminuria, has been reported in db/db mice and patients with diabetes (143, 185). The IL-6 receptor antibody (tocilizumab) can reduce proteinuria and glomerular mesangial matrix accumulation. Furthermore, the levels of IL-9 and TNF-α are positively correlated with the levels of urea and microalbuminuria (43, 185, 186). Albuminuria may be caused by TNF-α via alterations in the glomerular capillary wall and an increase in albumin permeability.

In addition, studies on IL-22 support the hypothesis that cytokines drive proteinuria. IL-22 can alleviate mesangial matrix expansion and proteinuria in mice (151, 172). IL-33 also represses microalbuminuria in DKDs (170, 187). Intriguingly, the increase in IL-33 levels in DN is only associated with diabetes but not with kidney injury (188). Therefore, the exact role of IL-33 in DKD remains controversial.

Notably, a novel cytokine (named "IL-233") possesses the activities of both IL-2 and IL-33 and protects against type-2 DN by promoting T-regulatory cells. Treatment with IL233 reduces hyperglycemia, plasma glycated proteins, and albuminuria, protecting mice from T2DN (189).

6 Promising novel therapies targeting T cells in DKD

Until now, the standard management strategy for DKD has prioritized strict glucose control and blood pressure with RAAS

blockade. However, the therapeutic means are limited to stopping or reversing the progression of DN. Therefore, new drugs targeting the pathological mechanisms of DKD, such as T cells and their products, have drawn increasing attention (Table 1).

6.1 Traditional drug therapy with T cells

First, there are some drugs that target the Th cells. Similar to triptolide, a well-known drug for DN, influences Th lymphocyte cells in rat models of DN by regulating the Th1/Th2 cell balance. DN is associated with the upregulation of Th1 cells and downregulation of Th2 cells; however, triptolide can alter this ratio in high-fat diets and STZ-induced rats (152). Concurrently, animal experiments have shown that miR-29b is a novel therapeutic agent for treating T2D that effectively rescues renal inflammation and fibrosis by inhibiting T-bet/Th1-mediated immune response (190).

Second, the expansion and activation of $CD4^+$ and $CD8^+$ T cells can be enhanced by Enalapril and γ-aminobutyric acid receptors in DKDs (191, 192).

In addition, combining anti-TNF-α therapy and the T-cell-specific antibody anti-TCR can reverse the diabetic metabolic state in a model of human T1D (193).

6.2 Tregs-targeted drugs

Researchers have discovered that administering drugs targeting Tregs can be beneficial in diabetic diseases. For instance, Paroxetine, a G protein-coupled receptor kinase 2 inhibitor, has been approved to rescue Treg differentiation and restore the population of circulating Tregs *in vitro* and *in vivo* (194). In obese WT and ob/ob (leptin-deficient) mice, a CD3-specific antibody or its F(ab')2 fragment can promote the predominance of $Foxp3^+$ cells over Th1 cells (166).

Ethnopharmacological relevance Diabetea teame was verified to promote the Treg/IL-17 ratio in clinical settings, suggesting the protective effect of DT against diabetes-related complications in the long term (195). In addition, Surfactin, a bacillus-produced natural immunomodulator, could increase $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs while simultaneously suppressing T cell proliferation and downregulating the activated $CD8^+$ T cells (196).

6.3 Recombinant human IL-2 and Tregs

IL-2 plays an essential role in the expansion of Tregs and can reduce tissue damage by limiting immune response. A single ultra-low dose of Aldesleukin (proleukin; recombinant human IL-2) has been demonstrated to regulate early altered trafficking and desensitization of Tregs in T1D (133). Simultaneously, low expression of mIL-2 also prevents the progression of diabetes by regulating Tregs in islets (135). Furthermore, combining low doses of IL-2 with exogenously administered Tregs leads to an increase in the number of Tregs, NK cells, mucosal associated invariant T cells, and clonal $CD8^+$ T cells (58).

6.4 Adoptive Treg immunotherapy

Recently, expanded Tregs have been used to treat deficits in the number and suppressive activity of Tregs in immune-related diseases. Two separate research groups have explored adoptive Treg immunotherapy and demonstrated its safety, tolerance, and efficacy in patients with DM (197, 198). Bluestone et al. reported a phase 1

TABLE 1 The therapeutic methods targeting to T cells in DKD.

The therapeutic methods	Target T	Potential mechanism	Reference
Triptolide (TP)	Th cells	Regulating the Th1/Th2 cell balance in DN	152
miR-29b	Th1	Rescues renal inflammation and fibrosis	190
Enalapril	$CD4^+$ and $CD8^+$ T	Promoting expansion and activation of T cells	191
TNF-α and TCR therapy	T cells	Reverse the diabetic metabolic state in T1DM	193
Paroxetine	Tregs	Rescued the differentiation and the population of Tregs	194
CD3-specific antibody	$Foxp3^+$	Promote the predominance of $Foxp3^+$ cells over Th1 cells	166
Diabetea teame (DT)	Tregs	Promote the Treg/IL-17 ratio	195
Surfactin	Tregs $CD8^+$ T	Increasing $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs Suppress CD8+ T cells	196
Recombinant human IL-2 and Tregs	Tregs	Regulating the trafficking and desensitization of Tregs in Type 1 Diabetes Increasing NK, MAIT, and $CD8^+$ T cell	58, 133, 135
Adoptive Treg Immunotherapy	Tregs	Improved insulin sensitivity Down-regulating the ACR in DN	165, 197, 198
MSCs	$CD8^+$T	Impairing the activation and proliferation of $CD8^+$T Preventing the exacerbation of kidney injury	64, 199

trial of adoptively transferred self-derived Tregs to repair or replace Tregs in patients with T1D. Simultaneously, adoptive transfer of $CD4^+$ $FoxP3^+$ Tregs significantly improved insulin sensitivity and decreased the albumin-to-creatinine ratio in DN (165).

6.5 Mesenchymal stem cells

In the last decade, MSCs have been widely used to treat DN. Intriguingly, MSC-CM pretreatment reduced $CD8^+$ T cell priming and proliferation capacities in the kidneys of DN rats (64). Furthermore, MSC transplantation not only impairs the activation and proliferation of $CD8^+$ T cells but also prevents the exacerbation of kidney injury, providing a new insight into the treatment of DN (199).

7 Conclusion

With the increase in the number of patients suffering from DKD, exploration of the function of T cells in DKD is increasingly important. After circulating T cells are recruited into the renal tissue or T cells are amplificated, differentiated, and activated in the kidney, T cells play protective or pathogenic roles through multiple pathways, including influencing insulin resistance, mediating podocyte damage, participating in fibrosis, and regulating proteinuria (Table 2 and Figure 1).

As Table 1 showed, based on the significant functions of T cells and cytokines, the application of T cell-associated therapies in DKD has been attempted and preliminary achievements have been made. Promising studies on T cell biology will unquestionably contribute to

TABLE 2 The fundamental function of T cells in DM and DN.

Regulatory factors	T cells population	Cytokine secretions	Key finds in DM and DN	Reference
STAT4↑ STAT1↑ T-bet↑ TIM3↓ IL-10↓ IL-4↓	Th1	IL-2↑ IFN-γ↑ TNF-α↑ T-bet↑	Activating macrophages Associated with proteinuria and creatinine clearance	17–25
GATA-3↑	Th2	IL-4↑ IL-5↑ IL-9↑ IL-10↑ IL-13↑	Suppress Th1 cell activation	26–33
JAK/STAT↑ STAT3↑ IL-2↓ STAT5↓ TGF-β↑ IL-1β↑ IL-6↑ IL-23↑	Th17	IL-17↑	Aggravating diabetic renal Regulating Th17/Th1 and Th17/ Treg Increase inflammatory Correlated with GFR	26, 34–46
	Th3	TGF-β↑		47
	Th9	IL-9 ↑	Associated with podocyte injury and ACR in T1DM	48
	Th22	IL-22↑		49
IL-6 and IL-21↑	Tfh	IL21↑	Manipulate the level of estimated creatinine, urea and urinary protein level, Fasting and postprandial blood glucose, Hemoglobin A1c in diabetic nephropathy	50–55
IL-2↑	Tregs	IL-2↑ IL-17↑ IL-10↓ IL-35↓ TGF-β↓	Maintaining the balance in the anti-inflammation and anti-inflammation in diabetes condition Limit the pro-inflammatory Th1 and Th17 Lessening glomerular hyperfiltration and albuminuria	56–63
	CD8+ T		Recruiting macrophages Ameliorating systemic insulin resistance Promoting podocyte injury Accelerating glomerulosclerosis	64–66
	NKT		Kidney damage through FasL pathway Taking part in the exacerbation of DM	67–72
	γδ T		Upregulated, but the mechanism is unknown	73
	MAIT	IL-2 GM-CSF IL-17	Influence the insulin resistance Promoting the level of HbA1c	74–78

a more profound understanding of DKDs, highlighting the need to identify new therapeutic approaches.

Author contributions

YLi and YLv conceived and drafted the review article. TZ, TH and YLa created the model figure. QS and YLi prepared the tables. ZK, YG, SL were involved in the compilation of the references. MY and YLu revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Platelet, a key regulator of innate and adaptive immunity

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Platelets, anucleate blood components, represent the major cell type involved in the regulation of hemostasis and thrombosis. In addition to performing haemostatic roles, platelets can influence both innate and adaptive immune responses. In this review, we summarize the development of platelets and their functions in hemostasis. We also discuss the interactions between platelet products and innate or adaptive immune cells, including neutrophils, monocytes, macrophages, T cells, B cells and dendritic cells. Activated platelets and released molecules regulate the differentiation and function of these cells *via* platelet-derived receptors or secreting molecules. Platelets have dual effects on nearly all immune cells. Understanding the exact mechanisms underlying these effects will enable further application of platelet transfusion.

KEYWORDS

platelet, monocyte, macrophage, T cell, B cell, dendritic cell

Introduction to platelets

The production of platelets from megakaryocytes (MKs) is a systematic process that is thought to occur in the bone marrow (1). Thrombopoiesis occurs from common myeloid progenitor (CMP) cells in the bone marrow, which differentiate into promegakaryocytes and then into MKs. After migrating into the vascular niche, mature MKs extend many proplatelets (PPTs) through the sinusoid vessel barrier (2–4). Then, PPTs interconvert into pre-platelets, and platelets are created after the fission of pre-platelets (5). Each MK can produce 1,000–3,000 platelets after multiple divisions (6, 7). A recent study proposed the lung as the main site of platelet release (8). The average lifespan of platelets is only 8–10 days. In the circulation, each individual has $150–400 \times 10^9$ platelets per liter of peripheral blood (9).

The primary roles of platelets are hemostasis and thrombosis. Hemostasis is the process that stops blood loss from a damaged vessel (10). Hemostasis involves multiple interlinked steps: primary hemostasis, secondary hemostasis, and tertiary hemostasis (11). Platelets are mainly involved in primary hemostasis, which is also called platelet clotting. In primary hemostasis, platelets stick to the damaged tissue and become activated, which recruits more platelets to form a platelet "plug" to stop blood loss from the damaged area. Primary hemostasis may also involve constriction of the blood vessel, which can occur due to substances released by platelets (12). In addition to hemostasis, platelet activation also contributes to thrombosis, which is a blood clot within a blood vessel that limits the flow of blood. Platelets play a significant role in the development of arterial thrombosis rather than venous thrombosis (13). Atherosclerosis allows the activation of platelets, causing adhesion and aggregation, which leads to the formation of a clot. Thus, the management of arterial thrombosis predominantly involves the use of antiplatelet agents for monotherapy or dual-antiplatelet therapy (14).

For long, Platelets are small, anucleate cell debris (15). Actually, platelets possess almost every feature of cells, except the nucleus. The role of platelets in hemostasis has long been known, but they have also been shown to be involved in defense against pathogens (16, 17), as well as in the acceleration of autoimmune diseases (18). Therefore, platelets are seen as a cellular component of the innate immune system (19). In the presence of certain infectious agents or inflammatory stimuli, platelets mediate hemostasis and thrombosis and activate innate and adaptive immunity *via* specific receptors (CD42, CD41 CD40, CD154, etc.) and/or granule release (CXCL4, CCL5, TGFβ, serotonin, β-defensin, etc.), RNA transfer, and mitochondrial secretion (20). Moreover, it was found that platelets can also release extracellular vesicles (EVs), including ectosomes (also called microvesicles or microparticles; 100–1,000 nm) and exosomes (40–100 nm) to regulate hemostasis, thrombosis and inflammation (21). In this review, we will summarize platelet-mediated regulation of innate and adaptive immune cells.

Influence of platelets on immune cells

Due to the haemostatic function of platelets, platelet transfusion is used to treat thrombocytopenia platelet function defecting disease in the clinic. Initially, platelet transfusions were thought to have no side effects, but recent findings have indicated that although the effects are not fatal, platelet transfusion can lead to febrile nonhemolytic transfusion reactions (FNHTRs), anaphylactic reactions, haemolytic transfusion reactions and other immune-mediated reactions (22). Guo et al. found that antibody-mediated immune thrombocytopenia (ITP) was resistant to allogeneic platelet transfusions, while the T-cell-mediated form of the disease was susceptible, suggesting that transfusion therapy might be beneficial for antibody-negative ITP (23). Moreover, it was reported that fresh platelets could induce transfusion-related immunomodulation (TRIM) independent of white cells (WBCs) due to their MHC antigen expression, whereas aging results in the loss of MHC and the ability to mediate TRIM (24). Ultraviolet B (UVB) radiation plus riboflavin treatment of WBC-enriched platelet-rich plasma (PRP) effectively blocks alloimmunization and modulates immune responses to subsequent exposures (25). These reports demonstrated that various reactions mediated by different WBCs led to limitations in the application of platelet transfusion. We detail the interactions between platelets and different WBCs below.

Neutrophils

As an indispensable member of the innate immune system, neutrophils are the first leukocytes to infiltrate the site of injury (26). Platelet derived P-selectin induces neutrophils to move to sites of thrombus formation by activating P-selectin glycoprotein ligand-1 (PSGL1), a receptor of P-selectin on neutrophils (27). The platelet-derived serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) also promotes neutrophil recruitment to inflamed tissue *via* the G-protein-coupled receptor 35 (GPR35) (28). In a murine model of *Klebsiella pneumoniae*-induced pulmonary inflammation, Toll-like receptor 4 (TLR4) deficiency in platelets decreased the number of neutrophils in the lung (29). Inhibition of platelet p110β (the catalytic subunit of phosphatidylinositol 3-kinase) prevented platelet–neutrophil interactions, diminishing neutrophil infiltration (30). It was also reported that activated platelet-derived nanovesicles could recruit neutrophils to exert anti-tumor effects (31). C-type lectin-like receptor (CLEC-2) was recently discovered as a platelet receptor. Blocking platelet CLEC-2 signaling enhanced liver recovery from acute toxic liver injuries by increasing tumor necrosis factor-α (TNF-α) production, which then improved reparative hepatic neutrophil recruitment (32). All these findings indicated that platelets regulate the movement of neutrophils.

Platelets also regulate neutrophil activation. Mac1 and LFA1 strengthen the attachment between platelets and neutrophils *via* junctional adhesion molecule 3 (JAM3) (33), Intercellular adhesion molecule 2 (ICAM-2) (34), CD42 (35) and Choline transporter-like protein 2 (CTL2) (36), which bind to platelet αIIbβ3 integrin, enhancing neutrophil activation. Clinical observational data showed that the levels of C-X-C chemokine receptor type 4 (CXCL4; also called platelet factor 4, PF4), CXCL7 (neutrophil activating protein-2, NAP2) and myeloperoxidase (MPO) were related to platelet activation and platelet–neutrophil interactions (37). Both CXCL4 and CXCL7 secreted by platelets can initiate neutrophil activation (38, 39). Similarly, inhibition of the chemokine receptors CXCR4 and CXCR7 on platelets and polymorphonuclear neutrophils (PMNs) was shown to reduce platelet–neutrophil complex (PNC) formation (40). Leukotriene B4 (LTB4) and leukotriene A4 (LTA4) derived from platelet-derived arachidonic acid (AA) can activate neutrophils (41). In addition, platelet-derived mitochondria induce the release of human neutrophil microvesicles that recruit additional immune cells to remove pathogens (42). Platelet-derived serotonin was shown to promote neutrophil degranulation, which increased the expression of the membrane-bound leukocyte adhesion molecule CD11b, enhanced inflammation in the infarct area and reduced myocardial salvage by inducing the release of myeloperoxidase and hydrogen peroxide (H_2O_2) (43).

An important function of neutrophils is to release neutrophil extracellular traps (NETs), which remove pathogens from the circulation. Platelet-derived exosomal high-mobility group protein 1 (HMGB1) and/or miR-15b-5p and miR-378a-3p promote excessive NET formation through the Akt/mTOR autophagy pathway during sepsis and subsequent organ injury (44). It was reported that both HMGB1 and the C3a component released by platelets could activate neutrophils to induce the formation of NETs (45–47). These stimuli significantly enhanced PSGL-1-induced neutrophil activation. Additionally, platelets interact with C3b attached to NETs (48). The P-selectin-PSGL-1 interaction was shown to induce the release of NETs, and clearing activated platelets *via* platelet-derived microparticle (100–1,000 nm) mediated neutrophil activation (49). Moreover, platelets were able to induce NOX-independent NET formation in a dengue virus non-structural protein 1 (NS1)-dependent manner (50). The inhibition of nuclear factor of activated T cells (NFAT) in platelets promotes interactions with neutrophils and NET induction, which might be harnessed in the clinic (51). In addition to influencing the infiltration of neutrophils, the TLR4-ERK5 axis in platelets facilitates NET formation to promote the capture of circulating tumor cells (52). Furthermore, a neutralizing anti-CXCL4 antibody significantly inhibited NET formation induced by NCA-associated vasculitis (AAV)-derived platelets (38).

Interestingly, NETs also induce a hypercoagulable state in platelets by upregulating phosphatidylserine and P-selectin on cells in the context of gastric cancer (GC) (53). Neutrophils can activate platelets by releasing antimicrobial cathelicidins *via* degranulation or as part of NETs. For example, cathelicidin LL-37 and its mouse homolog cathelicidin-related antimicrobial peptide (CRAMP) can bind glycoprotein VI (GPVI) on the platelet surface, further stimulating platelet and neutrophil activation (54). Citrullinated H3 histones, key markers of ongoing NETosis, have also been shown to activate platelets (48). During ischaemic stroke, neutrophils can rapidly bind platelets through P-selectin and glycoprotein Ibα (55), and neutrophils have been shown to undergo "plucking" on megakaryocytes to accelerate platelet production *via* CXCR4-CXCL12 signaling (56). Additionally, neutrophils activate platelets after pneumolysin exposure by releasing extracellular vesicles (EVs; 100–1,000 nm) (57). In acute myocardial infarction, the observed increase in S100A8/A9 levels in platelets was not due to an increase in synthesis but was due to uptake of proteins secreted by neutrophils (58). This result indicated that neutrophils were able to alter the platelet proteome.

Taken together, these findings support mutual regulation between platelets and neutrophils. Platelets can regulate infiltration, activation and NET formation in neutrophils (Table 1). In the clinic, depending on the specific conditions of a disease, the mechanism of mutual regulation between these cell types could be controlled or blocked.

Monocytes

Platelets mediate multiple types of immune responses, and many studies have shown that platelets can interact with innate immune cells during infection and inflammation. One study showed that platelet activation is the major initiator of platelet–monocyte aggregation (59). Platelets interact with monocytes through cluster of differentiation (CD)62p (known as P-selectin), which recognizes PSGL-1 expressed on the surface of monocytes to initiate aggregation (60). Platelet-derived hyaluronidase-2 (HYAL2) also causes aggregate formation (61). Platelets from severe COVID-19 patients were highly activated and induced the expression of tissue factor (TF) in monocytes from healthy volunteers (62). The increased expression of TF also drives platelet–monocyte aggregation (63), inflammatory activation and inflammatory cytokine secretion (64). However, activated platelet-derived EVs (40–100 nm) contribute to the suppression of TF expression by transferring hsa-miR-223-3p to monocytes, which inhibits aggregation (65). Therefore, the exact influence of platelets on platelet–monocyte aggregation needs to be further explored.

TABLE 1 Platelet-derived molecules affect the function of neutrophils.

Regulating aspects on neutrophils	Platelet components	Regulatory effect	References
Infiltration	P-selectin, 5-HIAA, p110β	Upregulated	(25, 26, 28)
	TLR4, CLEC-2	Downregulated	(27, 30)
Activation	αIIbβ3, CXCL4, CXCL7, MPO, LTB4, LTA4, mitochondria, serotonin	Upregulated	(35–37, 39–41)
NET formation	HMGB1, miR-15b-5p, miR-378a-3p, C3a, C3b, P-selectin, TLR4, CXCL4, NS1	Upregulated	(42–48)
	Nuclear factor of activated T cells (NFAT)	Downregulated	(49)

Platelets can induce the oxidative burst and inflammation in monocytes and neutrophils *via* direct interactions (66–70) and promote leukocyte adhesion and extravasation (71–73). In addition, platelet aggregability leads to monocyte extravasation into the infarcted myocardium and influences inflammation in patients with acute myocardial infarction (74). The SARS-CoV-2 spike protein can interact with the CD42b receptor to activate platelets and promote proinflammatory cytokine production by monocytes through the interaction of P-selectin/PGSL-1 and CD40L/CD40 (75). P-selectin was shown to contribute to the secretion of TNFα, IL-1β, IL-6, CXCL8, IL12 and CCL4 by autologous monocytes (76, 77). In addition, platelets were reported to potentiate the release of IL-8, mainly from monocytes (78). Thus, some researchers have concluded that platelet–monocyte aggregates can be used as a robust marker of platelet activation and monocyte inflammatory responses (79). Interestingly, platelets do not always activate monocytes to induce the production of proinflammatory cytokines, and platelet-monocyte interactions can actually decrease inflammation by increasing IL-10 levels and reducing TNF-α levels in monocytes through CD40L/CD40 (80).

Platelets also regulate the differentiation of monocytes. Both Sigrun Badrnya et al. and J H Phillips et al. reported that activated platelets increased CD16 expression, which induced monocytes to switch to an intermediate phenotype. $CD16^+$ monocytes produced transcripts for the gamma subunit of the high-affinity IgE FcR and could kill anti-CD16 hybridoma cell targets in the absence of CD3 zeta (81, 82). Similarly, it was reported that platelet-derived CXCL4 induced monocyte differentiation into macrophages (83). However, another study reported that inhibition of PSGL-1 or P-selectin did not attenuate platelet-mediated monocyte activation (84). This meant that there were other pathways activating monocytes. Zachary et al. found that platelet-derived β-2 microglobulin (β2M) induced monocyte proinflammatory differentiation through a noncanonical TGFβ receptor pathway (85) and regulated age-associated monocyte polarization. β2M was shown to maintain the balance between inflammatory and reparative signals. In addition, loss of β2M increases profibrotic cardiac responses (86). Thus, platelets have both pro- and anti-inflammatory effects on monocytes (Table 2).

Macrophages

The phenotype and function of macrophages are also affected by platelets. Haem-activated platelets promote the formation of macrophage extracellular traps (METs) *via* reactive oxygen species generation or histone citrullination, enhancing rhabdomyolysis-induced acute

TABLE 2 Platelet-derived molecules affect the function and differentiation of monocytes.

Platelets products	Receptors on monocytes	Effects on monocyte function	References
CD40L	CD40	IL-10 ↑, TNF-α ↓	(39)
P-selectin	PSGL-1	TNFα, IL-1β, IL-6,IL-8 CXCL8, IL-12, CCL4↑	(19, 35, 36)
Hyaluronidase-2 (HYAL2)	/	Platelet–monocyte aggregation ↑	(20)
Extracellular vesicles (EVs)	Tissue factor	Platelet–monocyte aggregation ↑	(24)
Platelet factor 4 (PF4)	/	Differentiating into macrophages ↑	(42)
β-2 micro globulin (β2M)	Noncanonical TGFβ receptor pathway	Proinflammatory differentiation ↑	(44)

kidney injury (87). Platelet-conditioned medium was also shown to induce an anti-inflammatory, pro-resolving phenotype in macrophages (88). Platelet-Treg cell aggregates in the lung induce macrophage polarization toward an anti-inflammatory phenotype and promote effective resolution of pulmonary inflammation (89). Ryoka et al. reported that platelet-rich plasma (PRP) suppressed M1 macrophage polarization and promoted M2 macrophage polarization (90). Platelet-rich fibrin could shift macrophage polarization from an M1 phenotype toward an M2 phenotype to induce an anti-inflammatory response (91) and reduce IL-1β release and caspase-1 production in macrophages that underwent pyroptosis by increasing NLR family pyrin domain containing 3 (NLRP3) ubiquitination (92, 93). Interestingly, both leukocyte-poor (LP) and leukocyte-rich (LR) PRP promoted the recruitment of M1 macrophages during the process of tendon healing, while the number of M2 macrophages was high only in the LP-PRP group (94). However, the presence of platelets skewed monocytes toward an M1 phenotype *via* the GPIb-CD11b axis in the presence of lipopolysaccharide (LPS) (95). Thus, platelets can affect macrophage polarization *via* different pathways.

Natural killer cells

Natural killer cells are also regulated by platelets. PLT-ectosomes (100–1,000 nm) can inhibit NK cell effector function in a TGF-β1-dependent manner, reducing the expression of surface receptors, like natural-killer group 2, member D (NKG2D), natural-killer p30 (NKp30) and CD226, and IFN-γ production (96). This result suggested that platelets can promote tumor dissemination by coating tumor cells (97, 98). Co-incubation of NK cells with platelets was shown to reduce NK cell cytotoxicity by reducing NK cell degranulation, IFN-γ production, NKG2D and natural-killer p46 (NKp46) expression and increased Killer cell immunoglobulin-like receptor 2DL1 (KIR2DL1) expression in NK cells (99). Furthermore, NK cell cytolytic activity was shown to be attenuated *via* tumor cell-induced platelet secretion (100). However, platelet-derived growth factor D (PDGF-DD)-activated IL-2-induced NK cells exert anti-tumor effects by binding with the NKp44 receptor (101), and PDGF-D-PDGFRβ signaling enhances IL-15-mediated human NK cell survival (102). In addition, platelet-derived CXCL4 induces human natural killer cells to synthesize and release interleukin-8 (103). Taken together, these findings show that platelets also have dual regulatory effects on NK cells.

T cells

In addition to innate immune cells, platelets also influence T-cell and B-cell responses. Norbert et al. found that platelets enhanced the differentiation and cytokine production of $CD4^+$ T cells *via* both direct cell–cell contact and multiple chemokines (platelet-derived CXCL4 and CCL5) (104). Platelets can produce many molecules, such as FasL, TNF-related apoptosis-inducing ligand (TRAIL), IL-7 and CD40L, which are all important for adaptive immune responses (105–107). In particular, platelet CD40L regulates $CD8^+$ T-cell response. Elzey et al. reported that depletion of platelets decreased the generation of cytotoxic T lymphocytes (CTLs) (108). In platelet-depleted mice, reconstitution of platelets increased the number of CTLs in the spleen and liver after lymphocytic choriomeningitis virus (LCMV) infection (109). Thus, platelets are important for the expansion of antigen-specific CTLs. Chapman et al. also demonstrated that platelets can process and present antigens *via* MHC class I and directly activate naive T cells in a platelet MHC class I-dependent manner (110). However, platelets were also shown to delay the infiltration of $CD8^+$ T cells into the liver, allowing increased viral replication *via* the release of serotonin, which might ultimately cause chronic hepatitis (111). In addition, Aslam et al. found that platelets suppressed $CD8^+$ T-cell function in a transfusion-related model and that transfusion of MHC-I bearing platelets prolonged allograft survival (24). Interestingly, the expression of MHC-I in platelets was shown to be significantly increased in humans and mice, which reduced the numbers and impaired the function of antigen-specific $CD8^+$ T cells during sepsis (112). Platelets promote protection against *C. albicans* airway mycosis by activating Th2 and Th17 responses *via* an antifungal pathway that includes candidalysin, GP1bα, and dickkopf WNT signaling pathway inhibitor 1 (Dkk-1) (113). However, activated platelets accumulate in the lung lesions of tuberculosis patients and inhibit T-cell responses and *mycobacterium tuberculosis* replication in macrophages (114). CD84-lacking platelets were shown to reduce cerebral $CD4^+$ T-cell infiltration and thrombotic activity, slowing neurological damage in an experimental model of stroke. In a clinical study, a high level of platelet CD84 expression resulted in poor outcomes in patients with stroke (115). Platelets block the immunosuppressive function of Tregs directly *via* the P-selectin/PSGL-1 axis, which induces Syk phosphorylation and an increase in intracellular calcium in systemic lupus erythematosus (SLE) patients (116). However, Jan et al. reported that interactions between platelets and Tregs *via* the P-selectin/PSGL-1 axis encouraged the release of the anti-inflammatory mediators IL-10 and TGFβ. Platelet-Treg cell aggregates induce macrophage polarization toward an anti-inflammatory phenotype in pulmonary inflammation (89). Rachidi

et al. found that platelets constrained T-cell immunity through a glycoprotein repetition predominant (GARP)-TGFβ axis, and platelet-specific deletion of GARP potentiated protective immunity against both melanoma and colon cancer (117). In addition, Hinterleitner et al. reported that platelet-derived GARP induced peripheral Treg cells by upregulating Foxp3 expression (118).

Platelet-derived CXCL4 was also shown to enhance Th1 cell responses and $CD4^+$ T effector memory cell responses *via* Akt-PGC1α-TFAM signaling-mediated mitochondrial biogenesis (119). Platelets exert dose-dependent regulatory effects on the effector responses of naive T cells *via* CXCL4-TGFβ. Low concentrations of CXCL4 reinforce TGFβ signaling, but high concentrations of CXCL4 have the opposite effect (120). In addition, knocking down the expression of CXCR3, the receptor of CXCL4, was shown to abolish Th1 and Treg cell responses (121). Platelet-derived mitochondria directly upregulate central memory (TCM) $CD4^+$ T cells and downregulate effector memory (TEM) $CD4^+$ T cells through C-X-C motif chemokine receptor 4 (CXCR4) and its ligand stromal cell-derived factor-1 (SDF-1) (122). However, CXCL4 expression is inversely correlated with T-cell function in advanced lung adenocarcinoma (LAC), leading to accelerated development of tumors (123). Pten-deficient platelets are hyperactive and overproduce multiple Tfh-promoting cytokines *via* the PDK1/mTORC2-AKT-SNAP23 axis, which promotes $CD4^+$ T-cell differentiation into Tfh cells. Pten deletion results in age-related lymphoproliferative diseases and humoral autoimmunity in mice (124).

PD-L1 is widely known as an inhibitor of the adaptive immune system. It can be transferred from tumor cells to platelets in a fibronectin 1-, integrin α5β1- and GPIbα-dependent manner in non-small cell lung cancer (NSCLC), and platelet PD-L1 possesses the ability to inhibit the function of $CD4^+$ and $CD8^+$ T cells (118). Christina et al. demonstrated that platelets decrease PD-1 and PD-L1 expression, T-cell proliferation and IFN-γ and TNF-α production (125). PD-L1-overexpressing platelets can rescue β-cells by suppressing the activity of pancreatic autoreactive T cells and increasing the percentage of Tregs in type 1 diabetes (T1D) (126). However, high expression of PD-L1 was found in platelets from COVID-19 patients, which inhibited the upregulation of CD25 expression and TNF-α and IFN-γ production by $CD4^+$ T cells (127). Activated platelet-derived IL-33, Dkk-1, and 5-HT or CD40L induce type 2 immune response or interact with TSLP-stimulated myeloid DCs to tune the sensitization stage of allergic asthma through RANK/RANKL signaling (128). In addition, platelet-$CD4^+$ T-cell aggregate frequency was positively correlated with HIV-1 viral load and was related to immune activation during HIV-1 infection (129). HIV-containing platelets result in dysfunction of glycolysis-mediated energy production in $CD4^+$ T cells. This result indicates that platelets might be a therapeutic target for immunological non-responders (130) (Figure 1).

B cells

Compared with those on T cells, the effects of platelets on B cells are less well studied. It was reported that the transfer of normal platelets into CD40L-deficient mice could transiently increase antigen-specific IgG production (131, 132). Fabrice et al. also reported that platelets could activate peripheral blood B cells and increase the production of immunoglobulins (133). These results indicated that platelet CD40L contributed to B-cell responses when $CD4^+$ T-cell-derived CD40L was absent. CD40L released from platelets contributes to the proliferation of tumor cells in intravascular large B-cell lymphoma (134). In addition, platelets secreting PF4 increase B-cell differentiation in the bone marrow environment by inducing the phosphorylation of STAT5 (135).

Since the start of the COVID-19 pandemic, vaccines for SARS-CoV-2 have been developed. In addition, numerous researchers have found that vaccine-induced immune thrombotic thrombocytopenia (VITT) occurs in individuals exposed to vaccines, especially adenoviral vector vaccines (136). VITT is an autoimmune condition characterized by antibodies that directly activate platelets, triggering thrombosis in the arterial and venous circulation. The pathophysiology of VITT is still incompletely understood (137). One hypothesis suggested that vaccines might bind to PF4, creating a novel antigen that is subsequently taken up by monocytes and trafficked to the lymph nodes, where it stimulates the proliferation of anti-PF4 memory B cells. Then, antibody binding to FcγRIII-A contributes to platelet clearance and thrombocytopenia (138). Additionally, platelets express human Fc receptors. The receptors for IgG, the Fc-γ receptor, and IgE, the Fc-ε receptor, are expressed on the platelet surface (139). FcγRIIA-expressing platelets activated by IgG immune complexes contribute to the severity of anaphylaxis (140). IgE antibody binding to platelets *via* the low-affinity IgE receptor (Fc epsilon RII/CD23) or high-affinity IgE receptor (Fc epsilon RI) led to immediate-type allergic reactions (141, 142). Thus, platelets and their secreted molecules can influence B cells in adaptive immunity. In addition, antibodies released by B cells are also able to regulate the numbers and activation of platelets.

Dendritic cells

Although many studies have indicated that platelets play critical roles in T-cell and B-cell adaptive immunity, the mechanism is still unknown. In addition to their ability to mediate T-cell and B-cell immune responses directly, platelets might also regulate these responses indirectly through dendritic cells (DCs). DCs are the primary antigen-presenting cells and can cross-present antigens to T cells to induce antigen-specific cell responses. Thus, changes in the number or phenotype of DCs influence cell immunity.

Platelets were reported to significantly inhibit proinflammatory (IL-12, IL-6, and TNFα) cytokine production and increase anti-inflammatory (IL-10) cytokine production in moDCs. In addition, platelet-derived soluble mediators inhibit T-cell priming and T helper differentiation toward an $IFNγ^+$ Th1 phenotype induced by moDCs (143). Platelet concentrates also downregulate the expression of CD40, CD80, CD83, and CD86 and IL-8, IL-12 and IL-6 secretion by $BDCA^+$ DCs (144, 145). Similarly, platelet-monocyte complex (PMC)-derived DCs were shown to exhibit reduced levels of critical molecules for DC function (CD206, CD80, CD86, and CCR7) and reduced antigen uptake capacity (146). Conversely, thrombin-activated platelets increased CD80 expression in DCs and induced DCs to produce tumor necrosis factor alpha (TNF-α), interleukin 12 (IL-12), and IL-6 after coculture of BMDCs and *staphylococcus aureus in vitro* (147). Furthermore, platelets have the ability to enhance the DC-mediated Th2 response and contribute to allergic inflammation through the RANK ligand (148). Platelets are necessary for efficient host

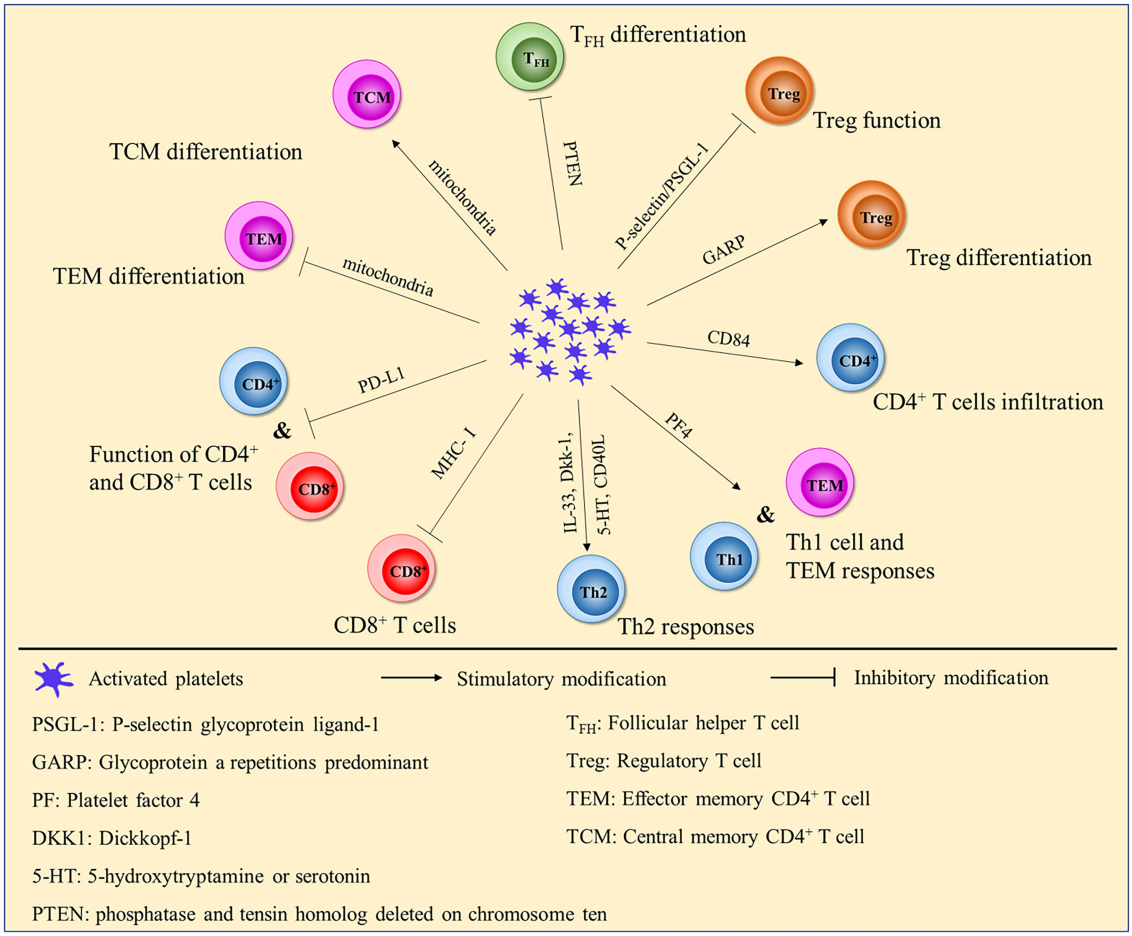

FIGURE 1
Platelets affect the function and differentiation of CD4⁺ and CD8⁺ T cells.

sensitization to allergens and increase the allergen sensitization of CD11c⁺ DCs (149).

Platelet-derived P-selectin interacts with PSGL-1 on the surface of monocytes and induces monocyte differentiation into DCs, which are more potent than cytokine-derived DCs during tumor-specific T-cell immune responses (150). Additionally, the interaction between platelets and DCs is mediated by Mac-1, which is upregulated on DCs by activated platelets in a PSGL-1-dependent manner (151). Moreover, platelet-derived CD40L can induce monocyte differentiation into DCs, promote DC maturation, increase the expression of costimulatory molecules (152, 153) and enhance interferon-α secretion by plasmacytoid dendritic cells in systemic lupus erythematosus (154). Sharmeen et al. also reported that platelets enhanced dendritic cell responses through CD40-CD40L during *staphylococcus aureus* infection (147).

Serotonin, another platelet molecule, had opposite effects on DC differentiation. As the expression of costimulatory molecules on DCs was reduced and IL-10 production was increased by serotonin, the antigen presentation function of DCs was repressed (155). Similarly, CXCL4 inhibited monocyte differentiation into CD1α⁺ DCs and increased the number of CD1α⁻ DCs, but CD1α⁻ DCs were not as effective as CD1α⁺ DCs in activating T cells (156). Moreover, CXCL4 enhanced monocyte-derived DCs to promote autologous CD4⁺ T-cell and CD8⁺ T-cell proliferation and the production of IFN-γ and IL-4 (157). In summary, platelets and their secreted molecules have different effects on the development or differentiation of DCs, and the exact mechanism still needs further exploration (Table 3).

Conclusion

In addition to supporting thrombosis, platelets release a number of mediators that regulate both innate and adaptive immunity. Due to the large number of platelets in the circulation, they and their products can efficiently interact with peripheral circulating cells directly, such as neutrophils, monocytes, T cells, B cells, DCs, macrophages, and NK cells, which modulates their differentiation. Platelets affect the functions of monocytes and neutrophils, including their receptors and soluble mediators. Moreover, platelet interactions with monocytes induce their differentiation into macrophages and regulate cytokine release. Depending on the severity of the disease, platelets can enhance or reduce leukocyte cytokine production, which maintains a balance to limit excessive inflammation during infection. Platelet-derived CD40L or other ligands can also modulate adaptive immunity. Additionally, both *in vitro* and *in vivo* evidence suggests that platelets also impact the development and functions of DCs to regulate T-cell

TABLE 3 Platelet-derived molecules affect the function and differentiation of dendritic cells.

Platelet products	Subtype of dendritic cells	Effects on dendritic cells	References
Secretome	mo-DCs	IL-12, IL-6, TNFα, CD206, CD80, CD86, CCR7↓; IL-10↑	(92, 95)
Platelet concentrates	$BDCA^+DCs$	CD206, CD80, CD86, CCR7, IL-8, IL-12 and IL-6 ↓	(93, 94)
Thrombin-activated platelet	BMDCs	CD80, TNF-α, IL-12, IL-6↑	(95)
RANK ligand	myeloid DCs	CD86, CD40, CD83, Th2 response↑	(97)
Platelets	$CD11c^+$ DCs	allergen sensitization↑	(98)
P-selectin	mo-DCs	Mac-1 expression↑	(100)
CD40L	mo-DCs	Maturation, costimulatory molecules, IL-12p40↑	(101, 102)
CD40L	pDCs	IFN-α↑	(103)
Serotonin	mo-DCs	costimulatory molecules↓; IL-10↑	(105)
CXCL4 (PF4)	mo-DCs	$CD1\alpha^+$DCs↓; $CD1\alpha^-$DCs↑; IFN-γ and IL-4↑	(106, 107)

and B-cell responses. According to previous reports, different products of platelets have different effects on DCs, thus changing their antigen presentation capacity.

All these reports indicated that in addition to hemostasis, platelets also play critical roles in the immune system, but the exact mechanism is still not clear. Given that platelet concentrates are widely used in clinical treatment and given the side effects of platelet transfusion, we need to consider the effects of platelets and their secreted molecules on immune cells, such as neutrophils, monocytes, B cells, T cells and DCs. Elucidating how platelets interact with these cells will contribute to broader application of platelet products and avoid adverse reactions.

Author contributions

CY and HW mainly drafted and revised this review. XF and JH conceived and provided lots of advises about this review. FZ conducted the overall writing and previewed the draft and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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