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Therapeutic potential of innate and innate-like effector lymphocytes in autoimmune and inflammatory diseases

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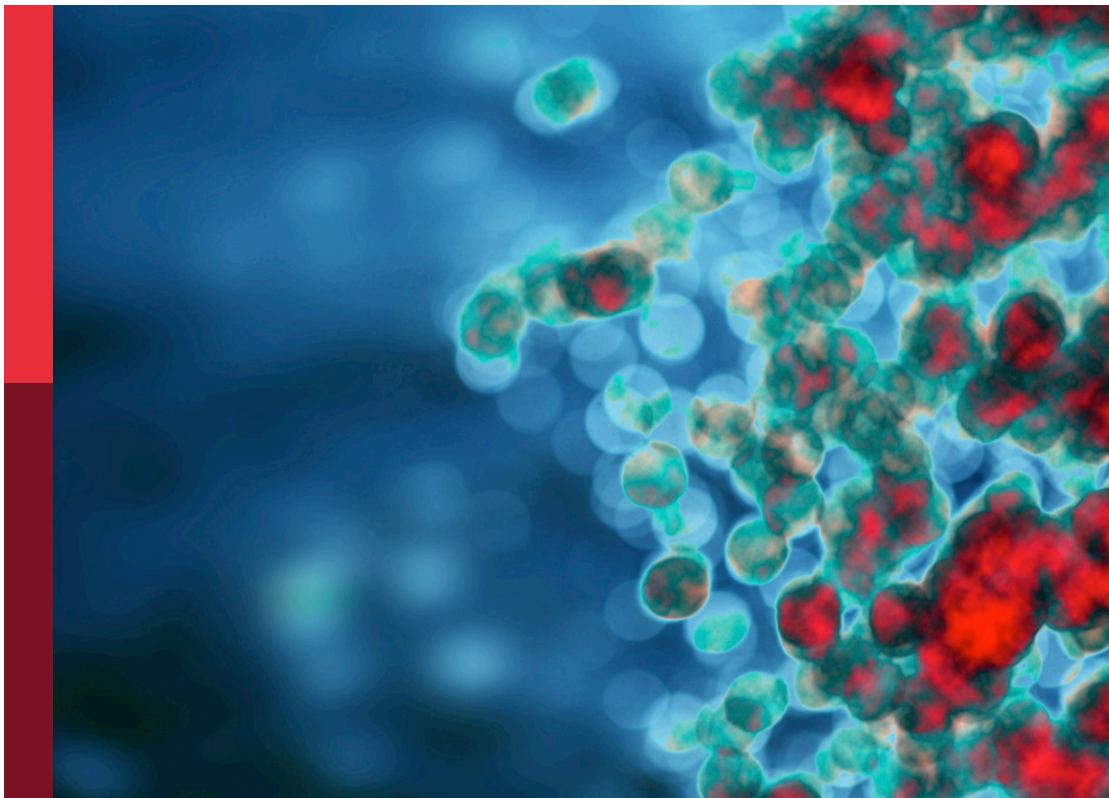

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Therapeutic potential of innate and innate-like effector lymphocytes in autoimmune and inflammatory diseases

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Table of contents

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Editorial: Therapeutic potential of innate and innate-like effector lymphocytes in autoimmune and inflammatory diseases

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Editorial on the Research Topic

Therapeutic potential of innate and innate-like effector lymphocytes in autoimmune and inflammatory diseases

Modulating the immune system holds great promise for treating a variety of autoimmune and inflammatory diseases, by stimulating desired immune responses and/or by inhibiting undesired immune responses. Traditional therapeutic modalities for autoimmune and inflammatory conditions exert global suppressive effects on immune responses and often impair overall immune competence, as they quell both pathogenic and protective immune responses. More recently developed biological therapies selectively suppress the pathogenic responses in autoimmune and inflammatory diseases by acting on specific immune cell subsets or the inflammatory mediators they produce. The latter treatments require a thorough understanding of the underlying disease pathology, especially the role of antigen-specific effector B and T lymphocytes of the adaptive immune system. However, comparatively little attention has been paid to the immunotherapeutic potential of innate and innate-like effector lymphocytes, which also make important contributions to the development and progression of autoimmune and inflammatory diseases. This Research Topic focuses on our emerging understanding of the roles of innate and innate-like lymphocytes in the pathogenesis of autoimmune and inflammatory diseases, and how this information might be exploited for the development of new and improved immunotherapies.

Innate and innate-like lymphocytes share a number of features that make them particularly attractive targets for immunotherapy. For example, their specificity is not impacted by polymorphic major histocompatibility complex (MHC) ligands and, therefore, uniform tools could be employed to elicit their therapeutic properties in genetically disparate individuals. Furthermore, their therapeutic activation or inactivation might not lead to widespread immune impairment and susceptibility to infection or cancer.

Among the innate lymphocytes, natural killer (NK) cells have been studied predominantly for their anti-tumor and anti-viral activities. Yet, these cells also play critical roles in a variety of other diseases. Wang et al. review the controversial role of NK cells in sepsis, where they might contribute to protective immune responses against the invading microbes, but also to the overall hyper-inflammatory phase of sepsis by producing cytokines and causing tissue destruction, and to the subsequent immune-suppressive phase of sepsis where they might adopt a hyporesponsive phenotype rendering the host susceptible to secondary infection. The original research article by Qi et al. explores the features of NK cells in patients with Alzheimer's disease, revealing quantitative and qualitative alterations in these cells, and the presence of a unique NK cell subset whose prevalence negatively correlates with patient cognitive functions. Although NK cells are best known for displaying innate effector functions, in some situations, such as during infection by human cytomegalovirus (HCMV), some NK cells might exert adaptive-like memory responses. The activating NKG2C receptor on such NK cells might engage with HCMV-derived peptides bound with the unconventional human leukocyte antigen (HLA)-E protein. The primary research article by Almazán et al. describes the identification of three peptides from HCMV that induce such NK cell-mediated memory responses. The investigators also generated synthetic versions of these HCMV peptides that could be potentially employed as therapeutic vaccines.

In addition to NK cells that were discovered nearly fifty years ago, work during the past two decades has identified multiple additional innate lymphocyte subsets. This growing family of innate lymphoid cells (ILCs) is typically partitioned into three groups: group 1 includes NK cells and ILC1 cells producing type 1 cytokines such as interferon (IFN)-γ, group 2 includes ILC2 cells producing type 2 cytokines such as interleukin (IL)-4 and IL-5, and group 3 includes ILC3 cells and lymphoid tissue inducer cells producing type 3 cytokines such as IL-17. Jia et al. review the contributions of distinct ILC subsets to the development of atopic dermatitis, with a focus on the pathogenic role of ILC2s. Zhang et al. discuss the controversial role of group 3 ILCs in intestinal diseases, which might be related to the capacity of these cells to respond to dietary metabolites and gut microbiota. Thomas and Peebles review IL-10-producing ILCs, which have been observed among all ILC subsets. These cells display a regulatory phenotype that promotes gut and lung homeostasis, with the potential for therapeutic applications in intestinal and lung diseases.

A unique subset of innate lymphocytes with T cell features and with the capacity for IFN-γ production and cytotoxicity has been identified within the intestinal epithelium. Hariss et al. present new findings on these so-called innate intestinal intraepithelial lymphocytes (iIELs) during infection by the intestinal, protozoon pathogen *Cryptosporidium parvum*, revealing the capacity of these iIELs to control parasite proliferation at early stages of the infection.

Multiple subsets of T lymphocytes display innate-like functions, including CD1d-restricted natural killer T (NKT) cells, MHC class I related-1 (MR1)-restricted mucosal-associated invariant T (MAIT) cells, innate subsets of T cell receptor (TCR) γδ-expressing T cells, and subsets of innate-like, TCR-expressing iIELs. Bharadwaj and Gumperz review different ways by which the anti-inflammatory properties of NKT cells might be harnessed to control pathological inflammation, and how differences in the functional properties between murine and human NKT cells will likely make this goal rather challenging. Lee et al. review the role of NKT cells to immunity in the skin, where these cells can exert either protective or pathogenic effects on inflammatory skin diseases, raising the possibility to modulate NKT cell effector functions for immunotherapy. The original research article by Imahashi et al. also focuses on skin inflammation, using a mouse model of allergic contact dermatitis, to explore the role of MR1-restricted MAIT cells to disease, showing that these cells are activated quickly following challenge with a contact allergen to suppress skin inflammation. Joyce et al. review the contribution of NKT cells and MAIT cells to a wide variety of infectious agents, arguing that these cells can integrate signals delivered by innate sensor cells responding to pathogens and then relay those signals to downstream innate and adaptive immune effector cells. Finally, Zhou reviews the role of NK cells, NKT cells, MAIT cells, and γδ T cells to early liver inflammation in various contexts, how such cells might trigger chronic liver inflammation and fibrosis, and how their depletion might be able to attenuate several liver diseases.

Similar to the T cell lineage, subsets of B lineage cells with innate-like functions have been identified. This includes marginal zone B (MZB) cells that reside at the interface between the circulation and lymphoid tissue, and B-1 B cells that reside primarily in the mesothelial cavities. Tandel et al. review yet another B cell subset, referred to as natural killer-like B (NKB) cells, with reported innate-like characteristics. While some research groups have confirmed that NKB cells express characteristic markers of both NK cells and B cells, other researchers have argued that these cells display phenotypic and functional characteristics of conventional B cells, prompting the need for further investigations of their origin and identity.

The articles included in this Research Topic provide elegant examples of the exciting, ongoing work on innate and innate-like lymphocytes, highlighting the potential of targeting these cells for immunotherapy of human autoimmune and inflammatory diseases.

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Conflict of interest

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Roles and therapeutic potential of CD1d-Restricted NKT cells in inflammatory skin diseases

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Natural killer T (NKT) cells are innate-like T lymphocytes that recognize glycolipid antigens rather than peptides. Due to their immunoregulatory properties, extensive work has been done to elucidate the immune functions of NKT cells in various immune contexts such as autoimmunity for more than two decades. In addition, as research on barrier immunity such as the mucosa-associated lymphoid tissue has flourished in recent years, the role of NKT cells to immunity in the skin has attracted substantial attention. Here, we review the contributions of NKT cells to regulating skin inflammation and discuss the factors that can modulate the functions of NKT cells in inflammatory skin diseases such as atopic dermatitis. This mini-review article will mainly focus on CD1d-dependent NKT cells and their therapeutic potential in skin-related immune diseases.

KEYWORDS

CD1d-restricted NKT cells, glycolipid antigens, atopic dermatitis, allergic contact dermatitis, psoriasis, UV-induced skin inflammation

Introduction

Inflammatory immune responses in the skin are attributed to exposure to allergic irritants (e.g., metals, fragrance chemicals, preservatives, antibiotics, and drugs), pathogens (e.g., *Staphylococcus aureus* and fungi), and ultraviolet (UV) radiation (1, 2). While many immune cell types contribute to the pathogenesis of inflammatory skin diseases, we focus here on the role of natural killer T (NKT) cells, a subset of innate-like T cells that co-express T and NK cell receptors. In general, NKT cells recognize glycolipid antigens presented by MHC I-like CD1d molecules. NKT cells can be further classified into two subsets based on their distinct TCR characteristics: type I (invariant TCRα chain, Vα14Jα18 in mice and Vα24Jα18 in humans) and type II (diverse TCR, non-Vα14Jα18/Vα24Jα18) NKT cells (3–6). Type I NKT cells are also called invariant NKT (iNKT) cells, owing to their unique expression of an invariant TCR alpha chain, and these cells react with the prototypical glycolipid antigen α-

galactosylceramide (α-GalCer). Both subsets of NKT cells make crucial contributions to skin inflammatory responses, playing either protective or pathogenic roles in animal models of inflammatory skin disorders (7, 8). Consistent with these animal studies, patients with inflammatory skin diseases (e.g., atopic dermatitis (AD), allergic contact dermatitis (ACD), psoriasis, and UV-induced skin inflammation) display functional alterations in CD1d-restricted NKT cells (7, 8). Functional heterogeneity of CD1d-restricted NKT cells may also contribute to the distinct outcome of various skin diseases. In particular, depending on the expression profile of CD4 and CD8 co-receptors, type I NKT cells can be subdivided into $CD4^+$ and $CD4^-CD8^-$ (double negative, DN) subsets. Furthermore, type I NKT cells are functionally subclassified by differential expression of transcription factors: T-bet for NKT1, GATA3 and PLZF for NKT2, and RORγt for NKT17 cells (3–6). This mini-review will discuss the immunomodulatory roles of CD1d-restricted NKT cells in various inflammatory skin disorders.

Atopic dermatitis (AD)

AD is a pruritic and chronic inflammatory skin disorder characterized by T helper type 2 (Th2)-dominant responses. It is elicited by pro-Th2 cytokines (e.g., thymic stromal lymphopoietin (TSLP), IL25, and IL33) released by keratinocytes and fibroblasts (9). Interestingly, AD's pathogenesis in humans closely correlates with quantitative and qualitative changes in iNKT cells among peripheral blood mononuclear cells (PBMCs) (10–17). Recently, several studies have reported that AD patients display phenotypic changes in CD1d-restricted NKT cells, suggesting their potential role in AD pathogenesis.

The frequencies of surface immune cell markers [i.e., CD4/CD8 (10, 12, 14, 15), CD161 (13), and CXCR4 (17)] among NKT cells of AD patients are altered. In addition, one study reported that AD patient-derived IgG antibodies induce selective expansion of the $CD4^+$ subpopulation in thymic but not splenic iNKT cells from non-atopic infants and such IgG-stimulated $CD4^+$ iNKT cells produced high amounts of IL4, IL17, and IL10 (18). Recently, Sun et al. reported that skin-resident $CXCR4^+$ iNKT cells recruited by fibroblast-derived CXCL12 aggravate AD through excessive secretion of both IFNγ and IL4 (17). Conversely, our study demonstrated that adoptive transfer of iNKT cells (mostly DN cells) from Vα14 TCR transgenic (Tg) NC/Nga (NC) mice effectively prevented spontaneous AD development in recipient NC mice by increasing IFNγ-producing $CD8^+$ T cells and regulatory T (Treg) cells (19). Furthermore, consistent with our report, previous studies have shown that DN iNKT cells can protect against airway hypersensitivity in a mouse model of asthma *via* expansion of Treg cells (20, 21). Moreover, based on studies that influenza infection or injection of Th1 cytokine-biasing glycolipids (e.g., α-C-GalCer and napthylurea-modified α-GalCer) during the neonatal period can induce preferential expansion of DN NKT cells in mice, expansion of DN NKT cells during early life might be effective in preventing AD development (20, 21). However, repeated injection of α-GalCer into Vα14 TCR Tg NC mice exacerbated AD pathogenesis, indicating that Th2-biased iNKT cells induced by repeated α-GalCer injection exhibit adverse effects on AD symptoms (22). This study therefore suggests that continuous exposure to pathogen-derived glycolipid antigens can dramatically influence AD development.

Pro-Th2 cytokines, including TSLP, IL33, and IL25, play a critical role in initiating Th2 immune responses in AD (9). It has been reported that enhanced expression of keratinocyte-derived TSLP in AD patients activates iNKT cells to secrete IL4 and IL13, which positively correlated with AD severity (23). Moreover, murine keratin-14^+ keratinocytes and $HMGB1^+$ fibroblasts in the skin express high levels of IL33 after intradermal injection of *S. aureus* (24). Although IL33- and IL25-induced iNKT cell activation has been shown to play an essential role in a mouse model of asthma (25, 26), it remains unclear whether CD1d-restricted NKT cells stimulated by IL33 and IL25 contribute to AD progression. It has been reported that the skin lesions of most AD patients are heavily colonized with *S. aureus* (27). In particular, the prevalence of multi-drug resistant *S. aureus* (MRSA) in children with AD has continued to increase for over ten years (28). Unlike α-GalCer, heat-killed *S. aureus* induces the secretion of substantial amounts of IFNγ rather than IL4 by iNKT cells *via* CD1d-dependent activation in the presence of DCs (29). In addition, an *S. aureus*-derived lipid fraction, containing a 60:40 ratio of PG (phosphatidylglycerol): lysyl-PG, stimulated type II NKT cells through CD1d-TCR engagement to produce IFNγ, resulting in protection against MRSA infection (30). However, treatment with sulfatide, a well-known endogenous ligand for type II NKT cells, significantly attenuated *S. aureus* sepsis *via* decreased secretion of TNFα and IL6 cytokines in the blood (31), suggesting that type II NKT cells might be involved in regulating *S. aureus* pathogenesis in the skin.

Epicutaneous and intradermal infection of *S. aureus* induces skin inflammation through MyD88-dependent signaling (32). Additionally, TLR-activated DCs can present self-lipid antigens (e.g., β-D-glucopyranosylceramide (β-GlcCer) and iGb3) to activate iNKT cells in a MyD88-dependent fashion (33). Furthermore, rapid up-regulation of Ugcg (ceramide glucosyltransferase) in DCs accompanied by *S. aureus* infection induces endogenous β-GlcCer accumulation in DCs, resulting in the CD1d-dependent presentation of β-GlcCer to iNKT cells. Notably, β-GlcCer C24:1 was the most potent β-GlcCer variant to activate iNKT cells in TLR-stimulated DCs (34). These findings support the notion that CD1d-restricted NKT cells contribute to regulating *S. aureus* infection-elicited immune responses *via* CD1d-dependent TCR engagement. It is well established that staphylococcal superantigens (SsAgs), such as staphylococcal enterotoxin B (SEB), contribute to the

pathogenesis of skin inflammation in AD (35). In addition, SsAgs expand Vβ8$^+$CLA (cutaneous lymphocyte-associated antigen)$^+$ memory T cells in PBMCs and induce their infiltration into skin lesions of AD patients (36). Since iNKT cells predominantly express a Vβ8 chain paired with a Vα14-Jα18 chain, *S. aureus* (strain COL)-derived superantigen SEB directly stimulated iNKT cells to release IFNγ rather than IL4 in an MHC II- but not CD1d-dependent manner (37).

Allergic contact dermatitis (ACD)

ACD, also called "contact hypersensitivity (CHS)", is considered a Type IV or delayed-type hypersensitivity (DTH) because it is mainly mediated by T cells. Many iNKT cells infiltrating ACD skin lesions display an effector phenotype with high levels of IFNγ and IL4, indicating that iNKT cells might play an essential role in ACD pathogenesis. Interestingly, the ratio of two cytokines, IFNγ and IL4, in the skin of these patients appears to diverge in a manner dependent on the allergen type (38).

Nickel allergy is the most prevalent metal-induced ACD. In the murine experimental setting of nickel allergy, iNKT cells are predominant in inflamed skin. They secrete high amounts of Th1-type cytokines (i.e., TNFα, IFNγ, and IL2) as well as cytolytic molecules (NKG2D, perforin, granzymes A and B, and FasL), suggesting that iNKT cells influence nickel allergy development (39). Notably, among iNKT cells in the ACD skin lesions, the DN iNKT subpopulation is over three times more abundant than the CD4$^+$ iNKT cell subset (39). Furthermore, since DN iNKT cells exhibit a Th1-like phenotype with high IFNγ and IL2 but low IL4 secretion in mice (19), and CD4$^-$ iNKT cells express high levels of NKG2D on their surface in humans (40), it is likely that DN but not CD4$^+$ iNKT cells play a pathogenic role in nickel allergy. Moreover, it has been reported that keratinocytes do not activate resting iNKT cells but could serve as targets for activated iNKT cells releasing cytolytic granules such as perforin and granzymes in ACD patients (41). Furthermore, the cytotoxicity of iNKT cells against keratinocytes was CD1d-dependent, consistent with a pathogenic role in ACD. However, the precise mechanism of iNKT cell activation in nickel allergy remains to be elucidated. Since TLR4 signaling (in humans) and MyD88/IL1 signaling (in mice) have been implicated in nickel-induced ACD (42–44), either immune cells (i.e., DCs and macrophages) or non-immune cells such as keratinocytes may mediate iNKT cell activation.

Different types of ACD can be induced by haptens (e.g., 2,4-dinitrofluorobenzene (DNFB), dinitrochlorobenzene (DNCB), and oxazolone). In murine DNFB-induced ACD, iNKT cells attenuate ACD pathogenesis *via* modulation of CD8$^+$ T cell activation but not Treg cell induction, suggesting a protective role of iNKT cells. These effects were attributed to IL4 and IL13 released from iNKT cells stimulated by hapten-loaded DCs through a CD1d-dependent pathway (45). A protective role of iNKT cells has also been reported in the DNCB-induced ACD mouse model, in a mechanism involving suppression of IFNγ production (46). These protective effects were strongly linked with increased IL10-producing regulatory B (Breg) cells constituting most of the CD1d^{hi}CD5$^+$ subset in the spleen and peritoneal cavity (46). However, a previous study demonstrated that iNKT cells play pathogenic roles in the oxazolone-induced ACD murine model. Oxazolone sensitization triggers iNKT cells to produce IL4 to co-activate innate-like B1 cells along with specific antigens for IgM antibody production, ultimately exacerbating ACD by recruiting effector T cells (47). Previous studies provide support for a critical role of CD1d-dependent cognate interactions between iNKT cells and B1 cells to induce B1 cell-derived circulating IgM in oxazolone-induced ACD (48–50). Moreover, the progression of oxazolone-induced ACD could be attenuated effectively by intraperitoneal injection of the iNKT cell antagonist α-ManCer (51), which provides support for the pathogenic role of iNKT cells in this model. Taken together, iNKT cells can play differential roles in ACD depending on the type of hapten employed in disease induction.

Psoriasis

Psoriasis is a chronic immune-mediated skin disorder characterized by red, scaly, thickened, inflamed, and itchy skin. Pro-inflammatory cytokines (i.e., TNFα, IFNγ, IL17, and IL22) are central in initiating psoriatic skin inflammation (52). Interestingly, Vα24$^+$Vβ11$^+$ NKT cells (53) or CD3$^+$CD56$^+$ NKT cells (54) in PBMCs were statistically decreased in number in psoriasis patients compared with healthy controls. In contrast, the relative frequencies of iNKT2 and iNKT17 cells in PBMC of psoriatic patients were increased compared with healthy controls, whereas total and CD69$^+$ iNKT cells were significantly decreased in number (55). Moreover, infusion therapy to psoriatic patients with CD3$^+$CD56$^+$ NKT cells (which likely consist of CD1d-restricted NKT cells) restored CD3$^+$CD56$^+$ NKT cell levels in patient PBMCs, leading to improved skin lesions in severe psoriasis (56). These studies indicate that CD56$^+$ NKT cells contribute to regulating psoriatic skin inflammation, possibly by producing Th2 cytokines such as IL4.

Conversely, psoriatic patients have significantly higher numbers of skin CD161$^+$ NKT cells in the pre-psoriatic skin than in normal skin (57). Importantly, CD1d-restricted CD161$^+$ NKT cells from psoriatic patients were capable of rapidly producing IFNγ upon recognition of glycolipid antigen presented by CD1d on keratinocytes (58). In addition, intradermal injection of these cells into pre-psoriatic human skin grafted on severe combined immunodeficiency (SCID) mice caused the development of psoriatic plaques (59). Furthermore,

injection of allogeneic blood-derived psoriatic lymphocytes induced psoriatic plaques in the skin of SCID mice receiving human skin xenografts, and increased $CD161^+$ NKT cell infiltration closely correlated with psoriasis pathogenesis (60). Another study also showed that CD1d-expressing keratinocytes could stimulate $CD161^+$ NKT cells to produce a more significant amount of IFNγ, resulting in exaggerated psoriasis (58). In addition, increased activity of PKCζ in TNFα-stimulated keratinocytes has been implicated in enhanced Vα24 and CD1d expression in psoriatic skin (61). Collectively, these studies suggest that $CD161^+$ NKT cells play a central role in the pathogenesis of psoriasis by inducing Th1-type cytokine production in a CD1d-dependent manner.

It is well known that patients with psoriasis show increased transepidermal water loss (TEWL), which reflects skin barrier abnormalities (i.e., increased permeability), accompanied by a reduction of epidermal ceramides (Cer) (62). In the upper epidermis, β-glucocerebrosidase (GlcCer'ase) was decreased in psoriatic skin compared with normal skin, suggesting that the decreased activity of GlcCer'ase may be responsible for GlcCer accumulation and a reduction of Cer in the lesional skin of psoriatic patients (63). In particular, the accumulation of 5-25% GlcCer in the stratum corneum (together with the concomitant loss of 5-25% Cer) has been implicated in increased TEWL in human skin (64). Since TNFα increases CD1d expression on keratinocytes and GlcCer-rich fractions activate NKT cells in a CD1d-dependent manner (61, 65), it will be worthwhile to investigate whether treatment with both a GlcCer'ase activator and a TNFα inhibitor (i.e., infliximab, adalimumab, or etanercept) can improve clinical symptoms by controlling pathogenic CD1d-restricted NKT cell activation in psoriatic patients.

UV-induced skin inflammation

CD1d-dependent iNKT cells play protective roles in UV-induced skin inflammation. For example, iNKT cell-deficient CD1d KO mice are more resistant to UV-induced apoptosis of keratinocytes and fibroblasts (66). Furthermore, Fukunaga et al. demonstrated UV irradiation suppresses DNFB-induced CHS in mice. Such immunoregulatory effects of UV exposure are associated with enhanced IL4 production by iNKT cells induced *via* CD1d-expressing Langerhans cells (LCs) in skin-draining lymph nodes (67). These studies identify CD1d-dependent NKT cells as therapeutic targets to modulate UV exposure-elicited Th1-type skin immune diseases such as CHS. Interestingly, two different NKT cell-deficient mouse models displayed distinct outcomes in response to UV-induced skin inflammation: Jα18 KO and CD1d KO mice generated pathogenic and protective responses, respectively. Although these results might reflect differential functions between type I and type II NKT cells (68), the effect of altered TCR repertoire diversity in Jα18 KO mice should be reassessed (69–71).

UVB irradiation induces the accumulation of sphingolipids such as GlcCer in the mouse epidermis (particularly the stratum corneum), resulting from markedly reduced GlcCer'ase activity, with a concomitant increase in TEWL (72). One previous study showed that *in vivo* glucosylceramide synthase (GCS)-dependent glycosphingolipid (GSL), in particular GlcCer, influences iNKT cell development in the mouse thymus (73). Because endogenous GlcCer is widely found in most mammalian tissues, the GlcCer-enriched lipid fraction could activate iNKT cells in a CD1d-dependent manner (34, 65). Therefore, these findings suggest that the CD1d-dependent immune suppressive effects of UV exposure might be attributed to iNKT cell recognition of CD1d loaded with endogenous glycolipids such as GlcCer. In addition, UV irradiation has beneficial effects on bacterial infection-induced pathology. For instance, in UV-irradiated mice, $CD4^+DX5^+$ NKT cells produce IL4 to inhibit *Candida albicans* infection-induced DTH immune responses in a CD1d-dependent manner (74).

Other skin-related diseases

In patients with scleroderma, also known as systemic sclerosis (SSc), numerical and functional defects of iNKT cells have been identified (75). B cells have been suggested as one of the key players in SSc pathogenesis. Scleroderma patients display significantly higher IL6 production by B cells, and suppression of B cell-derived IL6 was attributed to cell contact between iNKT and CD1d-expressing B cells *via* the CD1d-TCR axis (76). Furthermore, iNKT cells have been reported to play important roles in wound healing (77). For example, after skin wound induction, the healing process was delayed in iNKT cell-deficient Jα18 KO mice, which was associated with reduced IFNγ production. iNKT cells promote skin wound healing by preventing prolonged neutrophilic inflammatory responses (78). In addition, iNKT cells promote the clearance of *Pseudomonas aeruginosa* at the wound site during skin wound healing by inducing IL22, IL23, and antimicrobial peptide S100A9 after bacterial infection (79). Alopecia areata (AA) is a skin disorder that causes hair loss. A previous study showed that, in the human skin xenograft model, IL10-secreting iNKT cells prevent AA development, suggesting that their activities are related to suppression of $NKG2D^+CD8^+$ T cells, which are potential mediators of AA (80). In addition, vitiligo patients display defective frequencies and functions of iNKT cells in PBMCs (81).

Concluding remarks

CD1d-restricted NKT cells are critical immune mediators in regulating skin inflammatory responses (Figure 1 and Table 1). Thus, modulating the effector functions of NKT cells may be explored to develop therapeutics for skin

immune diseases. For example, the effects of NKT cell activation could be altered by the protocol employed to administer glycolipids [i.e., dosage (82), frequency (83), route (84, 85)]. Further, distinct types of NKT cell-stimulating glycolipids can contribute to the immune balance between Th1 and Th2 responses (86–88). Interestingly, it has been reported that the long-chain fatty acid palmitate (C16:0) directly activates iNKT cells to induce a decrease in IFNγ and IL4 (89), but an increase in IL10 production (90) *via* inositol-requiring enzyme 1α. Such iNKT cell-produced IL10 ultimately suppresses inflammatory responses, suggesting palmitate as a promising candidate to treat inflammatory skin diseases.

From the perspective of developing topical therapeutics for skin diseases, the skin barrier remains a significant challenge. Thus, there is growing interest in designing safe and effective drug delivery systems. One example is nanocarriers such as liposomes and micelles to help increase the penetration of drugs through the skin barrier (91). In particular, palmitate-containing liposomes may provide significant therapeutic benefits to iNKT cell-mediated skin inflammation (92). In addition, as increased β-GlcCer accumulation by the reduction of GlcCer'ase activity affects NKT cell activation (63, 93), extracellular vesicle-based delivery of GlcCer'ase represents a promising therapeutic approach (94). Furthermore, the smaller the nanoparticles, the higher their drug delivery efficiency to the skin (95). Recently, we have demonstrated that nano-sized graphene oxide (nGO) mediates anti-inflammatory responses *via* conversion of iNKT cells toward a regulatory phenotype (96). Thus, nGO could be a promising strategy to modulate iNKT cells for suppressing inflammatory skin diseases.

As already noted, iNKT cells are functionally divided into several groups depending on the expression of transcription factors. Despite emerging evidence on distinct roles of iNKT cell subsets in various immune responses, little is known about their involvement in inflammatory skin diseases. Thus, it will be important to explore the precise immunoregulatory mechanisms of the skin resident iNKT cell subsets to develop better therapeutic agents for skin inflammation.

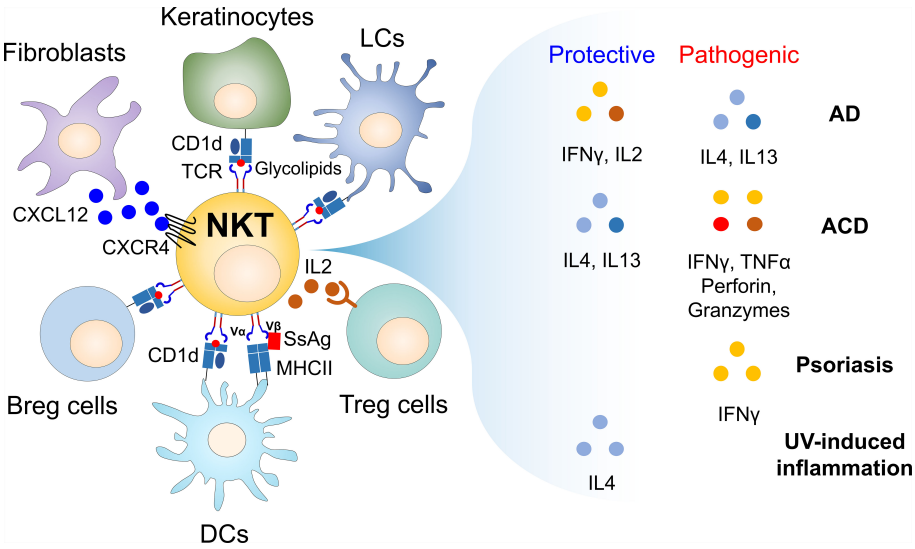

FIGURE 1
Cellular networks of CD1d-restricted NKT cells and their soluble factors in regulating skin inflammatory responses. Since the skin is constantly exposed to external stimuli such as pathogens and allergens, inflammatory immune responses occur when the skin barrier is broken. For example, during infection, endogenous glycolipids (i.e., β-GlcCer) induced by TLR signaling can stimulate CD1d-restricted NKT cells to produce large amounts of soluble factors such as cytokines that promote or regulate immune responses, contributing to maintaining skin homeostasis. Thus, CD1d-restricted NKT cells can link innate and adaptive immunity, despite the small number of these cells in the skin. In addition, CD1d-restricted NKT cells can regulate immune responses by interacting with non-immune cells (i.e., fibroblasts and keratinocytes) and immune cells (i.e., Langerhans cells, dermal DCs, Breg cells, and B1 cells) during skin inflammation. Furthermore, staphylococcal superantigens (SsAgs), such as staphylococcal enterotoxin B (SEB), bind to both MHC II expressed on APC and TCR Vβ8 chain of CD1d-restricted NKT cells, consequently bridging interaction between APC and NKT cells *via* antigen-independent manner. Thus, TCR Vβ8-expressing NKT cells might be involved in regulating *S. aureus* pathogenesis in the skin even without glycolipid antigens. Moreover, upon cross-talk with various cell types, CD1d-restricted NKT cells produce soluble factors (e.g., IFNγ, IL2, IL4, IL13, TNFα, perforin, and granzymes), which are either protective or pathogenic in inflammatory skin diseases. AD, atopic dermatitis; ACD, allergic contact dermatitis; APC, antigen-presenting cells; β-GlcCer, β-D-glucopyranosylceramide; Breg cells, regulatory B cells; DCs, dendritic cells; LCs, Langerhans cells; SsAgs, staphylococcal superantigens.

TABLE 1 Roles of CD1d-restricted NKT cells in various inflammatory skin diseases.

Diseases	NKT type	Subtype	Relative proportion	Species	Cellular source	Cytokines/Signaling molecules		Functions	References
						Increase	Decrease		
AD	I	$CD4^-$	↓	H	PBMC	-	-	-	(10)
	I	DN	↓	H	PBMC	-	-	-	(12)
	I	$CD161^+$	↓	H	PBMC	-	-	-	(13)
	I	$CD4^+$	↑	H	PBMC	-	-	-	(14)
	I	DN	↓	H	PBMC	IL4	IFNγ	-	(15)
	I	-	↑	H	PBMC, Skin	-	-	-	(23)
	I	$CXCR4^+$	↑	H	Skin	-	-	-	(17)
	I	$CXCR4^+$	↑	M	Skin	IFNγ, IL4, IL17	-	Pathogenic	(17)
	I	DN	↑	M	Skin	IFNγ, IL2	-	Protective	(19)
	I	DN	=	M	Spleen	IL4, IL10	IFNγ	Pathogenic	(22)
ACD	I	-	↑	H	Skin	IFNγ, IL4	-	-	(38)
	I	$CD4^+$, DN	↑	M	Spleen	IFNγ	-	-	(39)
	I	-	↑	H	Skin	Perforin, Granzyme B, K	-	-	(41)
	I	-	-	M	-	IL4, IL13	-	Protective	(45)
	I, II	-	-	M	-	-	-	Protective	(46)
	I	-	-	M	-	-	-	Pathogenic	(51)
Psoriasis	I	-	↓	H	-	-	-	-	(53)
	I	$CD69^+$	↓	H	PBMC	IL4, IL17, GATA3, RORγt	-	-	(55)
	I	$CD161^+$	↑	H	Skin	-	-	-	(59)
	I	-	↑	H	Skin	PKCζ	-	-	(61)
	I	$CD161^+$	↑	H	Skin	IFNγ	-	Pathogenic	(58)
UV-induced skin inflammation	I, II	-	-	M	-	-	-	Pathogenic	(66)
	I	-	-	M	Lymph nodes	IL4	-	Protective	(67)
	I	-	-	M	-	-	-	Protective	(68)
	I, II	-	-	M	-	-	-	Pathogenic	(68)
Scleroderma	I	-	↓	H	PBMC	IL17	-	-	(75)
Alopecia areata	I	-	↑	H	Skin	IL10	-	Protective	(80)
Vitiligo	I	$CD4^+$	↓	H	PBMC	-	-	-	(81)
Skin wound healing	I	-	-	M	-	-	-	Protective	(77–79)

I, type I; II, type II;-, not evaluated; DN, double negative; ↑, increase; ↓, decrease; =, no change; H, human; M, mouse; PBMC, peripheral blood mononuclear cells.

Author contributions

SL has done the literature search. SL and SH wrote the first draft. SL, SH, HP, and LVK edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

LVK is a member of the scientific advisory board of Isu Abxis Co., Ltd. (South Korea).

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

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Harnessing invariant natural killer T cells to control pathological inflammation

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Invariant natural killer T (iNKT) cells are innate T cells that are recognized for their potent immune modulatory functions. Over the last three decades, research in murine models and human observational studies have revealed that iNKT cells can act to limit inflammatory pathology in a variety of settings. Since iNKT cells are multi-functional and can promote inflammation in some contexts, understanding the mechanistic basis for their anti-inflammatory effects is critical for effectively harnessing them for clinical use. Two contrasting mechanisms have emerged to explain the anti-inflammatory activity of iNKT cells: that they drive suppressive pathways mediated by other regulatory cells, and that they may cytolytically eliminate antigen presenting cells that promote excessive inflammatory responses. How these activities are controlled and separated from their pro-inflammatory functions remains a central question. Murine iNKT cells can be divided into four functional lineages that have either pro-inflammatory (NKT1, NKT17) or anti-inflammatory (NKT2, NKT10) cytokine profiles. However, in humans these subsets are not clearly evident, and instead most iNKT cells that are $CD4^+$ appear oriented towards polyfunctional (T_{H0}) cytokine production, while $CD4^-$ iNKT cells appear more predisposed towards cytolytic activity. Additionally, structurally distinct antigens have been shown to induce T_{H1}- or T_{H2}-biased responses by iNKT cells in murine models, but human iNKT cells may respond to differing levels of TCR stimulation in a way that does not neatly separate T_{H1} and T_{H2} cytokine production. We discuss the implications of these differences for translational efforts focused on the anti-inflammatory activity of iNKT cells.

KEYWORDS

iNKT cell, CD1d, anti-inflammatory, immunotherapy, immuno-regulatory, immunomodulatory

Introduction

iNKT cells are innate T lymphocytes that are present in all individuals and use a unique "semi-invariant" TCR, comprised of a canonically rearranged TCRα chain (TRAV10-TRAJ18) paired with TCRβ chains utilizing TRBV25-1 in diverse rearrangements (1–3). The TCRs of iNKT cells are specific for CD1d, a non-classical antigen presenting molecule

that has minimal polymorphism at the amino acid level in human populations (4). CD1d molecules are constitutively expressed by professional APCs, including B cells, monocytes, macrophages, and DCs (5), and also by non-hematopoietic cells (particularly epithelial cells) in a variety of tissues (6). CD1d molecules are specialized for presenting lipidic antigens, which are structurally conserved molecules that are not highly mutable (7). Antigens recognized by iNKT cells derive from both self and microbial sources (8). Self-lipids recognized by iNKT cells are constitutively presented by $CD1d^+$ APCs, and may also be up-regulated during inflammation or cellular stress (9). Hence, because of their status as 'donor-unrestricted' T cells that recognize conserved antigens and do not mediate alloreactivity, iNKT cells are ideal candidates for allogeneic cellular immunotherapies. Due to their self-lipid recognition iNKT cells can be used for adoptive cellular immunotherapies without added antigens. Alternatively, they can be specifically activated by synthetic mimetics of their lipid antigens.

Extensive studies have demonstrated remarkable potency of iNKT cells in limiting T_{H1}-driven pathology in multiple settings, including autoimmune diseases, inflammation associated with obesity, and graft versus host disease (GVHD) [reviewed in (10–12)]. However, a central conundrum about iNKT cells is that they can also potently *promote* T_{H1} responses. Their T_{H1}-promoting functions have been associated with enhanced defense against infections and cancer (reviewed in (13, 14)), but also appear to play pathological roles in certain contexts, including atherosclerosis, sickle cell disease, and endotoxic shock (reviewed in (15–17)). Thus, in order to successfully exploit the potential of iNKT cells to treat inflammatory disease, it may be important to selectively engage their anti-inflammatory pathways.

How are the anti-inflammatory effects of iNKT cells mediated?

Two distinct mechanistic processes have been identified that may explain how iNKT cells limit T_{H1}-driven inflammation. The first is a regulatory axis characterized by iNKT cell production of T_{H2} (IL-4, IL-13) or regulatory (IL-10, TGFβ) cytokines, and by activation of anti-inflammatory cells including M2-polarized macrophages, myeloid-derived suppressor cells (MDSCs), and T_{regs} (Figure 1A). The second is a cytolytic pathway involving iNKT-mediated killing of inflammatory antigen presenting cells (APCs) that activate T_{H1} effectors (Figure 1B).

iNKT regulatory axis

Studies investigating insulitis in non-obese diabetic (NOD) mice were amongst the first to elucidate the regulatory activity of iNKT cells, with early work revealing a critical link to IL-4 and IL-10 production (18–21), and further analysis showing that they promote the differentiation of tolerogenic APCs that limit the activation of autoreactive T cells (22–25). A similar axis has been observed in murine models of diet-induced obesity, where adipose-resident iNKT cells play a powerful role in glucose tolerance by promoting macrophage polarization into a non-inflammatory M2 phenotype through secretion of IL-4 and IL-10 (26, 27), and by transactivating regulatory T cells *via* secretion of IL-2 (28). iNKT cells also contribute to the resolution phase of sterile inflammation in the liver by promoting monocyte transition into an anti-inflammatory phenotype through

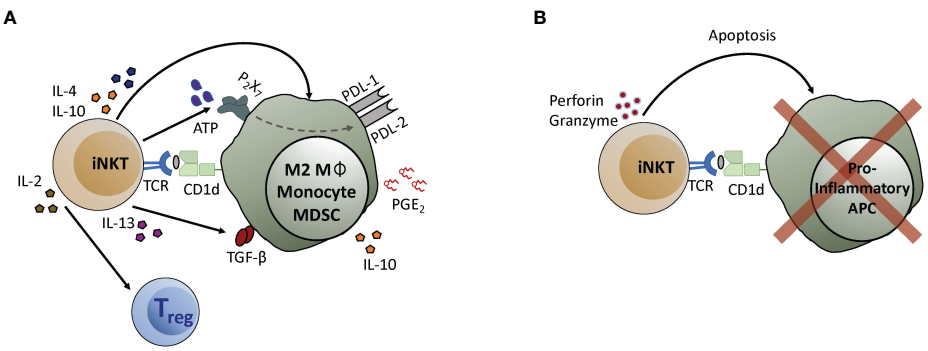

FIGURE 1
iNKT cell anti-inflammatory mechanisms. **(A)** iNKT cells interact with myeloid cell types to initiate the activation of regulatory pathways. Recognition of antigens presented by CD1d molecules expressed by myeloid cells induces iNKT cells to produce cytokines like IL-4, IL-10, or IL-13, that in turn act on the APCs. IL-4 and IL-10 promote macrophage differentiation into an M2 phenotype. IL-13 promotes monocyte differentiation into APCs that express suppressive cytokines such as IL-10 and TGF-β. Secretion of ATP by iNKT cells leads to upregulation of the checkpoint inhibitors PD-L1 and PD-L2, and iNKT interaction with monocytes induces secretion of PGE_2 by mechanisms that have not yet been determined. Additionally, IL-2 produced by iNKT cells helps to drive the expansion of T_{regs}. **(B)** iNKT cells can lyse pro-inflammatory APCs, leading to reduced T cell activation. In this case, recognition of antigens presented by CD1d molecules activates iNKT cells to release cytolytic granules that induce apoptosis of pro-inflammatory APCs.

secretion of IL-4 (29, 30). In murine models of allogeneic hematopoietic transplantation, iNKT cells protect against GVHD through IL-4 dependent mechanisms (31–33), and by promoting the regulatory functions of myeloid-derived suppressor cells (MDSCs) while driving T_{reg} expansion *via* secretion of IL-2 (34–36).

Analyses of human iNKT cells have suggested that they may participate in similar regulatory processes. IL-10 producing iNKT cells recently identified in the intestinal lamina propria of Crohn's Disease patients showed suppressive activity towards pathogenic $CD4^+$ T cells, and the frequency of IL-10 producing iNKT cells in colon tissue of these patients correlated inversely with T_{H1} and T_{H17} cell frequency, and was associated with reduced disease severity, higher *TGFB* gene expression, and lower levels of inflammatory proteins (37). Moreover, co-culture of human T_{regs} with iNKT cells led to increased T_{reg} FOXP3 expression, enhanced IL-10 secretion, and more profound inhibition of conventional T cell proliferation (38).

Human iNKT cells can also mediate potent suppression of T cell IFN-γ production by modulating the functions of monocytic cells. Our research group showed that GM-CSF and IL-13 secretion by human iNKT cells induced monocytes to differentiate into tolerogenic APCs that produced high levels of IL-10, expressed the checkpoint inhibitors PDL-1 and PDL-2, and potently suppressed T cell proliferation and IFN-γ secretion (39, 40). The regulatory phenotype of the APCs was due to iNKT cell release of extracellular ATP, which signaled through the P2X7 receptor on the monocytes to induce upregulation of PD-L1 and PD-L2 (41). This iNKT-monocyte interaction resembles a pathway observed in a murine model in which IL-13 secreted by CD1d-restricted T cells promoted monocyte expression of TGFβ, which led to suppression of T cell effector responses (42, 43), although the role of TGFβ in the human iNKT-monocyte pathway remains unclear.

We also used a xenotransplantation model of hematopoietic engraftment to investigate the impact of the human iNKT-monocyte pathway *in vivo*. The addition of allogeneic adult iNKT cells to human cord blood mononuclear cell grafts resulted in dramatically improved engraftment, which was due to iNKT cells inducing cord blood monocytes to secrete prostaglandin E_2, which potently suppressed T cell IFN-γ production (44). Since hematopoietic engraftment is suppressed by excessive IFN-γ (45), this analysis shows that human iNKT cells can engage powerful regulatory pathways that limit adverse effects of human T_{H1} activation *in vivo*.

iNKT cytolytic activity

A number of studies have suggested that iNKT cells may also control inflammation by eliminating pro-inflammatory APCs through a mechanism involving CD1d-dependent activation of the iNKT cells and lysis of APCs by cytotoxic granule deposition (46–50). Human iNKT cells were found to kill monocyte-derived DCs and blood DCs, but did not kill monocytes or plasmacytoid DCs, suggesting they specifically target certain types of APCs (46, 49). In another analysis, human iNKT cells preferentially eliminated monocyte-derived DCs that produced high levels of IL-12 while those that produced mainly IL-10 were spared, resulting in a DC population that limited T_{H1} activation (48). Together these studies suggest that this cytolytic pathway selectively targets pro-inflammatory APCs, and might thereby limit pathological inflammation. Consistent with this, in mice infected with a highly pathogenic strain of influenza A virus, iNKT cells were associated with reduced accumulation of inflammatory monocytes in the lungs (50). iNKT cell activity in this model was associated with reduced levels of MCP-1 (a chemokine that recruits monocytes and $CD4^+$ T cells), reduced damage to lung tissue, and improved survival even though viral loads were not affected (50). The effect of iNKT cells was thought to be due to their cytolytic activity against influenza-infected monocytes, suggesting that iNKT cells may limit pathological inflammation during viral infections by eliminating inflammatory APCs. However, an important note is that in all of these studies the iNKT cells were experimentally exposed to strong TCR stimulation prior to analysis of their cytolytic activity. Therefore, the physiological conditions that might lead to APC-targeted cytolytic activity by iNKT cells remain unclear.

How are iNKT cells activated, physiologically?

iNKT cells can be activated in two ways: either through TCR-mediated recognition of antigen presented by CD1d, or through TCR-independent pathways such as exposure to the cytokines IL-12 or IL-18, or LFA-1 ligation by high-density ICAM-1 (51–54). These TCR-independent pathways selectively induce iNKT cells to produce IFN-γ and not T_{H2} or regulatory cytokines (52, 54). Additionally, iNKT cells require a TCR signal for cytolysis of target cells (55–57). Thus, the anti-inflammatory activities of iNKT cells are probably highly dependent on TCR-recognition of antigens presented by CD1d molecules. Since it is clear that iNKT cells can mediate regulatory effects in the absence of infectious challenges, the antigens required for their anti-inflammatory pathways must be constitutively or chronically present. However, the sources and nature of the antigens that physiologically activate iNKT cells, and correspondingly the processes that contribute to their increased or decreased activation in different contexts, remain an ongoing area of inquiry.

Sources of antigen

Due to their shared use of a canonical TCRα chain, all iNKT cells recognize an unusual type of glycolipid in which the sugar head group is present in an α-anomeric configuration. Certain microbes

produce glycolipids of this type that are potent antigens for iNKT cells (reviewed in (8)). Recent studies indicate that bacterial species that can be found within the normal gut microbiota can produce similar antigenic lipids (58, 59), although these may be counter-regulated by related forms produced by other bacteria that are antagonists (60). These studies suggest that, particularly at mucosal sites, TCR-dependent activation of iNKT cells may fluctuate according to the composition of the microbial community.

iNKT cells can also recognize self-lipids as antigens. Mammalian cells do not directly synthesize the α-linked glycolipids recognized by iNKT cells, but the β-linked forms they produce may be converted at low frequencies to α-linked forms that are strongly antigenic (61, 62). Additionally, iNKT cells can recognize mammalian β-linked glycolipids as weak agonists (63). Some antigenic self-lipids, including lysophospholipids, glycosylated sphingolipids, and neutral lipids, are specifically upregulated during inflammation or cellular stress (64–70). Conversely, some non-antigenic self-lipids, such as sphingomyelin, can inhibit presentation of antigenic species (66). Together, the available data suggest that antigenic self-lipids are constitutively present, but are maintained in a manner that is only weakly agonistic for iNKT cells, and that during inflammation or cellular stress the abundance or nature of the antigenic self-lipids changes in a way that provides stronger TCR signals to iNKT cells. Additionally, as discussed below, activation by self antigens can be markedly enhanced by TCR-independent signals (71, 72).

What determines the nature of the functional response mediated by iNKT cells?

Exposure to inflammatory cytokines (IL-12, IL-18) or elevated levels of the adhesion ligand ICAM-1 selectively promotes iNKT cell IFN-γ secretion (51, 52, 54, 71, 72). Thus, when these signals are present, such as during inflammation, iNKT cells probably predominantly promote inflammatory responses. In contrast, the TCR-dependent activation pathway can promote either pro-inflammatory or anti-inflammatory outcomes (reviewed in (73)), and it has been of considerable interest to understand how TCR-mediated activation of iNKT cells leads to these contrasting effects. Two central factors have emerged: first, that the iNKT cell population contains multiple functionally distinct subsets; and second, that iNKT cell functional responses vary according to antigen characteristics.

Distinct subsets

In contrast to conventional T cells that become polarized into different effector phenotypes by priming in the periphery, iNKT cells are already cytokine competent as they exit the thymus (74). Murine iNKT cells are segregated into four functionally distinct subsets based on their expression of master-regulator transcription factors that govern cytokine production (Tbet, GATA3, RORγT, E4BP4) and on differences in expression levels of PLZF (promyelocytic leukemia zinc finger), a transcription factor that promotes cellular characteristics associated with innate lymphocytes (28, 75–78). NKT1 cells have a T_{H1} cytokine profile, often express a cytotoxic effector program, and are $PLZF^{lo}Tbet^{hi}$; NKT2 cells are characterized by high levels of IL-4 secretion and are $PLZF^{hi}GATA3^{+}$; NKT17 cells produce IL-17 and express RORγT with intermediate levels of PLZF; NKT10 cells produce IL-10, are preferentially found within adipose tissues, and are negative for PLZF but express E4BP4 (Figure 2A). NKT1, NKT2, and NKT17 lineages are generated during thymic selection, and are thought to home to distinct tissues (79). In contrast, NKT10 cells may originate from other subsets and differentiate into a regulatory phenotype as a result of exposure to factors in adipose tissues (27). The identification of these iNKT sub-lineages has led to the paradigm that the anti-inflammatory effects of iNKT cells are due to NKT2 or NKT10 cells, which become activated in different situations than NKT1 and NKT17 subsets as a result of differences in tissue localization.

In contrast, it has thus far not been straightforward to categorize human iNKT cells into NKT1, NKT2, and NKT17 lineages matching those in mice. Similar to their murine counterparts, most human iNKT cells express PLZF (80–82), and are characterized by an innate-like transcriptional profile that results in a "poised-effector" status allowing them to rapidly mediate functional responses (83). Multi-parameter flow cytometric analyses and gene expression studies have revealed human iNKT cells to express a diverse selection of cytokines and chemokines (84–88). Human iNKT cells can be segregated into two major subsets according to CD4 expression (84, 85). Those that express CD4 often appear to co-produce GM-CSF, IL-13, TNF-α, IFN-γ, IL-4, and IL-2, while those lacking CD4 appear more specialized for cytolysis (Figure 2C). These two major populations are sub-divided into further subsets characterized by additional markers (e.g. CD8α, CD161, CD62L) with distinctions in functional characteristics, but it is not clear that these subsets equate to the NKT1, NKT2, or NKT17 lineages observed in mice (89, 90). It is also not clear whether anti-inflammatory activity segregates according to $CD4^{+}$ or $CD4^{-}$ status of human iNKT cells, although $CD4^{+}$ iNKT cells are the ones that have been found to induce regulatory functions in monocytic cells, and the $CD4^{-}$ subset has appeared more likely to kill DCs.

Antigenic modulation

The prototypical iNKT antigen is called α-galactosylceramide (α-GalCer) (91), and synthetic forms of this lipid have proved

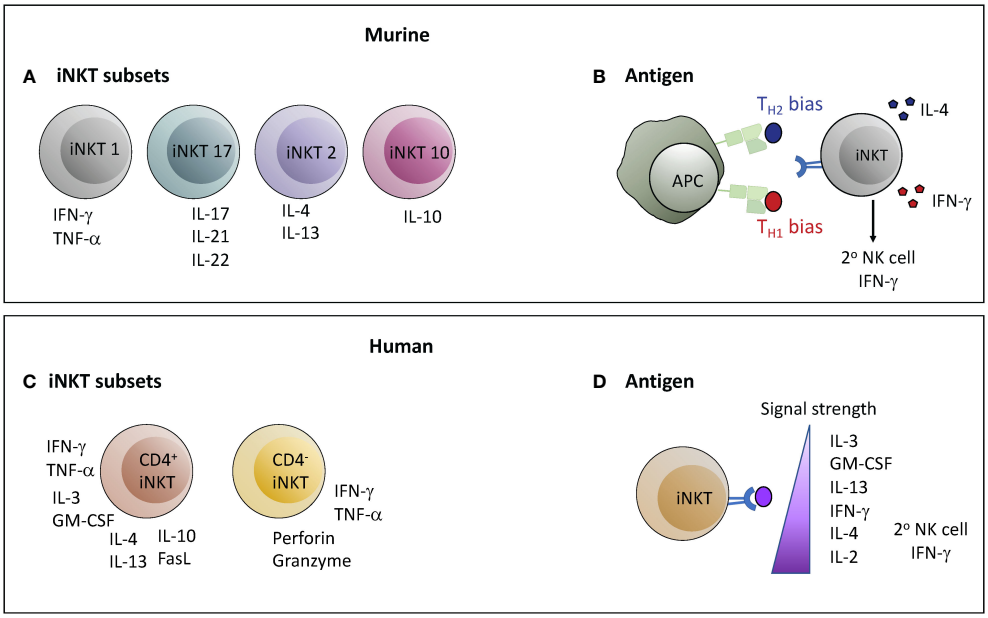

FIGURE 2
Determinants of the nature of the functional response mediated by iNKT cells. In both mice and humans the nature of the response mediated by iNKT cells may depend on the subset of iNKT cells activated or on the characteristics of the antigenic stimulation leading to activation. However, there are important differences between mice and humans in each of these parameters. **(A, C)** Murine iNKT cells can be classified into four lineages with functionally segregated cytokine profiles; whereas the two major subsets of human iNKT cells are characterized by comparatively polyfunctional cytokine production ($CD4^+$) or a more T_{H1}/cytotoxic profile ($CD4^-$). **(B, D)** Structural features of lipid antigens can bias murine iNKT cell responses towards either a T_{H1} or T_{H2} output, whereas human iNKT cell cytokine production proceeds in a hierarchical manner depending on the strength of the TCR signal. Antigens that stimulate a T_{H1}-biased response in mice typically also produce a strong secondary wave of IFN-γ production by NK cells, whereas strong agonists produce this effect from human iNKT cells.

extremely valuable as pharmacological agents that activate iNKT cells in a highly specific manner (92). Observations that structural variants of α-GalCer can produce substantially different immunological outcomes *in vivo* have led to interest in using these agents to selectively tune iNKT responses towards pro- or anti-inflammatory functions (92). Administration of α-GalCer to mice potently stimulates iNKT cells, and induces a mixed response where T_{H1}, T_{H2}, and regulatory cytokines are all produced, although with different kinetics (93). In contrast, certain analogues of α-GalCer have been shown to produce a T_{H2}-biased cytokine response (94, 95), while other variants produce a highly T_{H1}-biased response (96) (Figure 2B). The mechanisms underlying these differential responses appear complex. One component may be that certain variants induce biased cytokine production from iNKT cells themselves (97), while another important element likely relates to whether or not antigen-driven interactions between iNKT cells and APCs result in release of cytokines (e.g. IL-12) that activate a secondary IFN-γ response by NK cells (96, 98). A key factor may be the relative duration of antigen presentation by CD1d molecules, with more durable antigens being associated with T_{H1}-biased responses (99). Additionally, T_{H1}-biasing forms of α-GalCer may be selectively presented by APCs that produce IL-12, whereas T_{H2}-biasing forms may be more promiscuously presented and thus less likely to produce a secondary wave of IFN-γ production by NK cells (100). It is not clear whether antigen variants selectively activate different iNKT cell subsets, or bias the cytokine profile produced within a given subset (for example, by inducing higher IL-4 production by NKT1 cells, or increased IFN-γ by NKT2 or NKT17 cells), or whether any structural variants selectively promote IL-10 production. Interestingly, repeated administration of α-GalCer results in selective loss of its T_{H1}-promoting features, but under such "anergizing" conditions α-GalCer retains the ability to induce IL-4 secretion and to promote control of EAE pathology (101).

Whether human iNKT cell responses can be modulated similarly using α-GalCer structural variants remains an open question. It has become clear that TCR differences between murine and human iNKT cells result in significant discrepancies in TCR-signaling strength induced by lipid variants (102). Perhaps more importantly, polyfunctional human iNKT cells show a hierarchy of cytokine production in response to TCR stimulation that does not neatly segregate into clear T_{H1} or T_{H2} patterns. Weak TCR stimulation of human iNKT cells preferentially induces production of IL-3, GM-CSF, and IL-13, with increasing stimulation leading first to IFN-γ, then IL-4, then IL-2 (44, 103, 104) (Figure 2D). Secondary induction of NK cell IFN-γ secretion was associated with activation of human iNKT

cells by strong TCR agonists (104). It is therefore not clear that it will be feasible to selectively polarize human immune responses towards IL-4 production through the use of specific lipid antigen variants, although it may be possible to drive IL-13 production through administration of weak agonists.

Discussion

The potential of engaging iNKT cells therapeutically to treat T_{H1}-inflammatory pathology is well supported by pre-clinical studies in murine models, *in vitro* experiments using human cells, and *ex vivo* analyses of human subjects, but clinical data have been limited. Recently, however, a pilot clinical trial using allogeneic iNKT cells as a cellular immunotherapy to treat patients who were intubated with acute respiratory distress syndrome (ARDS) secondary to SARS-CoV-2 infection has shown highly promising results, with 77% survival of treated patients compared to a national average of 40% survival for other intubated SARS-CoV-2 patients during the same period of enrollment (105). Understanding whether such iNKT cell therapies work through one of the regulatory pathways shown in Figure 1A, or through elimination of inflammatory cells *via* cytolysis as depicted in Figure 1B, has important implications. For example, if APC killing is a key component it may be necessary to deliver a strong TCR signal to the iNKT cells to prime their cytolytic activity. Alternatively, if a regulatory pathway is involved it may be beneficial to generate iNKT cells that are biased towards production of T_{H2} cytokines or IL-10, depending on the pathway.

Also critical to developing effective iNKT cell therapies is to determine whether human iNKT cells include stable regulatory subsets, or whether polyfunctional iNKT cells are converted into a regulatory phenotype through particular signals. If a stable NKT10 lineage exists in humans, an attractive option might be to specifically engage these cells for immunotherapy. Alternatively, if human iNKT cells generally retain functional plasticity, it may be important to identify methods to specifically promote their regulatory functions. To this end, a recent study found that the presence of IL-7 during *in vitro* expansion of human iNKT cells resulted in a $CD4^+$ population with enhanced T_{H2} cytokine production (106), while exposure to short chain fatty acids, palmitate, or the mTOR inhibitor rapamycin may induce a regulatory phenotype (27, 37, 107). Another important consideration is that iNKT immunotherapy that engages T_{H2} pathways would likely be contraindicated in certain inflammatory diseases, including asthma, chronic obstructive pulmonary disease, and ulcerative colitis, where T_{H2} cytokine production by iNKT cells has been associated with disease-exacerbating effects (reviewed in (108–110)).

Overall, studies of human and murine iNKT cells over the last three decades clearly support the potential of this unique population to be utilized clinically to control inflammatory pathology. Key areas of further investigation will be to better understand the antigens that physiologically or pharmacologically activate human iNKT cells, and to determine the impact of iNKT cell antigenic activation in different tissues or by distinct APCs. For example, since lipid antigens can be retained locally at the site of administration (111), or distributed to distal sites through binding to lipid transport proteins (112, 113), it may be possible to control the location of iNKT cell activation. Additionally, since iNKT cells promote anti-inflammatory outcomes through interactions with multiple distinct APC populations, it may be possible to direct specific effects through engaging particular APC types, such as the regulatory B cells that ameliorate arthritic pathology (114). It will also be of importance to understand roles of non-invariant populations of CD1d-restricted T cells (often called type II NKT cells, reviewed in reference (115)), and to determine whether these other T cell populations promote or counter-regulate anti-inflammatory outcomes mediated by the "type I" iNKT cells discussed here. Finally, given the likely importance of TCR and CD1d structural differences, the difference in abundance between murine and human iNKT cells (common experimental mouse strains have ~100-fold higher frequencies of iNKT cells than humans), and of additional CD1 molecules (CD1a,b, c, and e) expressed in humans that may impact antigen availability or T cell responses (7, 116, 117), an important step for translating iNKT-based immunotherapies to the clinic may be the development of new animal models that better capture determinants that affect human iNKT cell functions.

Author contributions

NSB and JEG wrote the manuscript and generated the figures. 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

Author JG is a member of the Scientific Advisory Board of MiNK Therapeutics.

The remaining author declares 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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Development and function of regulatory innate lymphoid cells

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Innate lymphoid cells (ILCs) are a critical element of the innate immune system and are potent producers of pro-inflammatory cytokines. Recently, however, the production of the anti-inflammatory cytokine IL-10 has been observed in all ILC subtypes (ILC1s, ILC2s, and ILC3s) suggesting their ability to adopt a regulatory phenotype that serves to maintain lung and gut homeostasis. Other studies advocate a potential therapeutic role of these IL-10-expressing ILCs in allergic diseases such as asthma, colitis, and pancreatic islet allograft rejection. Herein, we review IL-10 producing ILCs, discussing their development, function, regulation, and immunotherapeutic potential through suppressing harmful inflammatory responses. Furthermore, we address inconsistencies in the literature regarding these regulatory IL-10 producing ILCs, as well as directions for future research.

KEYWORDS

regulatory, innate, lymphoid, IL-10, cell

Introduction

Innate lymphoid cells (ILCs) are an immune cell type that have cytokine production features of T lymphocytes but lack rearranged antigen receptors. As a result, ILCs lack antigen specificity and instead respond to alarmins released predominantly, but not exclusively, by epithelial and endothelial cells in response to damage caused by infection, injury, or disease. Currently, three groups of ILCs have been discovered and defined. Group 1 innate lymphoid cells (ILC1s), the counterpart to $CD4^+$ T helper (Th) type 1 cells, produce interferon gamma (IFN-γ) and express the transcription factor T-bet (1, 2). Group 2 Innate Lymphoid cells (ILC2s), analogous to $CD4^+$ Th2 cells, produce interleukin (IL)-5, IL-9, and IL-13, and express the transcription factor GATA binding protein 3 (GATA-3) (3–5). Group 3 innate lymphoid cells (ILC3s), that parallel $CD4^+$ Th17 cells, produce IL-17 and IL-22, and express the transcription factor retinoid-related orphan receptor gamma t (RORγt) (6–9).

In the field of allergy, ILC2s are a primary focal point due to their double-edged sword nature in both the pathogenesis, and possibly prevention, of allergic disease. In the respiratory and gastrointestinal tracts, epithelial cells can be challenged by infectious

agents or allergens that contain pathogen- or damage- associated molecular patterns, resulting in epithelial cell release of alarmins: IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which activate ILC2s (10–12). ILC2s respond by migrating to the challenged site where they proliferate and release the pro-inflammatory cytokines mentioned earlier at an amount that is 10-fold greater, on a per cell basis, than that released by their $CD4^+$ Th2 counterpart (13). As a result, ILC2s can participate in host protective roles, such as the eradication of helminthic parasites through IL-5-induced eosinophil recruitment and IL-13-induced goblet hyperplasia and peristalsis (14–16). However, when ILC2s are activated by alarmins in the setting of asthma, the IL-5 they produce can lead to eosinophil activation whose products damage the airway and exacerbate bronchoconstriction. IL-13 is a central mediator of asthma by promoting bronchial hyperresponsiveness and airway remodeling, as shown in Figure 1 (17). Additionally, IL-13 disrupts the integrity of the epithelial barrier by breaking down tight junctions (18) and promoting TSLP release, leading to corticosteroid resistance in ILC2s (19).

To promote immunologic tolerance, T regulatory cells (Tregs) derived in the thymus or extrathymically from $CD4^+$ naïve T cells release the anti-inflammatory cytokines transforming growth factor beta (TGF-β) and IL-10 (20, 21). Interestingly, recent studies reveal a unique ability for ILCs to adopt a regulatory phenotype, similar to Tregs, through production of IL-10. Herein we review the development, function, regulation, pathogenic and potential immunotherapeutic roles of IL-10-producing ILCs, as well as address controversies and directions for future research.

Regulatory innate lymphoid cells (ILCregs)

Using IL-10-green fluorescent protein (GFP) reporter mice, a small subset of Lin^- $CD45^+$ $CD127^+$ $IL\text{-}10^+$ ILCs were identified in the small intestinal lamina propria (sLP) at baseline. Sample analysis of human intestinal biopsies using flow cytometry also confirmed the presence of these $IL\text{-}10^+$ ILCs in the sLP of humans at baseline (22). These cells were named regulatory innate

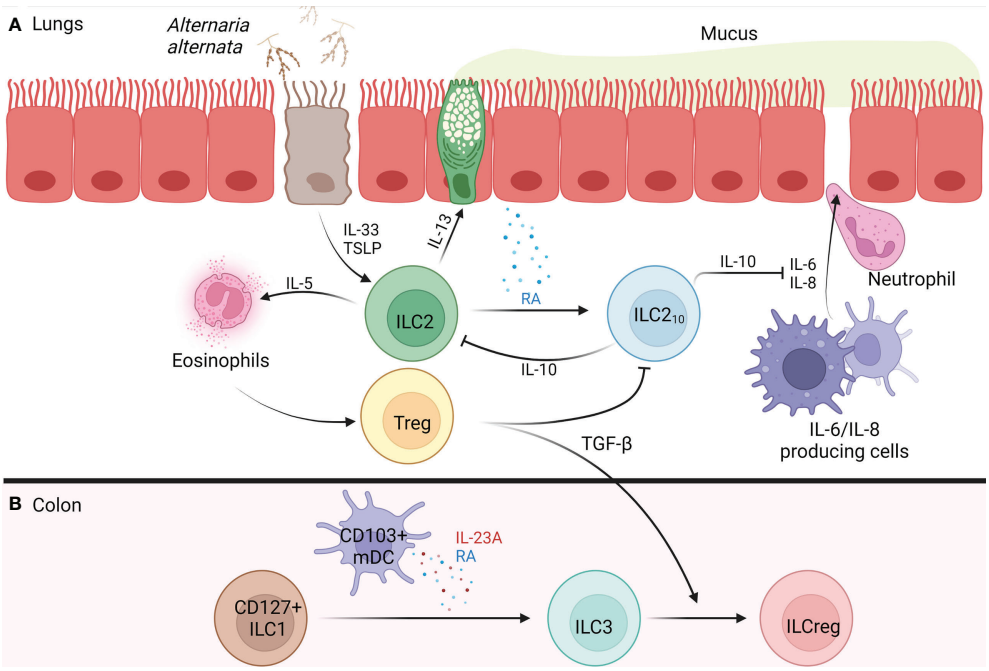

FIGURE 1
Development of $IL\text{-}10^+$ ILCs in the Lung and Colon of Humans. **(A)** *Alternaria alternata* activates airway epithelium 2a) activated airway epithelium releases TSLP and IL-33 2a) IL-33 activates ILC2s, causing release of IL-5 and IL-13, while TSLP confers corticosteroid resistance 3a) IL-5 recruits and activates eosinophils 4a) IL-13 causes goblet cell hyperplasia, AHR, and release of RA from airway epithelium 5a) RA promotes the trans differentiation of ILC2s into $ILC2_{10}$s 6a) $ILC2_{10}$s release IL-10 which inhibits ILC2-mediated type 2 inflammation and maintains barrier integrity through the inhibition of IL-6 and IL-8 which function to increase barrier permeability, resulting in neutrophil transmigration 7a) Tregs form and regulate type 2 inflammation through release of TGF-β which blocks $ILC2_{10}$s. **(B)** In the colon, $CD103^+$ mDCs release RA and IL-23A, promoting $CD127^+$ ILC1s trans differentiation into ILC3s 2b) Tregs release TGF-β promoting the trans differentiation of ILC3s into ILCregs. AHR, airway hyperreactivity; ILC, innate lymphoid cell; ILC1, type 1 innate lymphoid cell; ILC2, type 2 innate lymphoid cell; ILC3, type 3 innate lymphoid cell; $ILC2_{10}$s, $IL\text{-}10^+$ type 2 innate lymphoid cell; ILCreg, regulatory innate lymphoid cell; IL, interleukin; mDC, monocyte-derived dendritic cell; RA, retinoic acid; TGF-β, transforming growth factor beta; Treg, regulatory T cell; TSLP, thymic stromal lymphopoietin. Figure 1 was created using BioRender.com.

lymphoid cells (ILCregs) due to their absence of ILC1 markers NK1.1, NKp46, and *Tbx21* (encodes T-bet); ILC2 markers ST2, killer cell lectin-like receptor subfamily G member 1 (KLRG1), and GATA-3; and ILC3 markers NKp46, CD4, and RORγt. Thus, these IL-10$^+$ ILCs were deemed to be a new kind of ILC subset (22).

Interestingly, while ILCregs exhibited similarities to Tregs, such as their ability to produce IL-10 and TGF-β, they lacked expression of the Treg transcription factor Foxp3 (23). Unlike ILC1s, ILC2s, and ILC3s, ILCregs originate from the common helper-like innate lymphoid precursor (CHILP)-α4β7$^+$Id2^{high} and express *Id3* which is required for their development/maintenance (1, 22). Due to the constitutive presence of ILCregs in the intestines and their expansion seen during dextran sodium sulfate (DSS)-induced colitis in Rag$^{-/-}$ mice (22), ILCregs have been conjectured to maintain gut tolerance through production of IL-10. When activated ILC1s and ILC3s were adoptively transferred into DSS-induced colitis Rag1$^{-/-}$ *Il2rg*$^{-/-}$ (ILCreg deficient) mice, severe colitis resulted, an effect that was attenuated upon ILCreg reconstitution (22). However, severe colitis resulted upon transferring IL-10Rα$^{-/-}$ ILC1s and ILC3s into Rag1$^{-/-}$ *Il2rg*$^{-/-}$ mice reconstituted with WT ILCregs, revealing that ILCregs protect against colitis through IL-10 (22). Notably, Tregs isolated from *Foxp3*-DTR (human diphtheria toxin receptor)-GFP mice adoptively transferred into ILC1/ILC3 reconstituted Rag1$^{-/-}$ *Il2rg*$^{-/-}$ mice had no effect on intestinal inflammation even after the depletion of Foxp3$^+$ Tregs using diphtheria toxin (DT) treatment (22). However, when ILCreg^{DTR} cells were depleted in the intestines of mice following DT treatment, severe inflammation ensued (22). Importantly, these studies distinguish ILCregs as having a unique protective function in the intestines of mice.

In addition to the sLP, ILCregs have been discovered residing in the kidney's interstitium of both humans and mice at baseline. These ILCregs produce large amounts of IL-10 and TGF-β that protect against renal ischemia/reperfusion injury (IRI), an effect that was abolished by neutralizing IL-10 and TGF-β antibodies (24). Interestingly, administration of an IL-2/anti-IL-2 monoclonal antibody complex (IL-2c) expanded ILCregs in the kidney of IRI Rag$^{-/-}$ mice, reducing tubular epithelial cell apoptosis and improving renal function (24). Importantly, depletion of these renal ILCregs using PC61 (an anti-CD25 antibody) showed greater kidney injury in IRI Rag$^{-/-}$ mice, revealing their critical role in renal protection (24). Adoptive transfer of ILCregs expanded *ex vivo* with IL-2c into IRI C57BL/6 mice further confirmed their protective role by restoring kidney function through the suppression of ILC1 and neutrophil infiltration and enhancing M2 macrophage generation (24). Notably, ILCregs in the kidneys reduced the frequency of ILC1s but not ILC2s or ILC3s, suggesting a pathogenic role of ILC1s in renal IRI (24) as well as differences in ILCreg function dependent on anatomical location (see Table 1).

Regulatory phenotype of ILC2s

There is also evidence that ILC2s have the capacity to produce IL-10 and may have immunoinhibitory potential. For instance, the hypoxic microenvironment of pancreatic ductal adenocarcinoma tumors (PDAC) can promote ILC2s to become regulatory IL-10$^+$ ILC2s through the upregulation of hypoxia-inducible factor 1-alpha (HIF-1α) which binds to the *Il10* promoter (27). Importantly, reoxygenation or neoadjuvant chemotherapy caused IL-10$^+$ ILC2s to convert back into ILC2s, suggesting a regulatory plasticity. Unlike the previously described ILCregs, IL-10$^+$ ILC2s maintained their ILC2 phenotype through the expression of Il1rl1 (ST2), KLRG1 (26) and *Gata3* (28); thus, they have been termed ILC2$_{10}$s (see Table 1).

TABLE 1 Differences between mouse and human ILCregs and ILC2$_{10}$s.

Cell	Location (Ref)	Species	Phenotype	% Of Total IL-10$^+$ ILCs at Baseline	Express *Id3*	Express GATA-3/ KLRG1/ST2?	TGF-β	Function
ILCregs	Kidneys (24)	Human	Lin$^-$ CD127$^+$ CD161$^+$ IL-10$^+$	~4.4%	Yes	No	Stimulatory	Suppresses ILC1s
		Mouse	Lin$^-$ CD127$^+$ IL-10$^+$	~2.7%				
	Intestines (22)	Human	Lin$^-$ CD45$^+$ CD127$^+$ IL-10$^+$	~15%				Suppresses ILC1s and ILC3s
		Mouse		~13%				
ILC2$_{10}$s	Lungs (25, 26)	Human	Lin$^-$ CD45$^+$ CD127$^+$ CD161$^+$ IL-10$^+$	0%	No	Yes	Inhibitory	Suppresses ILC2s
		Mouse	Lin$^-$ CD45$^+$ Thy-1.2$^+$ IL-10$^+$	~0.4%				

GATA-3, GATA binding protein 3; Id3, inhibitor of DNA binding 3; ILCs, innate lymphoid cells; ILC1s, type 1 innate lymphoid cells; ILC2s, type 2 innate lymphoid cells; ILC3s, type 3 innate lymphoid cells; ILC210s, IL-10+ type 2 innate lymphoid cells; ILCregs, regulatory innate lymphoid cells; KLRG1, killer cell lectin-like receptor G1; Ref, reference; sLP, small intestine lamina propria; ST2, soluble interleukin 1 receptor-like 1; TGF-b, transforming growth factor beta.

ILC2s treated with the common Treg polarization factors TGF-β, vitamin D, or retinoic acid (RA), became $ILC2_{10}$s only in the presence of RA (25). Notably, administration of a pan-retinoic acid receptor (RAR) inhibitor blocked $ILC2_{10}$s generation in a dose-dependent manner, revealing that RA acts through RAR to induce the $ILC2_{10}$ phenotype (25). In an *in vitro* study using air liquid interface (ALI) cultures of primary bronchial epithelial cells treated with IL-5, IL-13, and IL-33, from patients with chronic rhinosinusitis with nasal polyps (CRSwNP), only IL-13 promoted RA generation (25). This result suggests that IL-13 derived from ILC2s upregulates $ILC2_{10}$s by promoting RA generation from epithelial cells, which in turn downregulates the ILC2-induced type 2 inflammatory response through IL-10 release (see Figure 1). This implies that ILC2s have a mechanism to autoregulate the inflammation that they induce.

In a model of allergic lung inflammation in mice induced by either four daily intranasal administrations of IL-33 or chronic papain exposure, a population of IL-10 producing Lin^- $Thy1.1^+$ ILC2s emerged (26). Interestingly, the same population of $IL\text{-}10^+$ ILC2s was induced by treating ILC2s *in vivo* with IL-2c (26). However, ILC2 production of IL-10 is not restricted to the lungs. When treating small intestinal ILC2s from naïve mice with IL-2, IL-4, IL-10, IL-27, and neuromedin U (NMU) together, these ILC2s began producing IL-10 (29). Interestingly, IL-2 and IL-4 enhanced IL-10 production by ILC2 when these cytokines were administered individually in culture (29). As a result, these experiments collectively suggest that ILC2 trans-differentiation into $ILC2_{10}$s is a self-amplifying process instructed by their cytokine milieu and environment.

Regulation of IL-10 producing ILCregs and ILC2s

Immune suppression is not always beneficial, as in the case of PDAC tumors where $IL\text{-}10^+$ ILCs promote tumor growth (27). As a result, regulation of IL-10 by ILCs is crucial. A study conducted on ILC2 from WT and C3a receptor knockout ($C3ar^{-/-}$) mice reported that genetic deletion of the C3a receptor resulted in significantly less IL-13, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) production, while C3a signaling inhibited IL-33-induced IL-10 production from $ILC2_{10}$s (30). Thus, the anaphylatoxin C3a combined with IL-33 stimulation enhanced the pro-inflammatory ILC2 phenotype through inhibiting *Il10* transcription and promoted ILC2 antigen-presentation to $CD4^+$ T cells, resulting in Th2 differentiation (30). Additionally, tumor necrosis factor-like cytokine 1A (TL1A) strongly abrogated IL-10 production in $ILC2_{10}$s while increasing IL-5 and IL-13 production (29). Collectively, these results reveal that the regulatory phenotype adopted by ILC2s is reversible and influenced by environmental conditions.

Cytokines can also downregulate IL-10-expressing ILCs. In human $ILC2_{10}$s from patients with systemic sclerosis (SSc), treatment with TGF-β dramatically decreased the production of IL-10 and reduced KLRG1 expression, an ILC2 surface marker found to be required for IL-10 production (29, 31, 32). However, unlike $ILC2_{10}$s, ILCregs rely on TGF-β signaling for their survival and expansion (see Table 1), as seen through the effects of deleting TGF-β receptors on ILCs using $Tgfbr2^{flox/flox}$; CreERT2 mice (22, 33). This finding reveals differences between ILCregs and $ILC2_{10}$s, potentially revealing the presence of two regulatory ILC subtypes.

Regulatory phenotype of ILC3s and ex-ILC1s

Several pieces of evidence suggest that ILC3s are plastic and can become ILCregs. A study investigating colorectal cancer (CRC) tumor infiltrating ILCs from azoxymethane/dextran sodium sulfate (AOM/DSS)-induced colitis models revealed that ILC3 numbers decreased, while ILCreg numbers increased, during CRC tumor progression (34). At the late-stage of CRC tumors, fate mapping using Rosa26-STOP-tdTomato;Rorc-Cre;IL-10-GFP lineage tracing mice followed by AOM/DSS treatment revealed former ILC3s (exILC3s) producing IL-10 and expressing *Id3* (34). Using TGF-β receptor knockout mice treated with AOM/DSS, ILCreg numbers decreased while ILC3 numbers increased, causing tumor growth suppression (34). Furthermore, ILC3 treatment with a TGF-β inhibitor prevented the conversion of ILC3s to ILCregs, a result that was consistent in both the AOM/DSS-induced CRC mice and patient derived xenograft (PDX) tumors (34). Collectively, TGF-β drives the trans-differentiation of ILC3s towards ILCregs in both humans and mice. This important finding reveals that IL-10 production from ILCs is not limited to $KLRG1^+$ ILC2s, as previously thought (29, 32), and brings to question whether ILC1s can adopt a regulatory phenotype.

$CD127^+$ ILC1s that lost their ability to proliferate contained the capacity to reversibly differentiate into ILC3s (exILC1s) in the presence of IL-2, IL-23, and IL-1β when administered together (35). Further analysis revealed that exILC1s lost their T-bet expression and IFN-γ production, but began expressing RORγt and producing IL-22, committing to an ILC3 phenotype (35). Notably, in the presence of IL-2 and IL-12, ILC3s and exILC1s lost their RORγt and IL-22 expression while upregulating T-bet expression and IFN-γ production, committing to an ILC1 phenotype (35). In addition to the mentioned cytokines, RA signals through its receptors (RARA, RARG, and RXRG) present on $CD127^+$ ILC1s to accelerate the

differentiation of ILC1s into ILC3s (35, 36). Human monocyte derived dendritic cells (mDCs) treated with RA upregulated CD103 expression and began producing RA and IL-23A under basal conditions and lipopolysaccharide (LPS) stimulation, revealing a role $CD103^+$ mDCs play in $CD127^+$ ILC1s differentiation toward ILC3s (35). As a result, it is possible ILC1s can become ILCregs through their commitment to an ILC3 phenotype in the presence of $CD103^+$ mDCs (Figure 1). However, *ex vivo* stimulation with IL-12/IL-15 markedly increased IL-10 production in human ILC1s revealing their direct ability to adopt a regulatory phenotype (33). These findings reveal a regulatory plasticity within all ILC subtypes, and potential crosstalk between DCs and ILCs which should be further investigated in future research.

Immunotherapeutic potential of IL-10 producing ILCs through stimulation or inhibition

Through *in vivo* generation and stimulation in the lungs, $ILC2_{10}$s show promise as potential therapeutics for allergic airway inflammation. Using CRSwNP patient nasal epithelial cells, ALI cultures co-cultured with $ILC2_{10}$s and challenged with grass-pollen allergen revealed that the addition of the $ILC2_{10}$s prevented allergen-induced epithelial barrier disintegration, an effect that was diminished upon the addition of anti-IL-10 neutralizing antibodies (32). Elevation of IL-10R surface expression on epithelial cells occurred upon allergen exposure, enhancing the $ILC2_{10}$-induced epithelial barrier restoration (32). As a result of this restoration, grass-pollen sublingual allergen immunotherapy (GP-SLIT) was investigated in allergic individuals. In groups treated with GP-SLIT, frequencies of $ILC2_{10}$s increased compared to the placebo-treated group, negatively correlating with clinical symptoms (32). This result shows promise in using GP-SLIT to induce $ILC2_{10}$s in atopic individuals, which function to restore barrier integrity and attenuate type 2 inflammation through IL-10 production. Furthermore, an *in vitro* study on nasal epithelium from allergic individuals co-cultured with $ILC2_{10}$s revealed that IL-10 served to maintain epithelial and endothelial barrier integrity by blocking IL-6 and IL-8, both of which promote neutrophil translocation by increasing barrier permeability as shown in Figure 1 (37, 38). In mice, IL-10 attenuated Th2-mediated allergic airway inflammation by downregulating Th2 survival through restoring granzyme B expression in $CD4^+$ $IL\text{-}10^{-/-}$ cells (39).

To further investigate the immunosuppressive role of $ILC2_{10}$s in allergic diseases such as asthma, $ILC2_{10}$s and ILC2s in a 1:1 mix were adoptively transferred into $Rag^{-/-}$ $\gamma c^{-/-}$ (T-cell, B-cell, and NK cell deficient) mice intranasally challenged with IL-33. In doing so, ILC2-dependent allergic airway hyperreactivity (AHR) was downregulated, a result that was abrogated upon the intraperitoneal administration of anti-IL-10R (40). $ILC2_{10}$s-induced AHR attenuation was further confirmed in mice challenged with *Alternaria alternata* that were adoptively transferred the same 1:1 $ILC2_{10}$s/ILC2s mix. The role of IL-10 was confirmed when administration of anti-IL-10R antibodies abrogated this effect (40). Collectively, *in vivo* generation of $ILC2_{10}$s in the lungs attenuates type 2 allergic responses through IL-10 production.

Another potential therapeutic role of $ILC2_{10}$s is the promotion of islet allograft survival in mice as measured through improved glucose tolerance (41). $ILC2_{10}$s were delivered to recipient mice either intravenously or through co-transplantation with the graft. Interestingly, allograft survival was increased in only the co-transplantation group, revealing a need for $ILC2_{10}$s to be within the graft to achieve maximal graft protection (41). Further investigation is needed to determine how these findings translate into clinical practice.

ILCregs and $ILC2_{10}$s – The same cell or are they different?

In this review we discussed ILCregs as those cells that express *Id3*, are stimulated by TGF-β, and arise from the $\alpha4\beta7^+Id2^{high}$ CHILPs or from ILC3s in the presence of TGF-β. Separately, we defined $IL\text{-}10^+$ ILC2s as $ILC2_{10}$s as a consequence of their sustained expression of GATA-3 and suppression by TGF-β (see Table 1). However, whether these cells are the same or different remains to be fully defined. Notably, ILCregs arose in the gut and kidneys at steady state and during inflammation (22), while $ILC2_{10}$s arose in both the gut and lungs in the presence of inflammation only (25, 27). As such, further studies should be directed towards the molecular comparison of ILCregs and $ILC2_{10}$s to determine if their GATA-3 expression and response to TGF-ß is cell type specific or influenced by their environment/location.

As previously discussed, ILCregs devoid of all ILC markers were expressed in the sLP of mice (22). However, upon repeat of this experiment by a different group, no such cell population was found (29). Interestingly, this group discovered that only Lin^- $CD127^+$ $Thy1^-$ ILC2s expressed IL-10 in the small intestine (29). This finding revealed inconsistencies surrounding the presence and identification of ILCregs in the sLP. One reason for the inconsistent result was suggested to be caused by genetics and/or environmental factors. However, even controlling for these factors by purchasing C57BL/6 mice from three different vendors, no ILCregs were identified (29). As a result, the existence of ILCregs in mice are non-generalizable. Further studies need to investigate the contributions of other environmental influences such as inflammation or autoimmunity on the presence of ILCregs, in both the intestines of mice and humans.

Through studying the suppressive function of ILCregs in a mouse model of colitis, IL-10 inhibited the activation of both

ILC1s and ILC3s, as previously discussed. However, in an *in vitro* study investigating the suppressive role of TGF-β and IL-10 in human ILC subsets, IL-10 inhibited cytokine production from pre-stimulated ILC2s while having no effect on pre-stimulated ILC1s (33). As a result, further studies are needed to determine the differential role of ILCregs in repressing the function of ILC1s, ILC2s, and ILC3s between mice and humans, and to determine whether this difference is influenced by the inflammatory environment.

Other roles of $ILC2_{10}$s remains to be investigated, such as its ability to suppress lung eosinophilia. Through treating $Rag^{-/-}$ mice with IL-33 and IL-2c, a significant reduction in IL-33-induced lung eosinophilia occurred with extensive generation of $ILC2_{10}$s (26). However, no inhibitory studies using anti-IL-10 antibodies or IL-$10^{-/-}$ ILC2s were performed to prove the role of $ILC2_{10}$s in attenuating eosinophil migration to the lungs. As a result, *in vivo* delivery of IL-2c should be further investigated in its efficacy as an immune-targeted therapy that could reduce eosinophilia in atopic patients as well as protect against renal IRI, colitis, allergic airway inflammation, and allograft rejection due to its ability to generate $ILC2_{10}$s both *in vivo* and *in vitro*.

Interestingly, a cross-sectional study comparing grass-pollen allergic (GPA) and house dust mite-allergic (HDMA) individuals to a non-atopic healthy control (NAC) revealed that ILC2s from atopic individuals fail to adopt an IL-10-producing regulatory phenotype (32). This finding reveals a possible limitation in treating allergic disease through $ILC2_{10}$ generation. As a result, the regulation of the IL-10 promoter in ILC2s from GPA and HDMA patients should be investigated as it could further explain the lack of immune regulation seen in atopic patients.

Conclusion

There is increasing evidence that the IL-10 produced by ILCs suppresses immune responses and could be helpful, such as in allergic disease, or harmful, such as in the setting of cancer, to patients. However, due to the limitations regarding the specific deletion of IL-10^{+} ILCs *in vivo*, these cells remain an enigma as their exact role in human or mouse disease remains unknown. For instance, there are no specific surface markers for ILCregs for which antibody depletion could target to determine their role in regulating inflammatory processes. This is an emerging field that is certainly ripe for further investigation to understand the full nature and importance of these suppressive ILCs in human health.

Author contributions

CT wrote all drafts of the manuscript and RP edited the manuscript drafts. 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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Alzheimer's disease alters the transcriptomic profile of natural killer cells at single-cell resolution

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Alzheimer's disease (AD) is the most common dementia without an effective cure at least partially due to incomplete understanding of the disease. Inflammation has emerged as a central player in the onset and progression of AD. As innate lymphoid cells, natural killer (NK) cells orchestrate the initiation and evolution of inflammatory responses. Yet, the transcriptomic features of NK cells in AD remain poorly understood. We assessed the diversity of NK cells using web-based single-cell RNA sequencing data of blood NK cells from patients with AD and control subjects and flow cytometry. We identified a contraction of NK cell compartment in AD, accompanied by a reduction of cytotoxicity. Unbiased clustering revealed four subsets of NK cells in AD, i.e., $CD56^{bright}$ NK cells, $CD56^{dim}$ effector NK cells, adaptive NK cells, and a unique NK cell subset that is expanded and characterized by upregulation of CX3CR1, TBX21, MYOM2, DUSP1, and ZFP36L2, and negatively correlated with cognitive function in AD patients. Pseudo-temporal analysis revealed that this unique NK cell subset was at a late stage of NK cell development and enriched with transcription factors TBX21, NFATC2, and SMAD3. Together, our study identified a distinct NK cell subset and its potential involvement in AD.

KEYWORDS

Alzheimer's disease, natural killer cell, single cell sequencing, cytotoxicity, innate immunity

Introduction

Alzheimer's disease (AD) is the most common dementia type with limited therapeutic options partially due to the incomplete understanding of disease. Inflammation has emerged as a major contributor to AD pathogenesis (1–3). Mounting evidence has demonstrated that microglia and hematogenous myeloid cells

participate in β-amyloid pathology and cognitive decline (4–6). In contrast, the involvement of lymphocytes in the etiology of AD is less studied.

As innate lymphoid cells, natural killer (NK) cells are critical players that control the initiation and progression of brain inflammation (7, 8). NK cells are generally divided into $CD56^{bright}$ and $CD56^{dim}$ subsets. As a less numerous subset, $CD56^{bright}$ cells are the primary source of NK cell-derived regulatory cytokines, whereas $CD56^{dim}$ cells are mainly cytotoxic effector cells producing IFN-γ upon stimulation. However, the phenotype and function of NK cells in AD remain poorly understood. To address this question, we assessed the transcriptomic alterations of NK cells in AD by analyzing web-based single-cell RNA sequencing data of blood NK cells. As a result, we found reduced number and cytotoxic activity of blood NK cells in AD patients versus control subjects. In particular, we identified an increase of a unique NK cell subset that is at a late stage of development and enriched with transcription factors (TFs) TBX21, NFATC2 and SMAD3, and negatively correlated with the cognitive decline in AD.

Materials and methods

Single-cell RNA sequencing data collection

Single-cell RNA sequencing data of 36,830 cells from a recent published study on human peripheral blood mononuclear cells (PBMCs) from three patients with AD (two men and one woman, 22,770 cells) and two control subjects (one man and one woman, 14,060 cells) were accessed from the GEO public database (GSE181279) (9). There was no significant statistical difference regarding the age between two groups (AD vs. control: 67.7 ± 8.6 vs. 71.0 ± 8.5 years, $p = 0.699$). Low-quality cells with <200 genes, >20,000 UMI, and >10% mitochondrial genes as well as genes that expressed less than three cells were filtered out. The remaining 36,561 cells were finally included in the analysis.

Data integration, dimensionality reduction, clustering, and visualization

Seurat (v 4.1.0) (10) was used for dimensionality reduction, clustering, and visualization. For each sample dataset, we used the filtered expression matrix to identify cell subsets. The filtered gene expression matrix was normalized using the NormalizeData function, in which the number of UMIs of each gene was divided by the sum of the total UMIs per cell, multiplied by 10,000, and then transformed to log scale (in UMI-per-10,000+1). After normalization, the data were scaled with the ScaleData function, and the top 2,000 highly variable genes were identified by the FindVariableFeatures function and used for the following principal component analysis (PCA). Subsequently, the harmony v1 integration method was used to correct the potential batch effect and then clustering with top 20 principal components and resolution 0.5 was performed by graph-based clustering and visualized using t-Distributed Stochastic Neighbor Embedding (t-SNE) with Seurat functions RunTSNE. After the identification of cell types, NK cells were extracted and subclustered for further detailed analysis. The subclustering was performed *via* Seurat with top 13 principal components and a resolution of 0.4. To identify cell types in sample datasets, we used sets of marker genes for each of those cell types and annotated each cell type based on their average expression and expression ratio as previously described (11, 12).

Differential gene expression analysis

Differentially expressed genes (DEGs) in a given cell type compared with all other cell types were determined with the FindAllMarkers function from the Seurat package (Wilcoxon rank-sum test, *p*-values adjusted for multiple testing using Bonferroni correction). The FindMarkers function was used to compute the DEGs between groups. We set min.pct = 0 and logfc.threshold = 0 to obtain all the DEGs and finally filter by *p*-value < 0.05 to draw the DEGs' volcano plot.

Enrichment analyses of differentially expressed genes

The enrichGO and enrichKEGG (cutFC = 0.5) functions of the RNAseqStat R (https://github.com/xiayh17/RNAseqStat) package were used to calculate and visualize the enrichment results of the whole NK cells' DEGs between AD and controls; the Gene Ontology (GO) enrichment mainly displayed the enrichment results of upregulated genes. The Database for Annotation, Visualization, and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) was used to annotate and analyze the associated GO terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways of the DEGs (*p*-value < 0.05 and LogFoldChange > 0.25). GO terms and KEGG and Reactome pathways with adjusted *p*-value < 0.05 were considered significant.

Gene set module score analyses

To further verify the identity of each NK cell cluster, the AddModuleScore function was used to calculate the gene set score as previously defined (13–16): (i) blood $CD56^{dim}$ NK (FGFBP2, GZMB, GZMA, SPON2, S100A4, CST7, FCGR3A, IGFBP7, GZMH, and CFL1); (ii) blood $CD56^{bright}$ NK (GZMK, CD44, PPP1R14B, CXCR3, RPL36A, SCML1, COTL1, NCF1, XCL1, and HLA-DRB1); and (iii) adaptive NK (KLRC2, CD52,

IL32, CD3E, CD3D, CD3G, B3GAT1, TTC16, CADM1, SGCD, VIM, and CCL5). Owing to the similarity between NK and ILC1 cells, we identified ILC1 by scoring the gene signatures of CXCR3, IFNG, LTA, IL12RB1, TBX21, IKZF3, LEF1, ZBP1, JUNB, TSHZ2, SP140, BCL11B, PRDM1, IL6R, IL6ST, IL18BP, SOCS3, IFNG-AS1, GZMM, GZMK, GZMA, SH2D1A, CD6, CD27, CD5, CCR7, CD28, TNFRSF1B, TNFSF8, TNFRSF10A, CCL5, LAG3, CD3D, CD3E, CD3G, CD4, CD8A, CD8B, TRAV13-1, TRAV8-2, TRAV4, TRBV5-1, TRAV9-2, TRAV2, TRBV2, TRAV41, TRBV20-1, TRAV26-2, and TRAV8-4 as previously described (17). Cell subclusters with high score were deemed as ILC1 and excluded.

Pseudo-time analysis

For NK cell subclusters, we performed pseudo-time analysis with Monocle2 (v2.18.0). The ordering was based on the 3,801 DEGs between clusters. Then, the data space was reduced by DDRTree algorithm into two dimensions. The cells were finally ordered in pseudo-time and clusters with a high score of $CD56^{bright}$ NK signatures were considered as the start point of the trajectory.

To identify significantly branch-dependent genes, we used the BEAM algorithm function and gene significance was set to *q*-value < 1E-04. The selection of branch-dependent TFs was according to the intersection between 682 branch-dependent genes and human TF sets from the Human TFDB database.

Protein–protein interaction network construction

After the identification of TFs, STRING (https://cn.string-db.org/) was used to construct the protein–protein interaction network.

Human peripheral blood samples

Peripheral blood samples were collected from seven patients with AD (three men and four women) and 11 control subjects (six men and five women). There was no statistically significant difference regarding the age (AD vs. Control: 61.6 ± 7.8 vs. 66.5 ± 6.7, $p = 0.202$) and sex ($p = 0.280$) of AD patients vs. control subjects. For AD patients, participants were diagnosed with AD (IWG-2) and had positive amyloid PET imaging. Control subjects were generally healthy with normal laboratory test results.

Flow cytometry

PBMCs were isolated from whole-blood specimens and stained with fluorescent-labeled antibodies. For the staining of the intracellular molecules, cells were fixed and permeabilized using commercial kit (eBioscience) according to the manufacturer's instruction. All antibodies were purchased from BioLegend (San Diego, CA, USA), including CD3 (UCHT1), CD56 (HCD56), CD69 (FN50), CD27 (O323), NKG2D (1D11), CX3CR1 (2A9-1), and IFN-γ (4S.B3). Flow cytometry was performed using FACS Aria III (BD Bioscience, San Jose, CA, USA). Data were all analyzed by FlowJo v10.8.1.

Statistical analysis

All statistical analyses of single-cell sequencing data were performed using R software, version 4.1.3. Data represent mean ± SEM. The statistical significance of module gene set analysis was assessed by the Wilcoxon rank sum test with continuity correction or by the Kruskal–Wallis test with Dunn's multiple comparisons test, with *p*-value adjustment by the Benjamini–Hochberg method. Unpaired Student's t test was employed to compare flow cytometry data from AD patients in comparison to controls. $p < 0.05$ was considered to be statistically significant.

Results

Single cell transcriptomic analysis of PBMCs from patients with AD

We analyzed the single-cell RNA-sequencing data of PBMCs (GEO database: GSE181279) from three patients with AD and two control subjects. A total of 36,561 cells were included for assessment. Among these cells, 22,582 were from AD patients and 13,979 were from control subjects (Figure 1A, Supplementary Figures 1A–C). Unbiased clustering identified eight major cell subsets based on specific markers: $CD4^+$ T cells (CD3D, CD3G, and CD4), $CD8^+$ T cells (CD3D, CD3G, and CD8A), double-positive T cells (CD3D, CD3G, CD4, and CD8A), NK cells (identified as expression of NKG7, KLRD1, and NCR1, and lack of expression of CD3), B cells (CD19, CD79A, and CD79B), plasma cells (CD19, CD79A, TNFRSF17, and CD38), monocytes (CD14), and platelets (PPBP and PF4) (Figure 1B). The cellular distribution of each group is shown in Figure 1C. The dot and violin plots displayed the expression of specific genes in each cluster (Figures 1D, E).

Reduced number and cytotoxicity in blood NK cells from patients with AD

In the determination of cellular composition and distribution, we found a reduction of blood NK cells in patients with AD versus control subjects (Figures 2A–C, Supplementary Figure 1D). NK cells from patients with AD displayed upregulation of DUSP1 and

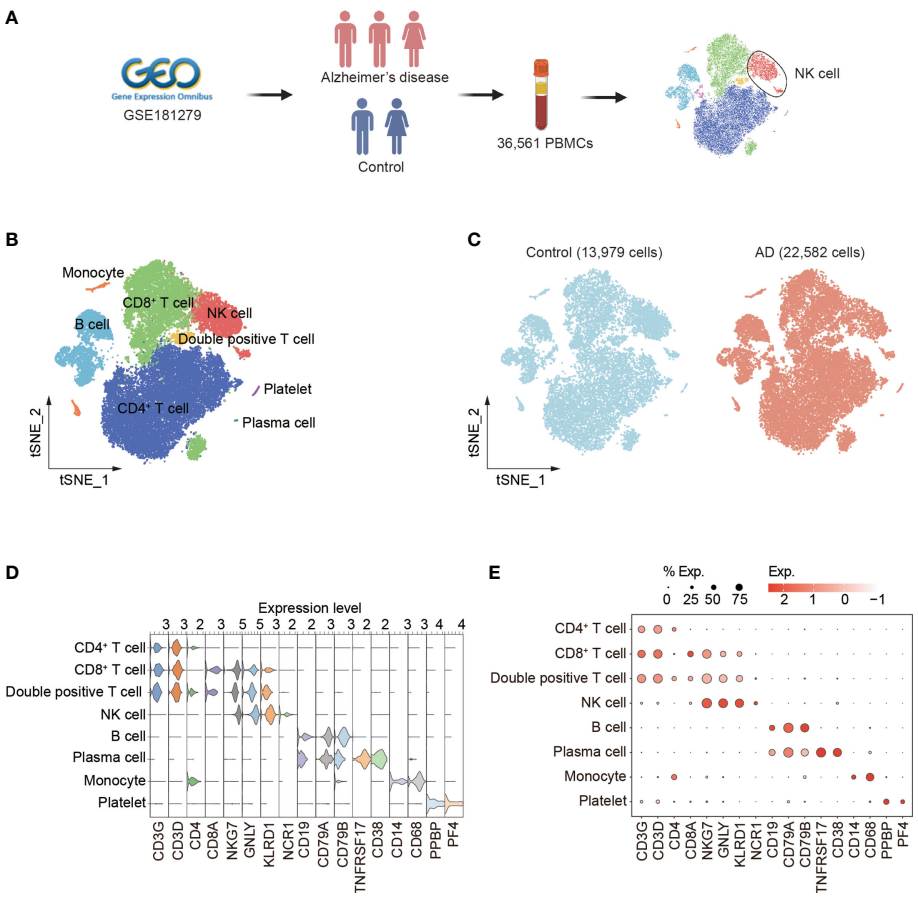

FIGURE 1
Single-cell transcriptomic analysis of PBMCs from patients with AD. **(A)** Schematic of experimental design. **(B)** tSNE plot of 36,561 single cells from total PBMCs of three AD patients and two control subjects. **(C)** tSNE plots of 13,979 single cells from control subjects and 22,582 single cells from AD patients, colored by group. **(D, E)** The violin **(D)** and dot **(E)** plots showed expression levels of known cell type-specific markers. PBMCs: peripheral blood mononuclear cells; tSNE: t-distributed Stochastic Neighbor Embedding.

DUSP2 that are regulators of the ERK signaling pathway and the RNA-binding protein ZFP36L2 that is related to immunosuppression as well as TBX21 involved in NK cell maturation (Figure 2D). We also observed a decrease in cytotoxicity genes (FCER1G, CTSW, GZMB, GNLY, KLRF1, SPON2, FGFBP2, and PRF1) and activation markers (CD69 and KLRB1) in NK cells from patients with AD (Figure 2E). The KEGG analysis also revealed a reduction of NK cell-mediated cytotoxicity (Figure 2F). GO analysis revealed the DEGs in NK cells regarding lymphocyte activation, cell adhesion, and related intracellular pathways (Figure 2G).

To verify the above findings, we conducted flow cytometry analysis of peripheral blood from 7 AD patients and 11 control subjects. In line with scRNA-seq results, we found a reduction of NK cell number and percentage in peripheral blood from AD patients, though the difference was not significant (Supplementary Figures 2A, B, D). We also found reduced expression of CD69, CD27, NKG2D, and IFN-γ in NK cells from peripheral blood of AD patients (Supplementary Figures 2C, D). Together, these results suggest reduced number and cytotoxicity in blood NK cells from patients with AD.

Subclustering analysis revealed expansion of a unique NK cell subset expressing CX3CR1, TBX21, MYOM2, DUSP1, and ZFP36L2 in patients with AD

Next, subclustering analysis was performed to assess the transcriptomic alterations in blood NK cells from patients with AD after exclusion of ILCs *via* module score analysis. Unsupervised clustering of the remaining 2,897 NK cells (Control: 1,842 cells, AD: 1,055 cells) revealed four subsets: NK0, NK1, NK2, and NK3 (Figure 3A, Supplementary Figure 1E). As shown in Figure 3B, the expression of NK cell signatures (CD7, NKG7, GNLY, KLRD1, and KLRF1) was identified in these subsets.

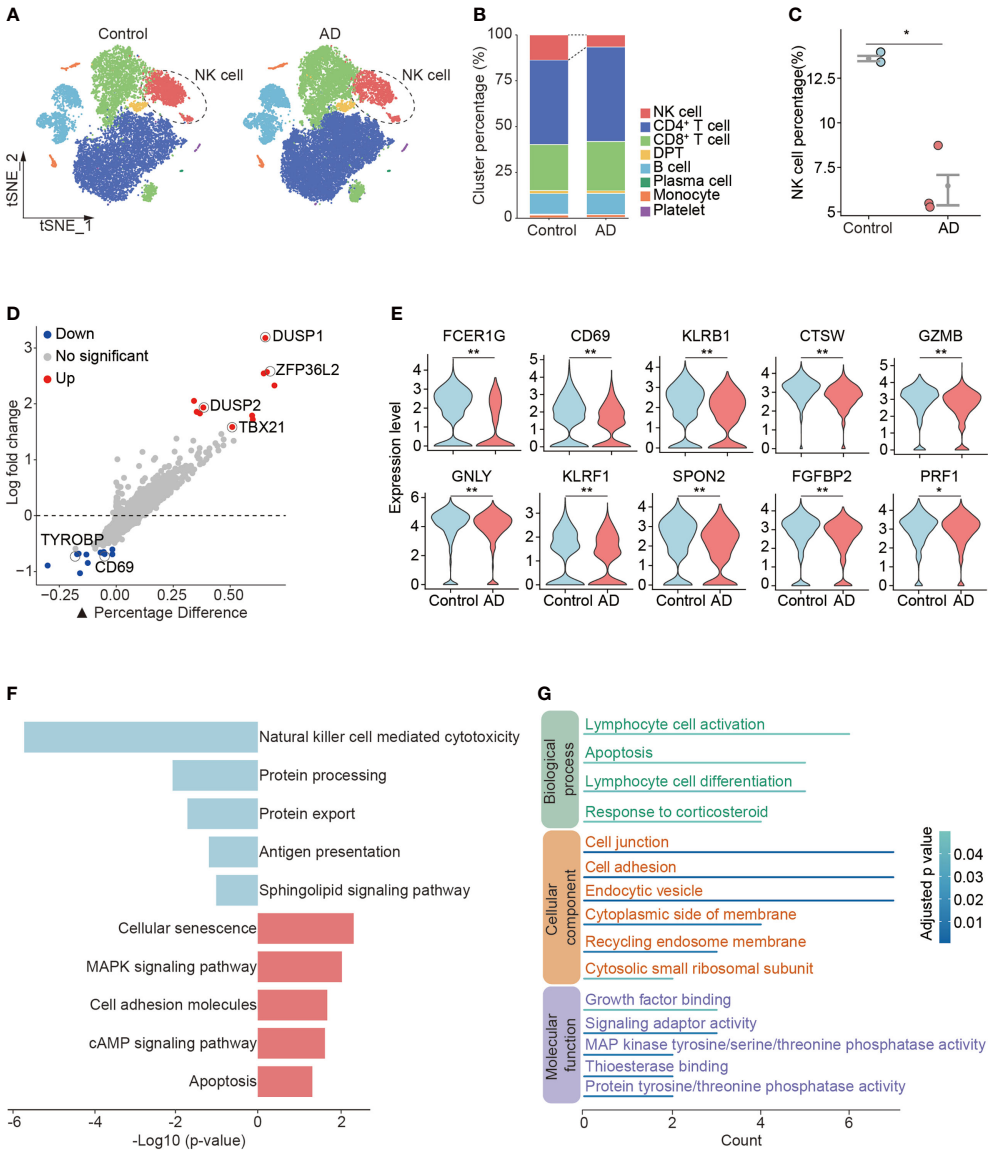

FIGURE 2
Reduced number and cytotoxicity in blood NK cells from patients with AD. **(A)** tSNE plots of total PBMCs from controls and patients with AD. NK cells were labeled with ellipse tag, which were reduced in the AD group. **(B)** The distribution of cell clusters in AD and control groups. **(C)** NK cell percentage in individual level. Data are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$. **(D)** Assessment of differentially expressed genes (DEGs) using log-fold change expression versus the difference in the percentage of cells expressing the gene in blood NK cells from patients with AD versus controls (▲Percentage Difference). Genes labeled were chosen based on log-fold change > 1.5 (Up) and log-fold change < −0.5 (Down), adjusted *p*-value from Wilcoxon rank sum test < 0.05. **(E)** Violin plots show the expression levels of cytotoxicity and activation markers in blood NK cells from patients with AD versus controls. **(F)** KEGG pathway analysis of DEGs in blood NK cells from patients with AD versus controls. Blue: downregulated pathways, pink: upregulated pathways. **(G)** GO enrichment analysis of DEGs in NK cells.

Thereafter, we assessed the top genes that were expressed among these four NK cell subsets (Figure 3C). We found that the NK0 cluster expressed genes associated with cytotoxic factors (FCER1G, SPON2, GZMM, and GZMB) and activation markers CD69 and CD160, resembling $CD56^{dim}$ effector NK cells. The NK1 cluster displayed an enrichment of genes including CX3CR1, TBX21, MYOM2, DUSP1, and ZFP36L2. The expression of CX3CR1, TBX21, and KIR2DL2 suggests that the NK1 subset was in the late stage of NK cell development as previously described (14). The NK2 cluster expressed CD3E, GZMH, CCL5, IL32, VIM, and KLRC3 as well as HLA molecule-encoding genes (HLA-DRB1, HLA-DPB1, and HLA-DPA1) related to previously reported adaptive NK cells (14, 15). The NK3 cluster expressed

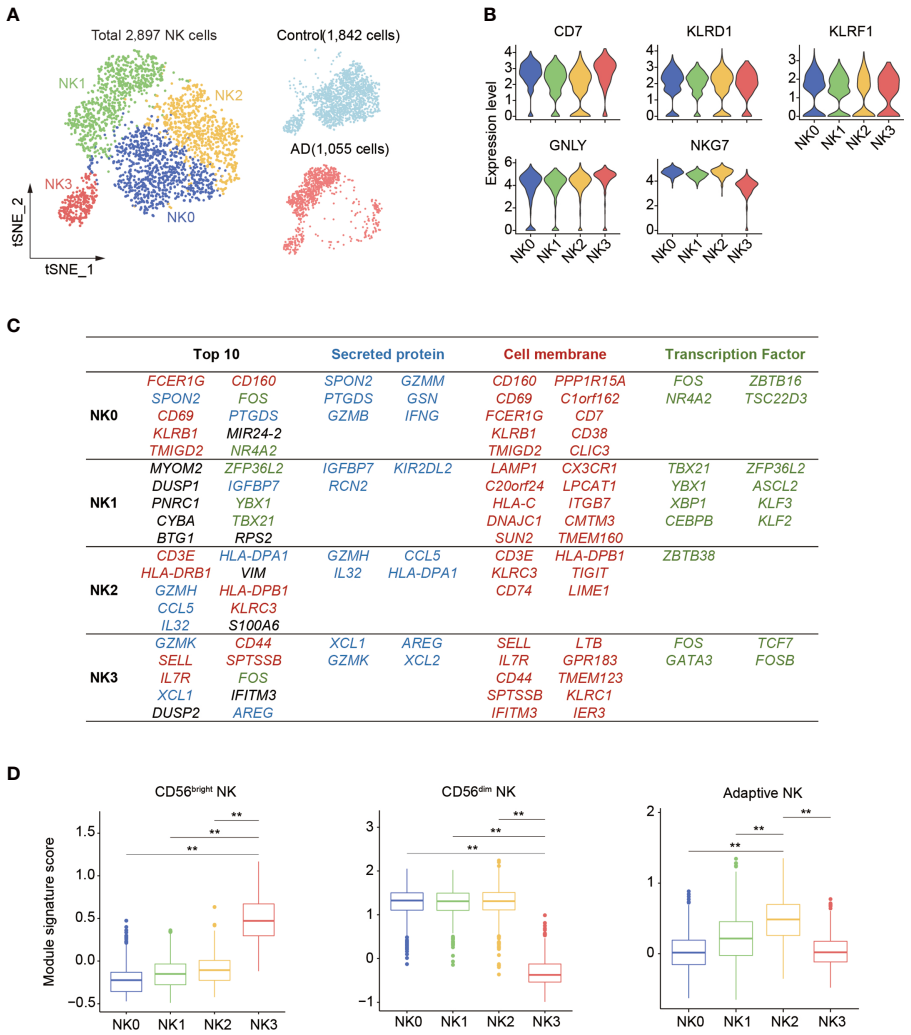

FIGURE 3
Subclustering of blood NK cell in patients with AD. **(A)** tSNE plot of 2,897 NK cells from three patients with AD and two control subjects. **(B)** Violin plots display the expression of NK cell lineage markers in each cluster. The *y*-axis represents normalized expression value. **(C)** Top 10 most enriched genes among the total gene set and among upregulated genes encoding secreted proteins, cell membrane markers, and transcription factors that are different among four NK cell subpopulations. Blue: secreted protein, red: cell membrane, green: transcription factors. **(D)** The boxplots showing the distribution of the module score for blood $CD56^{dim}$ as well as $CD56^{bright}$ NK cells and adaptive NK cells among each NK cell subset. $^{**}p < 0.01$.

GZMK, IL7R, SELL, XCL1, XCL2, KLRC1, and CD44, resembling $CD56^{bright}$ NK cells (13–15).

Gene signature module score analysis revealed that the NK0, NK1, and NK2 clusters resemble $CD56^{dim}$ NK cells (13–15), whereas the NK3 cluster resembles $CD56^{bright}$ NK cells (Figure 3D). Of note, the NK2 subset shared the highest adaptive NK cell gene set score (Figure 3D). GO, KEGG, and Reactome enrichment analysis revealed that the NK0 cluster had an enrichment of cytotoxicity (Figure 4A). The NK1 subset was enriched in apoptotic process and cellular senescence, accompanied by upregulation of CX3CR1 and KLF2 (Figure 4A). In contrast, NK2 displayed an enrichment in adaptive features of NK cells (Figure 4A). The NK3 cluster was enriched in cytokine signaling and pathways related to immune regulatory function (Figure 4A).

Correlation analysis was performed to measure the similarity among these four NK cell clusters. As shown in Figure 4B, the NK0 cluster was similar to the NK2 cluster. The NK1 cluster had a weak similarity with NK0 and NK2 clusters. In contrast, NK3 represented a distinct subset to other NK cell clusters. Correlation analysis of DEGs revealed the NK1 subset as an enriched subset in AD relative to controls (Figures 4C, D, Supplementary Figure 1F), accompanied by a contraction of the NK0 subset and the NK2 subset (Figures 4D, E).

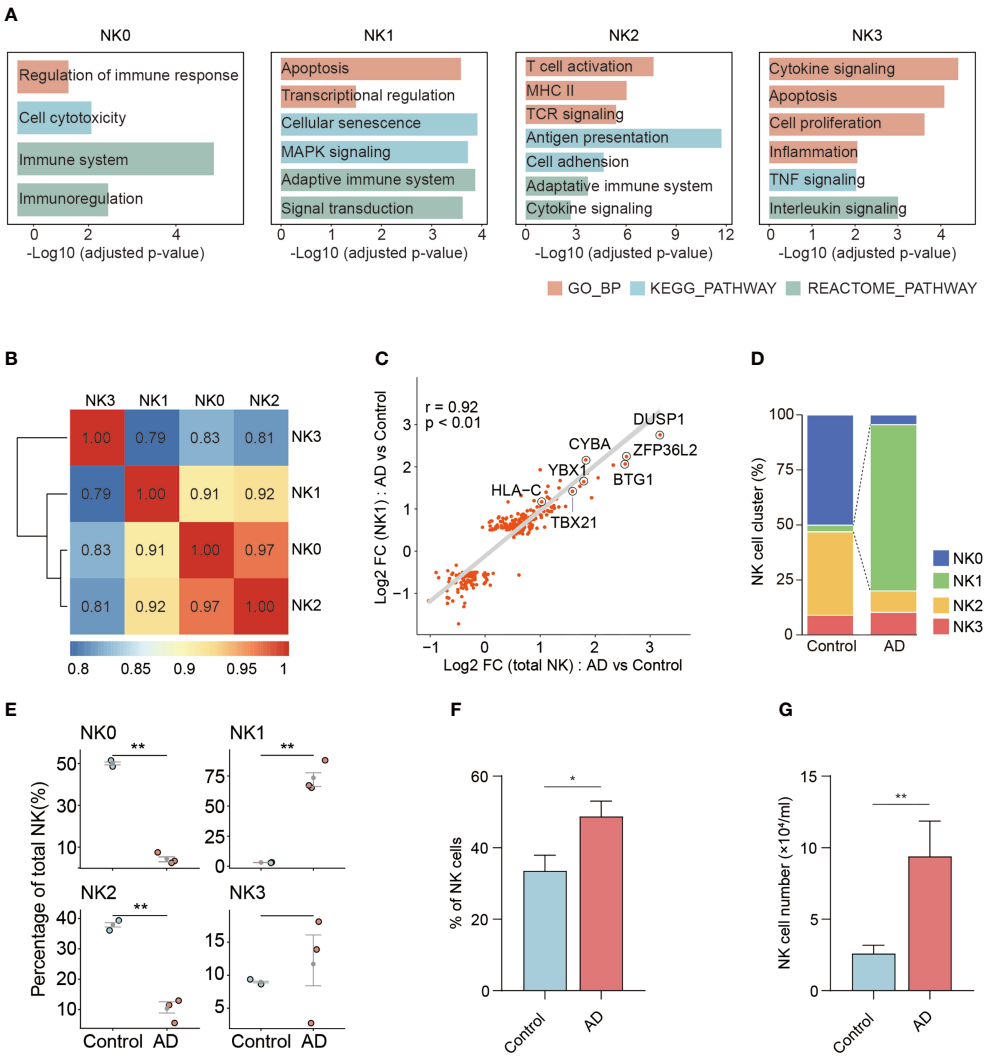

FIGURE 4
The functional characteristics and distributions of four NK cell subsets. **(A)** Bar plots show the selected GO and KEGG as well as Reactome terms enrichment for each of the four NK cell subsets in patients with AD versus controls. The significance threshold was set to $p < 0.05$. **(B)** Correlations among four NK subsets. **(C)** Scatter plot showing the correlation between total fold changes of NK cells' gene expression from AD versus control (*x*-axis) against NK1 subset fold changes from AD versus control analysis (*y*-axis). Selected top genes are shown. **(D)** The distribution of four NK subsets in patients with AD and controls. **(E)** The relative proportion of each cluster was calculated in each sample. **(F)** Bar plot showed the CX3CR1 expressing NK cell percent in AD and control groups. **(G)** Bar plot showed the number of CX3CR1 expressing NK cell in groups of AD patients and control subjects. In **(F, G)**, AD: $n = 7$, control: $n = 11$. Data are presented as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

Similarly, flow cytometry results show that the percentage and number of NK cells expressing CX3CR1 were increased in AD patients (Figures 4F, G). A negative correlation was seen between the number of CX3CR1-expressing NK cells and the severity of cognitive impairment (Supplementary Figure 2E).

These results demonstrate the expansion of a distinct blood NK cell subset expressing CX3CR1, TBX21, MYOM2, DUSP1, and ZFP36L2 in AD patients and its relation to cognitive impairment.

Pseudo-temporal ordering of blood NK cells reveals a branched trajectory with a significant shift toward the NK1 subset in patients with AD

Since the enrichment of CX3CR1 and TBX21 in the NK1 subset suggests augmented maturation of NK cells, we next conducted pseudo-temporal analysis with Monocle2. The pseudo-time analysis ordered cells along a trajectory that

segregates 2,897 NK cells into two major branches of cell fate 1 and cell fate 2, highlighting a specific developmental trajectory of NK cells. As shown in Figures 5A, B, the cell fate 1 branch was mostly constituted by the $CD56^{dim}$ effector NK cell subset and the adaptive NK cell subset, whereas the cell fate 2 branch was mainly constituted by the NK1 subset. $CD56^{bright}$ NK cells were mainly distributed in the top trajectory of the pre-branch that represents the initial state of NK cells. Notably, the trajectory in AD displayed an evident shift toward the NK1 subset in the cell fate 2 branch (AD: 89.1%, Control: 5.0%) (Figure 5C).

We identified 682 branch-dependent genes during the cellular state transition from the pre-branch to cell fate 1 and cell fate 2 through branched expression analysis. Hierarchical clustering of these genes revealed three gene modules. Representative TFs for each gene module are shown in Figure 5D. Among these modules, most of the genes in module 2 were concentrated in the cell fate 2 branch cells. Notably, TBX21, NFATC2, and SMAD3 are representative TFs of module 2 and may serve as hub genes in control of NK cell alterations in AD (Figures 5E, F). Meantime, these genes' expression level was enriched in patients with AD versus controls (Figure 5G).

These results demonstrate that the blood NK1 subset in patients with AD is at the late stage of NK cell development and key TFs including TBX21, NFATC2, and SMAD3 may play a vital role in this subset expansion.

Discussion

The major goal of this work was to address the alterations of human NK cell transcriptome in AD from an unbiased transcriptome-wide perspective, and identify an NK cell subset that may be linked to disease pathogenesis. As documented here, we found reduced numbers and cytotoxic activity of blood NK cells in AD. Among identified NK cell subsets (i.e., NK0, NK1, NK2, and NK3), a unique NK1 subset is expanded in AD and characterized by expression of CX3CR1, TBX21, MYOM2, DUSP1, and ZFP36L2. Pseudotime analysis identified that this distinct NK cell subset is at a late stage of NK cell development, accompanied by an increased expression of TFs of TBX21, NFATC2, and SMAD3. Flow cytometry analysis of blood NK cells from AD patients revealed this subset, together with its association with cognitive impairment.

In this study, we subclustered NK cells at single-cell resolution and identified four different subsets. We found reductions of cytotoxic (NK0) and adaptive (NK2) subclusters in the blood of AD patients. Previous single-cell studies have demonstrated the adaptive features of NK cells expressing KLRC2 (NKG2C), CD52, and IL32 (15), along with expression of antigen presentation and T-cell activation markers (14). Consistent with the above studies, we also found adaptive features of NK cells such as the lack of CD3 and the expression of NK cell markers (NKG7, KLRD1, and NCR1). The lower distribution of cytotoxic (NK0) and adaptive (NK2) subsets in the blood suggests that these NK cells may be mobilized and recruited into other organ compartments in AD. Another explanation could be a result from altered neurogenic innervations toward these NK cell subsets, leading to their reduction, although other possibilities cannot be excluded. These postulations await future investigations.

CX3CR1 is involved in the chemotaxis of leukocytes; a previous study revealed a beneficial role of $CX3CR1^+$ NK cells in experimental autoimmune encephalomyelitis (EAE), in a mouse model of multiple sclerosis (MS) (18). Another study suggested that CX3CR1 could identify a late stage of NK cell development characterized by decreased effector function (19). In this study, we found reduced NK cell cytotoxicity and expansion in a unique subset of $CX3CR1^+TBX21^+$ NK cells associated with cognitive impairment in AD patients. Trajectory analysis suggests that the expansion of this NK cell subset in AD patients may have resulted from augmented differentiation from the NK3 subcluster, although enriched apoptotic processes were noted in this subcluster. Nevertheless, the discrepancy between previous studies and our findings may involve distinct features of NK cells across different disease conditions, i.e., MS vs. AD, and the potential discrepancies of NK cells in humans vs. mice. Future studies are required to pinpoint NK cell features and their precise contributions to AD progression.

Although a few studies have suggested the involvement of NK cells in AD patients and mouse models (20, 21), it is still early to conclude the precise impact of NK cells on the initiation and progression of AD pathology. NK cells participate in CNS inflammatory injury once they sense danger signals and are receptive to neurogenic innervations in brain disorders (22, 23). It is reasonable to postulate that the alterations of NK cell signatures are likely, at least partially, the result of the brain pathology in AD. On the other hand, the altered features of NK cells may be involved in AD pathology, albeit further studies are required to better understand the role of NK cells during disease progression. Additionally, the small sample size in a Chinese cohort of both single-cell analysis and flow cytometry tests is a limiting factor to interpret our findings. Future studies are required to verify these results among large populations including Chinese and subjects from other countries.

In summary, our study demonstrated the alterations of transcriptomic profile in blood NK cells from patients with AD and identified a distinct NK cell subset related to cognitive decline. These new results provide additional support to the involvement of NK cells in AD pathology.

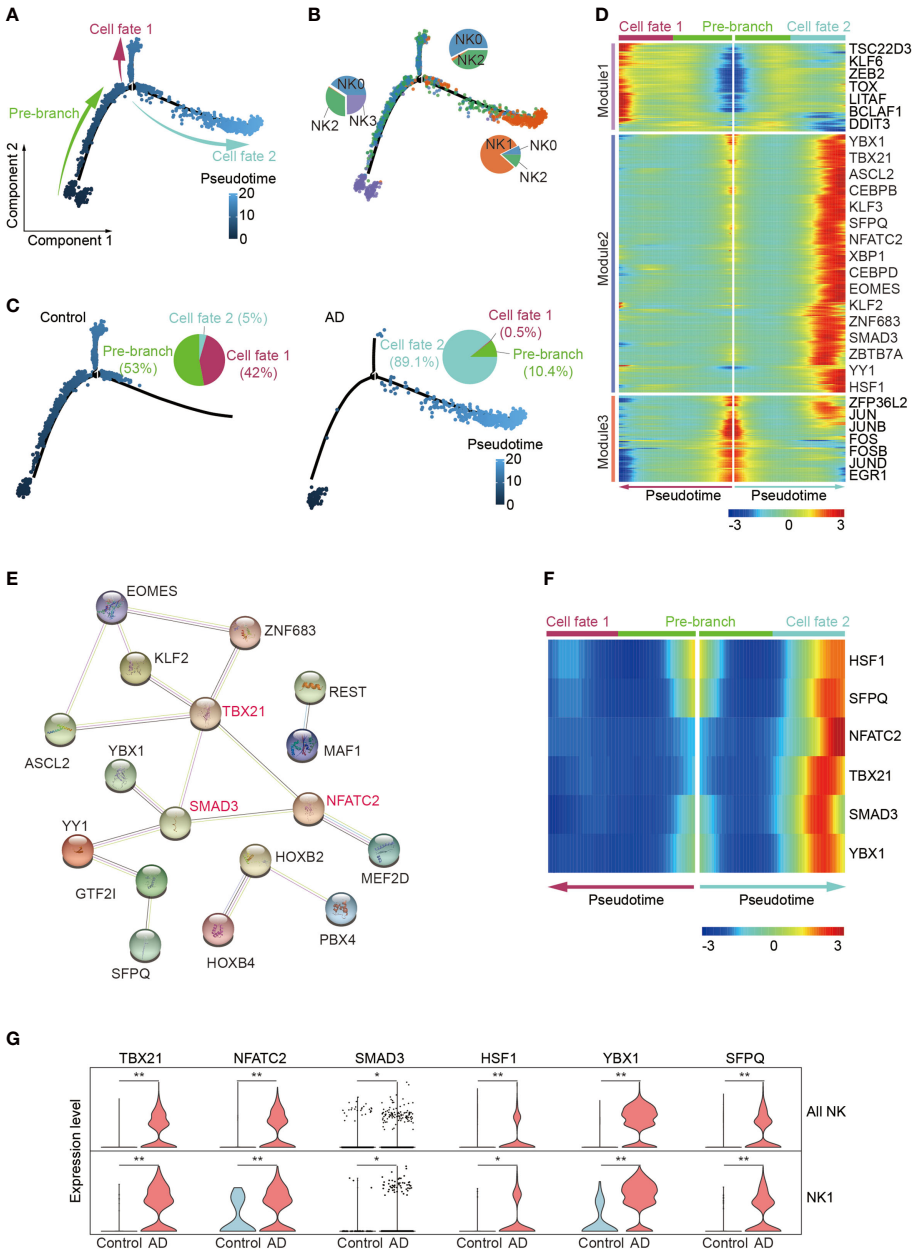

FIGURE 5
Pseudo-temporal ordering of NK cells reveals a branched trajectory with a significant shift toward the NK1 subset in AD. **(A)** Pseudo-time ordering of NK cells shows a branched trajectory. **(B)** The distribution of the four NK subsets among each branch. **(C)** Evident shift toward a NK1 phenotype (cell fate 2) in AD (right panel) versus control (left panel). **(D)** Hierarchical clustering of the branch-dependent genes reveals three gene modules. The significance threshold was set to a *q*-value of the branched expression analysis modeling test < 1e-04. The transcription factors involved in each module are shown. **(E)** The protein–protein interaction network of the module 2 transcription factors was constructed by STRING. TBX21, SMAD3, and NFATC2 are colored red and serve as hub genes. **(F)** Six key TFs displaying branch dependence are from gene module 2; most of them are enriched in cell fate 2. **(G)** Violin plots show the expression levels of six specific transcription factors in AD and control group. *$p < 0.05$, **$p < 0.01$.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181279.

Author contributions

QL, HL, and CQ designed research. NZ, FL, and YH help to collect peripheral blood samples or finish the flow cytometry experiments. CQ, WZ, and HL analyzed data and drafted the manuscript. QL edited the manuscript. All authors read and approved the 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.2022.1004885/full#supplementary-material

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NKB cells: A double-edged sword against inflammatory diseases

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Interferon-γ (IFN-γ)-producing natural killer (NK) cells and innate lymphoid cells (ILCs) activate the adaptive system's B and T cells in response to pathogenic invasion; however, how these cells are activated during infections is not yet fully understood. In recent years, a new lymphocyte population referred to as "natural killer-like B (NKB) cells", expressing the characteristic markers of innate NK cells and adaptive B cells, has been identified in both the spleen and mesenteric lymph nodes during infectious and inflammatory pathologies. NKB cells produce IL-18 and IL-12 cytokines during the early phases of microbial infection, differentiating them from conventional NK and B cells. Emerging evidence indicates that NKB cells play key roles in clearing microbial infections. In addition, NKB cells contribute to inflammatory responses during infectious and inflammatory diseases. Hence, the role of NKB cells in disease pathogenesis merits further study. An in-depth understanding of the phenotypic, effector, and functional properties of NKB cells may pave the way for the development of improved vaccines and therapeutics for infectious and inflammatory diseases.

KEYWORDS

innate lymphoid cells, inflammation, IFN-γ, NKB cells, Th1 cells, IL-18, IL-12, infectious diseases

Abbreviations: ACE, Aceclofenac; ALD, Alcoholic liver disease; AsC, Asymptomatic HBV carrier; BCR, B-Cell receptor; BMMSCs, Bone marrow derived mesenchymal stem cell; CDR3, Complementarity-determining region 3; CHB, Chronic hepatitis B; CIA, Collagen induced arthritis; GSF, Gingival crevicular fluids; HBV, Hepatitis B virus; HBV-ACLF, HBV-associated acute-on-chronic liver failure; HC, Healthy control; HIS, Humanized immune system; IFN-γ, Interferon-γ; ILCs, Innate lymphocyte cells; IL-18BP, IL-18 binding protein; LN, Lymph nodes; LPS, Lipopolysaccharide; LPHNPs, Lipid-polymer hybrid nanoparticles; MLNs, Mesenteric lymph nodes; MTX, Methotrexate; MZ, Marginal Zone; NK cells, Natural killer cells; NKB, Natural killer-like B cells; NKBP, NKB precursor; NLCs, Nanostructure lipid carriers; PBMCs, Peripheral blood mononuclear cells; RA, Rheumatoid arthritis; SIV, Simian immunodeficiency virus.

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NKB cells and their immune functions

Technological advances in cell biology have allowed investigators to better understand the phenotypic and functional characteristics of individual immune system cell types. Further, *in vitro* and *in vivo* investigations have identified the contributions of various immune effector cells to infections and inflammatory diseases (1–4). Moreover, advancements in immunobiology have allowed increased understanding of the mechanisms that underlie the signaling pathways responsible for pathogen elimination (2, 5–10).

B and T cell populations in the adaptive immune system share functional similarities with cells in the innate immune system, including natural killer T (NKT) cells (11), γδT cells (12, 13), and B1 B cells (14–16). The latter cell subsets play regulatory roles by stimulating acquired and innate immune responses in the host in order to fight pathogens. Furthermore, the characteristic features of NK cells and ILC subsets have been well studied with respect to IFN-γ production in response to host cell invasion by infectious agents (17–20).When analyzing NK cells in the spleen and mesenteric lymph nodes (MLNs) of mice, a population of cells co-expressing the NK cell markers NK1.1 and NKp46, as well as the B cell markers CD19 and IgM, were identified (21). Distinct from conventional NK and B cells, these cells uniquely expressed CD106 and CD63, and lacked expressions of common lineage marker (CD3, CD4, CD8, CD11b, and CD11c). This novel cell population, with properties comparable to NK and B cells, was referred to as "natural killer-like B (NKB) cells" ($CD19^+NK1.1^+$) (21). Giemsa staining, electron microscopy, immunofluorescence staining, imaging flow cytometry, and immunohistochemistry investigations confirmed that NKB cells exhibit a morphology similar to lymphocytes. These cells contained a small amount of endoplasmic reticulum and lacked cytotoxic granules in their cytoplasm.

NKB cells are primarily localized to the marginal zone (MZ) of the spleen; $CD19^+NKp46^+$ NKB cells were observed in the human spleen (∽ 2.7%) as well as in the MLNs (∽ 2.3%) (21). Gene expression profile studies revealed that NKB cells predominantly express components of the B-cell receptor (BCR), members of the Ly49 family of NK cell receptors, the major histocompatibility complex-I and II (MHC-I and II), CD40, CD83, and a higher expression of IL-18 as well as the proliferation marker Ki67. NKB cells expressed elevated levels of the B cell transcription factor *Pax5* and low levels of the NK cell transcription factor *Id2*. These cells also expressed high levels of CD63 and CD106 but lacked expressions for several characteristic dendritic cell (DC) markers (CD11c), ILC markers (CD127 or IL-7Rα), and T cell markers (CD3). Studies involving several knock-out mouse models ($Rorc^{-/-}$, $\mu MT^{-/-}$, $Id2^{-/-}$, $Rag1^{-/-}$, and $Il2g^{-/-}$) confirmed the roles played by the IL-2R common γ chain as well as *Rag* recombinase signaling in NKB cell development and/or maturation (21). Furthermore, common γ chain-associated cytokines (IL-2, IL-15, and IL-4) were required for NKB cell expansion and longevity.

NKB cells were unable to secrete two major effector cytokines, namely, IFN-γ and TNF-α, and did not exhibit NK-like cytotoxic activity. Analysis of the complementarity-determining region 3 (CDR3), the most hypervariable region of the BCR and TCR, revealed a non-Gaussian distribution in the length of CDR3 sequences and a restricted BCR repertoire in NKB cells. However, conventional B cells exhibited a Gaussian distribution of CDR3 sequences with a broad BCR repertoire. These findings suggest the distinct nature of NKB cells as compared with B and NK cells. NKB cells were phenotypically characterized following the microbial infection of mice. The NKB cells expanded for up to 24 h post-infection and these cells secreted a variety of cytokines (IL-6, IL-12, IL-15, IL-1β, and IL-18), suggesting important functions. Moreover, infection progression led to a gradual increase in IL-18 production, whereas higher levels of IL-12 production were observed during the early phases of infection. When exposed to microbial agents *in vitro*, NKB cells were able to transactivate Th1 cells and ILCs to produce IFN-γ. NKB cells that were adoptively transferred into $Rag1^{-/-}$ mice became activated, and expanded in response to *Listeria monocytogenes* infection, suggesting immune activation independent of conventional B and T cells. Furthermore, IL-18 appeared to be the signature cytokine produced by NKB cells (21–23). Co-culture assays demonstrated the rapid activation of both NK cells and ILCs that secrete IL-18 and IL-12 to clear bacterial infections.

The developmental origin of NKB cells was revealed by transferring either NK cell progenitors (NKP) or pro-B cells to B cell-deficient μMT mice. The transferred pro-B cells differentiated into NKB and B cells, whereas the NKP cells did not further differentiate. The $Lin^-CD122^+CD19^+NK1.1^+$ cell lineage facilitated the conversion of NKP cells to NK cells *via* IL-15 (binds with CD122), and the former cells further differentiated into $Lin^-CD122^-CD19^+NK1.1^+$ NKB cells. Hence, the subpopulation of $Lin^-CD122^+CD19^+NK1.1^+$ cells was termed NKB precursor (NKBP) cells. This was further confirmed by transferring NKBP cells into NKB-deficient mice, after which NKBP cells differentiated into NKB cells. However, the transfer of different B cell populations into μMT mice failed to produce NKB cells. In short, the $Lin^-CD122^+CD19^+NK1.1^+$ lineage was proposed as the relevant precursor to NKB cells. These cells exhibit the *bonafide* NKB cell development pattern as well as their functional responses to microbial infection (21).

The frequencies, locations of tissue residence, and phenotypic markers of NKB cells were defined using genetic murine models. The majority of IgM^+ cells failed to display stains for other markers of NK cells. $NK1.1^+NKP46^-CD19^+$ and $NK1.1^-NKp46^+CD19^+$ cells resembled conventional B cells

rather than the distinct NKB cell population (24). Sorted NK1.1$^+$NKP46$^-$CD19$^+$ cells that were stimulated with lipopolysaccharide (LPS) for three days rapidly differentiated into CD138$^+$Blimp-1$^+$ plasmablasts. Further, the proliferation and survivability of splenic NK1.1$^+$NKP46$^-$CD19$^+$ cells were assessed in the presence of *bonafide* NK cell homeostasis factors. The majority of the NK1.1$^+$NKP46$^-$CD19$^+$ cells did not survive in the presence of IL-15, and several cells exhibited an NK1.1$^+$NKp46$^+$ phenotype. Additionally, anti-NK1.1 and -NKp46 monoclonal antibodies were bound to the relevant antigens present on BCR$^+$ cell subsets. Nevertheless, it was suggested that the novel NKB cell population is part of the conventional B cell lineage, rather than a distinct population. Although the possible role of NKB cells in the pathogenesis of infectious and inflammatory disorders has been explored, the precise identity of this cell type requires further validation using antibody-based identification methods (24).

Kerdiles et al. utilized the *Ncr1*-driven *Cre* model, where inefficient expression of *Cre* may be a possible explanation for the low or absent expression of NKp46. Furthermore, Wang and colleagues reported that bone marrow-derived pro-B cells were the primary source of NKB cells that originated from NKBP cells. The transcription factors involved (as stated earlier for NKB cells), and the regulatory mechanisms by which pro-B cells are converted to NKBPs (the results of an adoptive transfer experiment carried out on µMT mice), followed by their maturation into NKB cells is of particular interest. Moreover, LPS stimulation failed to induce the production of IgM by NKB cells. These conflicting results may be attributed to the detection method employed by Kerdiles et al. Finally, NKB cells are distinct from conventional B cells and thus represent a unique subset of innate B cells that may play an important role during microbial infection. The conversion of pro-B cells to mature NKB cells requires further investigation (25).

NKB cells and their role in infectious and inflammatory diseases

NKB cell studies in SIV-infected non-human primates

Based on the discovery of novel NKB cell populations in rodents, Manickam et al. investigated NKB cells in macaques and humans (26) (Figures 1A, B). The peripheral blood mononuclear cells (PBMCs) and tissue mononuclear cells from various organs of uninfected and HIV-infected humans, as well as those from uninfected and simian immunodeficiency virus (SIV)-infected macaques, were analyzed. Similar NKB cell frequencies were identified in both groups of primates, and their enrichment in the spleen validated earlier findings carried out in rodents (Figures 1A, B). A broad distribution was also noted in other human organs such as the tonsils, colon, jejunum, and lymph

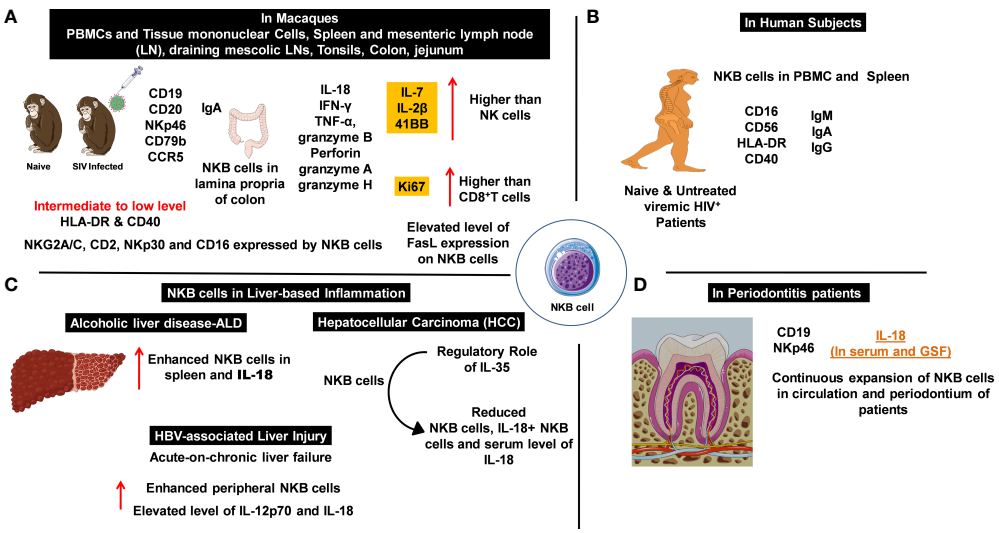

FIGURE 1
Natural killer-like B cells (NKBs) play a crucial role in the pathogenesis of infectious and inflammatory diseases. The expression profile of NKB cells with cytolytic granules and other signaling and defense molecules which help to understand inflammatory disorders. **(A)** NKB cells and their expression profile in the periphery and deep-seated secondary lymphoid organs of naïve and simian immunodeficiency virus (SIV)-infected macaques. Expression of inflammation mediators and cytolytic granules in cytotoxic immune cells. **(B)** The numbers and characteristics of NKB cells that were studied in the circulation and lymphoid organs of patients with viral infections. **(C)** The increased number of NKB cells is proportional to the elevated expression of IL-18 during inflammation observed in liver pathologies. **(D)** An increase in NKB cells mediates enhanced IL-18 production in patients with periodontitis.

nodes (LN). These cells expressed NKp46, moderate levels of CD16, and low levels of both HLA-DR and CD40. Molecular studies confirmed the expression of several NK cell markers (CD2, NKp30, NKG2A/C, and CD16) by NKB cells in both species (Figures 1A, B) (26).

Manickam and colleagues identified this unique NKB cell population in both naïve and chronically SIV-infected macaques. The distributions of NKB cells in different deep-seated organs varied in the uninfected macaques. NKB cells were differentiated from other cell types depending on their $CD3^{-}NKG2A^{+/-}NKp46^{+/-}CD20^{+}CD127^{-}$ phenotype, as characterized by fluorescence-activated cell sorting (FACS). In healthy humans, NKB cells expressed CD40 and HLA-DR, and the levels of these markers did not significantly differ in HIV-infected individuals following antiretroviral therapy (ART). The expression pattern of surface immunoglobulin differentiated NKB cells from other cell lineages. High expressions of IgM and IgA and a low expression of IgG were observed in uninfected macaques and humans (Figure 1B) (26). It was estimated that IgG expression levels were increased 10-fold in both the spleen and MLNs of SIV-infected macaques, compared with the levels of PBMC in HIV-infected patients (26).

The role of NKB cells in gut-associated inflammation during mucosal immune responses to SIV infection was investigated (27). NKB cells present in the lamina propria of SIV-infected rhesus or cynomolgus macaque colons were compared with uninfected controls. RNA sequencing and flow-cytometry analyses showed that the NKB cells display receptors, markers, and functions similar to NK and B cells (27). The NKB cells were the primary source of IL-18 production in the colon following SIV infection. This was confirmed by staining lamina propria lymphocytes with anti-IL-18 antibodies and NKB cell marker antibodies. *In vitro* studies confirmed that NKB cells were the "natural source" of IL-18. Additional findings showed that almost 68% of the NKB cells (from the colon tissues of six infected subjects) produced IL-18, whereas none of the B or NK cells produced IL-18. Since IL-18 and IL-1β are canonically produced in response to inflammasome activation, IL-1β was investigated. IL-1β was produced by NK and B cells; however, no production was observed by the NKB cells (27). Thus, the NKB cells were concluded to be the primary source of IL-18, whose expression is highly regulated by a non-canonical pathway that mounts inflammatory responses in the SIV-infected colon. Transcriptional analyses demonstrated that NKB cells possessed a unique transcriptome, compared with that of NK and B cells. The NKB cells expressed both MS4A1 (CD20) and NCR1 (NKp46), whereas the B and NK cells only expressed MS4A1 and NCR1, respectively.

NKB cells expressed increased levels of granzyme H transcripts compared with NK and B cells. In NK cells, the Fas ligand (FasL) targets and destroys virus-infected cells. An increased expression for FasL in NKB cells was observed during viral infection (27). Furthermore, over 84% of the NKB cells expressed FasL, compared with 10% and 14% of NK and $CD8^{+}$T cells, respectively. Protein expression confirmed elevated IFN-γ production (46.7% of NKB cells vs. 7.95% of NK cells and 8.14% of $CD8^{+}$ T cells) in the infected macaque colons. These findings suggest that NKB cells may have originated from NK cells that were extracted from the colon. However, these findings contradict earlier results which showed that NKB cells originated from pro-B cells. The immunoglobulin (Ig) loci in the NK cells did not undergo *de novo* V(D)J recombination since expressions for μ heavy as well as λ and κ light chains inside the cytoplasm were observed. In addition, over 40% of the NK cells (excluding the $CD56^{-}CD16^{+}$ phenotype) expressed intracellular IgM. Hence, other B-cell molecules began to appear during SIV infection. CD79b expression plays an important role in surface Ig expression, depending on its interaction with the Ig heavy chain and CD79 (28). Neither NK cells nor CD79b expressions were observed in the SIV-infected colon, despite the intracellular expression of IgM. The NKB cells expressed IgA, similar to gut B cells, except for the co-expression of CD56 and CD20. B cells produce IgA with J chains upon conversion to plasma cells, therefore, CD79b and Ig are crucial for NKB cell signaling pathways. The co-expression of CD79b and IgA may be important since CD79b acts as a signal transducer and Ig may trigger the production and activation of NKB cells. The interaction between CD79 and the Igα heavy chain ruled out the possibility of poly-IgA playing a role, resulting in the monomeric expression of IgA. The expression mechanism for Ig in NKB and NK cells present in infected colons, which can allow confirmation of whether the NKB cells originate from NK or pro-B cells, warrants further investigation (27). These findings suggest that the expression of CD79b in NKB cells functions differently to conventional B cells. Furthermore, affinity maturation in the germinal center (GC) and downregulation of CD79b are essential for the selection of B cells in GCs, followed by antigen presentation to T follicular helper cells.

The signature pro-inflammatory cytokine IL-18 is produced by NKB cells together with IFN-γ and TNF-α. The production of granzyme B and perforin confirmed the cytotoxic nature of NKB cells (27). Moreover, significantly increased production of granzyme A was found in NKB cells, compared with NK and $CD8^{+}$T cells. In addition, two subpopulations of granzyme A-producing NKB cells were observed, although their prevalence and phenotypic and functional characterization requires further investigation. IL-18 binds to IL-18Rβ secreted by NKB cells and facilitates both IFN-γ and TNF-α production during the pathogenesis of infectious and inflammatory diseases. The expressions for IL-7, IL-2β, and 4-1BB compared with NK cells, the increased Ki67 expression (proliferation marker) compared with $CD8^{+}$ T cells (Figure 1A), and RNA-sequencing analyses demonstrated the enhanced proliferation of NKB cells compared with NK and $CD8^{+}$ T cells. In brief, these studies presented the presence of newly identified NKB cell populations in the colons of SIV-infected macaques and other

deep-seated tissues. The phenotypic characteristics shared by these cells with NK and B cells, together with their functional properties and enhanced proliferative activity (compared with NK and $CD8^+$ T cells) indicates their important role during infectious pathologies. The origin of NKB cells, their role in SIV pathogenesis, pathways for both cytokine production and cytolytic activity, and the role of FasL on the surface of these cells (for loss of $CD4^+$ T cells – primarily Th17 cells) (Figure 1A) all require further study (27).

NKB cells in liver pathologies

Role of NKB cells in alcoholic liver disease

The studies discussed above suggest that NKB cells play a role in microbial infection and inflammation. A recent study on alcohol-induced liver disease (ALD) and intestinal damage, based on a chronic-binge alcohol abuse model, explored the therapeutic mechanisms of pre-activated (with toll-like receptor 3; TLR3) bone marrow-derived mesenchymal stem cells (P-BMMSCs) (29). Interestingly, elevated numbers of NKB cells in the spleen and significantly higher IL-18 serum levels were observed in alcohol-treated mice. These cells were shown to activate NK cells and ILC1s, leading to the aggravation of ALD. This was followed by chronic inflammation and increased lipid deposition (Figure 1C). Treatment with BMMSCs resulted in a reduction of NKB cell numbers and serum IL-18 levels (29).This confirmed the pathogenic role of NKB cells in ALD, which may be relevant to other inflammatory diseases. Additionally, P-BMMSCs indicated the involvement of TLR ligands in the immunosuppressive activities of NKB cells. However, the mechanism of action and the immunosuppressive activity of NKB cells are not fully understood. Nevertheless, NKB cells may be used to target infectious and inflammatory diseases and to develop therapeutic interventions (Figure 1).

NKB cells in hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the leading causes of mortality in cancer patients. The reasons for an impaired immunological network in HCC are not fully understood (30). IL-35 has been studied for its immunosuppressive activity towards both the hepatitis B virus (HBV) and HBV-associated HCC (31). Upregulated/activated IL-35 restricts the anti-tumor activity of $CD8^+$ T cells in the tumor microenvironment. Additionally, IL-35 produced by the regulatory T cells (T_{reg}) drives T cell exhaustion in the tumor microenvironment, as indicated by the increased expression of inhibitory receptors (PD-1, TIM-3, and LAG-3) (32). These interactions regulate the activity of Th9 cells in HCC (33). Moreover, IL-35 also regulates the tumor microenvironment in conjunction with other negative regulators [IL-18-binding protein (IL-18BP-an antagonist of IL-18)] (34, 35). This suggests that IL-35 may play an immunomodulatory role for NKB cells in HCC. This hypothesis was tested by studying both the peripheral and liver infiltrating NKB cells obtained from HCC patients (30). The number of NKB cell ($CD3^-CD19^+CD56^+NKp46^+$) populations that produced IL-18 in the peripheral and liver infiltrating sites of HCC patients were downregulated.

IL-35, IL-12, and IL-18 serum levels were quantified, revealing increased IL-35 and reduced IL-18 levels in HCC patients and confirming the regulatory role of IL-35 (30). Additionally, IL-35 serum levels were well correlated with the frequencies of peripheral NKB cells, $IL\text{-}18^+$ NKB cells, and IL-18 serum levels (Figure 1C). The effect of NKB cells on $CD8^+$ T cells was revealed using co-culture experiments (autologous $CD8^+$ T cells cultured with HepG2 cells in the presence/absence of NKB cells or recombinant human IL-18BP). The cytotoxic activity of $CD8^+$ T cells in the control group was augmented, compared with the HCC patient group. Human IL-18BP directly suppressed the NKB cell-mediated cytotoxicity of $CD8^+$ T cells. Elevated levels of IFN-γ and TNF-α were identified in the supernatant of $CD8^+$ T cells in the control group. Hence, the NKB cells promoted the cytotoxic activity of $CD8^+$ T cells *via* IL-18 signaling.

Intrahepatic lymphocytes (IHLs) were stimulated with IL-35 (1 ng/ml) for 24 h and reduced frequencies of NKB and $IL\text{-}18^+$ NKB cells were observed in both patients and healthy controls. Reduced IL-18 supernatant levels confirmed these results, and no changes were observed in IL-18BP secretion (30).The continuous secretion of IL-18BP neutralizes IL-18, decreasing its levels. Therefore, the regulatory effect of IL-35 towards NKB cells does not appear to be associated with IL-18BP. In order to study the role of IL-35 in regulating NKB cell activity, autologous $CD8^+$ T cells were co-cultured with HepG2 cells in the presence/absence of NKB cells for 12 h and the cytotoxicity of $CD8^+$ T cells was determined. The results suggest that IL-35 plays an immunoregulatory role in the NKB cells, mediating $CD8^+$ T cell cytotoxicity and inducing tumor progression. The observed immunosuppressive activity of IL-35 in HCC patients raises several questions for future studies. For example, a major focus of this study was to determine the role of IL-18 on NKB and $CD8^+$ T cells present in HCC patients, with similar levels of IL-12 observed in both HCC patients and healthy controls. However, the presence and functional activities of IL-12-producing NKB cells in the livers of both healthy individuals and HCC patients requires further *in vitro* and *in vivo* investigation.

Role of NKB cells in hepatitis B virus associated liver injury

The hepatitis B virus (HBV) is one of the etiologic factors for chronic liver disease, which can lead to acute-on-chronic liver failure (ACLF). The change from low-grade inflammation to chronic, pathogenic HBV-associated ACLF was studied (36). The authors performed assays on HBV patients to evaluate the regulatory properties of NKB cells and to determine the roles of IL-12 and IL-18 in HBV-associated liver injury. A phenotypic

characterization of different lymphocyte (B, T, NK, and NKB cells) populations was performed on blood collected from patients (36). The gating strategies utilized for B and T cells were $CD3^-CD19^+$ and $CD3^+CD19^-$, respectively, while the $CD16^+56^+$ gate within the double negative population of $CD3^-CD19^-$ cells was considered to be NK cells. NKB cells were gated and differentiated using the phenotypic markers for $CD3^-CD16^+56^+NKp46^+CD19^+$ cells. The major lymphocyte populations (primarily T, B, and NK cells) were largely unaltered in all experimental groups. However, a significant difference was observed in the peripheral NKB cells of HBV-ACLF patients, compared with chronic hepatitis B (CHB) patients, asymptomatic HBV carriers (AsC), and healthy controls (HC). A significantly elevated level of IL-12p70 was observed in the plasma of HBV-ACLF and CHB patients, compared with the AsC and the HC, whereas IL-18 levels were slightly altered in only the HBV-ACLF patients (36). The frequency of NKB cells positively correlated with IL-18 levels, but did not correlate with IL-12 levels in the HBV-ACLF surviving patients. Following therapy, a significant reduction in the number of NKB cells was observed in HBV-ACLF patients. Surprisingly, there was neither a change in IL-12p70 and IL-18 plasma levels, nor a correlation between the frequency of NKB cells and the liver injury index of HBV-ACLF patients. Therefore, the role of NKB cells in HBV-ACLF pathogenesis was not fully established.

IL-12 and IL-18, the signature cytokines of NKB cells, were used to stimulate PBMCs that were isolated from HBV-ACLF patients. Previous findings on infectious diseases and cancer (36) provided evidence for the increased frequencies of T and NK cells at two (1 and 10 ng/ml) IL-12 concentrations. No differences in the B and NKB cell frequencies were observed between unstimulated and IL-12-stimulated PBMCs (at 1 and 10 ng/ml). Nevertheless, a noticeable increase in the frequency of NKB cells in HBV-ACLF patients upon stimulation with a high concentration of IL-18 (10 ng/ml) was reported, but no changes were observed at a low concentration (1 ng/ml). Minor changes were observed in the T, B, and NK cell lineages when PBMCs were stimulated with IL-18. IL-18 was continuously produced by the NKB cells, and IL-12 production was monitored during the early phases of microbial infection (21). The HBV-X protein induced IL-18 expression in the liver which incurred hepatic damage during HBV infection. IL-12 plays a vital role in promoting central memory $CD8^+$ T cells by reversing the exhaustion of virus-specific $CD8^+$ T cells. Furthermore, *in vitro* stimulation assays using IL-12 and IL-18 suggested that IL-18 plays a direct role (positive feedback) in the signalling mechanism of NKB cells.

IL-18R signalling primarily activates two major pathways (MyD88 and STAT/MAPK) *via* NF-κB (phosphorylation). Reduced amounts of phosphorylated NF-κB p65 were observed upon stimulation with 10 ng/ml IL-18, compared with stimulation using 1 ng/ml (36). IL-18 levels were significantly reduced in the supernatant of cells stimulated with 10 ng/ml, while only a slight reduction was observed when cells were stimulated with 1 ng/ml IL-18. Thus, a lower IL-18 concentration did not allow complete neutralization of IL-18 by IL-18BP, resulting in phosphorylation of NF-κB. This directly enhanced the production of NKB cells in HBV-ACLF. The signature cytokines IL-12 and IL-18, and their role in NKB cell activation during microbial infections require further investigation. Further studies that explore the impact of NKB cells on B and T cells in different pathogenic settings may be useful in developing next-generation therapeutics.

Pathogenic role of NKB cells in periodontal infection

Detailed pathogen-host interaction studies are required to better understand the role of cytokines and chemokines in the progression of periodontitis (2). The accumulation of IL-18 has been reported in patients with acute and chronic periodontitis (Figure 1D) (37). IL-18 knock-out mice exhibited a loss in periodontal bone during periodontitis caused by *P. gingivalis* (38). The presence of NKB cells and the production of inflammatory cytokines (IL-18) have been reported in patients with periodontitis. The pathogenic nature of NKB cells was confirmed in the *P. gingivalis*-induced periodontitis murine model (37). $CD3^-CD19^+NKp46^+$ NKB periodontium cells were found to be the major source of IL-18 production in both the serum and the gingival crevicular fluid (GSF) of periodontitis patients; this was not the case in healthy individuals. No physiological changes were observed in the periodontal ligaments upon stimulation with recombinant IL-18. The neutralization of IL-18 suppressed bone loss, the infiltration of non-immune cells, and cytokine production (37). The continuous expansion of NKB cells in both the circulation and the periodontium of patients and mice with periodontitis suggests that NKB cells play a pathogenic role in intraoral infection. Following periodontal therapy, periodontal-infiltrating NKB cells were not observed, raising the possibility of a distinct lineage of lymphocytes (37). Due to the small sample sizes and the unclear sources of IL-18 secretion in these studies, the potential of NKB cells as a therapeutic regimen for periodontitis remains to be established.

Role of NKB cells in rheumatoid arthritis

Rheumatoid arthritis (RA) is a heterogeneous, systemic autoimmune disease, characterized by synovitis, progressive bone damage, loss of joint function, and extra-articular manifestations (39, 40). Genetic analyses in experimental animals with RA and clinical investigations have revealed the

genetic and environmental risk factors associated with RA and the ultimate propagation of chronic inflammation. The unregulated production of inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-6, IL-15, and IL-18) is responsible for various disease complications (41) as well as the disruption of immune homeostasis (42).

Our *in vitro* and *in vivo* findings (43, 44) suggest the therapeutic potential of combination therapy using the anti-inflammatory drugs aceclofenac (ACE) and methotrexate (MTX). The intravenous delivery of MTX *via* lipid-polymer hybrid nanoparticles (LPHNPs) (45, 46), together with the topical application of ACE using nanostructured lipid carriers (NLCs) (44, 47), suggests that the induction of apoptosis in proinflammatory RA cells was regulated by NF-κB and FOXO1 transcription factors. Therefore, MTX+ACE-loaded nanoscale carrier-based co-therapy approaches can modulate RA-induced inflammation and can induce apoptosis in pathogenic cells. Our group has been elucidating both the mechanism underlying RA pathogenesis, as well as the capability of MTX +ACE combination therapy to modulate RA-induced inflammation and to establish immune homeostasis by maintaining the immune cell Th1 phenotype.

Collagen-induced arthritis in mice possessing a humanized immune system ($CD34^+$ cells reconstituted immunodeficient (NSG) mice repopulated with a human immune system; HIS) may be employed to confirm the role of NKB cells during RA. NKB cells can be adoptively transferred to these CIA-HIS mice, followed by treatment with ACE+MTX (Figure 2A). This may subsequently be followed by immunophenotyping for Th17 (IL-6 and IL-23A) and Th1 (IFN-γ, IL-2, IL-10, and TNF-α/β) marker expressions using cells extracted from deep-seated lymphoid organs (the spleen and the bone marrow). We expect a skewed immunological balance towards an immunoregulatory phenotype (Th1), as well as modulation of RA-induced chronic inflammation. In another approach, CIA-HIS mice may receive co-therapy treatment followed by adoptive transfer of NKB cells (Figure 2B). Immune homeostasis may be analyzed to determine whether the immunological balance is tipped towards the Th1 cytokine expression profile. These proposed future studies aim to confirm whether NKB cell expansion is inhibited in the lymphoid organs of CIA-HIS mice receiving co-therapy (Figure 2B) in order to maintain the immunoregulatory phenotype (48–51).

The low expression of CD40 during stimulation fails to activate Akt (a downstream effector of PI3K-Akt signaling), thus limiting the translocation of NF-κB from the cytoplasm to the nucleus due to its intact inhibitor IκB. We believe that human THP-1 macrophages (phorbol myristate acetate (PMA)-differentiated human THP-1 monocytes) receiving co-therapy treatment are regulated by the FOXO1 transcription factor. We also believe that they mediate the pro-apoptotic protein Bim expression, which drives programmed death of LPS-stimulated human macrophages (Figure 2C).

CIA-HIS mice may prove to be a viable preclinical tool to confirm whether co-therapy can suppress the inflammation-dependent conversion of Th1 to Th17 cells (Figures 2A, B). The CIA-HIS mice that receive co-therapy may exhibit reduced inflammation controlled by expanding NKB cells, inducing programmed death of pathogenic RA cells. The maintenance of an immunoregulatory phenotype by controlling the conversion of Th1 to Th17 cells, using our proposed co-therapy regimen in CIA-HIS mice, should provide a better understanding of the role played by NKB cells during RA.

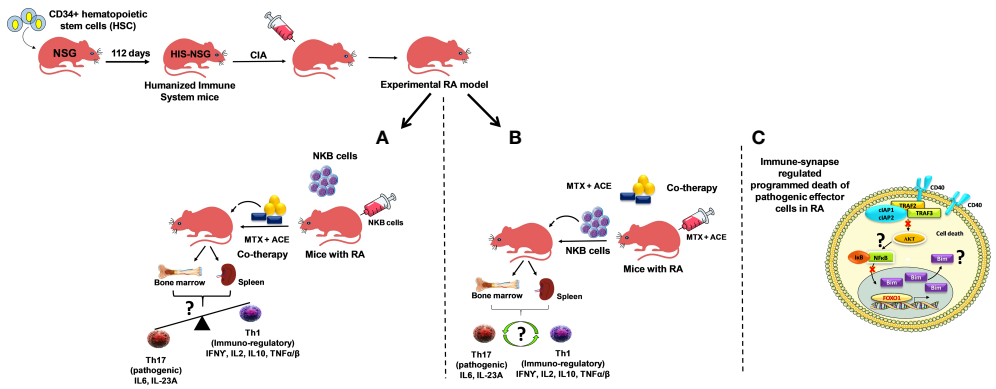

FIGURE 2
Experimental humanized immune system mice (NSG mice reconstituted with hematopoietic stem cells (HSCs); $CD34^+$ cells repopulating the human immune effectors and referred to as HIS mice). Arthritis induced by collagen treatment (collagen-induced arthritis; CIA) in HIS mice (CIA-HIS) to study the role of combined therapy in the modulation of arthritis-induced inflammation. **(A)** CIA-HIS mice may be adoptively transferred with NKB cells followed by aceclofenac- and methotrexate-based combination therapy (co-therapy) to assess the immunoregulatory (Th1) phenotype in immune cells, allowing the progression of RA to be determined, and **(B)** co-therapy treatment in CIA-HIS mice followed by adoptive transfer of NKB cells. **(C)** CD40-mediated and NF-κB-controlled downstream effectors of the PI3K-Akt pathway (Akt1) and pro-apoptotic protein (Bim) induction during the signaling pathway to drive programmed death in pathogenic effector RA cells.

Conclusions

The proposed novel immune cell lineage, namely, NKB cells, is of significant interest in the development of therapeutic interventions for infectious and inflammatory diseases. The protective role played by NKB cells during infectious diseases, and their role in maintaining immune homeostasis by promoting an immunoregulatory environment, suggests that they can critically contribute to health and disease. The maintenance of an immunoregulatory (Th1), rather than a pathogenic (Th17), immune cell phenotype in HIS mice may provide an important impetus to develop therapeutic interventions for systemic inflammatory diseases. Finally, the interactions of NKB cells with other immune cells may be further explored to expand the current knowledge on host-pathogen interactions.

Author contributions

Conceptualization: NT and RKT; resources and information collection: NT; writing—original draft preparation: NT, SN, and RKT; writing—review and editing: NT and RKT. 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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Natural killer cells in sepsis: Friends or foes?

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Sepsis is one of the major causes of death in the hospital worldwide. The pathology of sepsis is tightly associated with dysregulation of innate immune responses. The contribution of macrophages, neutrophils, and dendritic cells to sepsis is well documented, whereas the role of natural killer (NK) cells, which are critical innate lymphoid lineage cells, remains unclear. In some studies, the activation of NK cells has been reported as a risk factor leading to severe organ damage or death. In sharp contrast, some other studies revealed that triggering NK cell activity contributes to alleviating sepsis. In all, although there are several reports on NK cells in sepsis, whether they exert detrimental or protective effects remains unclear. Here, we will review the available experimental and clinical studies about the opposing roles of NK cells in sepsis, and we will discuss the prospects for NK cell-based immunotherapeutic strategies for sepsis.

KEYWORDS

natural killer cells, sepsis, immunotherapy, protective effect, detrimental effect

1 Introduction

Sepsis is a life-threatening multiple-organ dysfunction syndrome caused by localized or systemic infections, which is one of the major causes of death to patients in the hospital worldwide (1–3). It has been estimated that approximately 750,000 people suffer from sepsis every year in the United States and an estimated 20-30% patients die from it (4, 5). However, there is no specific, standardized treatment strategy for sepsis (6). Numerous studies have shown that dysregulation of innate immune responses is a major contributing factor to the incidence and development of sepsis (7, 8). For example, studies on monocytes, macrophages, neutrophils, and dendritic cells have provided insight into their roles in both the inflammatory and immunosuppressive phases of sepsis (9–14). Natural killer (NK) cells, which were discovered in the early 1970's (15, 16), are a heterogeneous group of innate lymphocytes with the capacity to regulate both innate and adaptive immune responses. They are best known for their roles in fighting infections and tumors, mainly relying on their cytotoxicity and immune regulatory properties (17).

Recent studies have implicated NK cells in the pathological process of sepsis, suggesting that they might be employed as prognostic biomarkers or therapeutic targets (2, 18).

However, seemingly contradictory conclusions about NK cells playing beneficial or harmful roles in sepsis have been obtained (19). Hence, we will review these reports to discuss whether NK cells are friends or foes in sepsis, and we will further discuss the prospects of NK cell-based immunotherapy for sepsis.

2 The immunological characteristics of sepsis

Sepsis has previously been used to describe severe disease caused by infection (20). However, this definition cannot accurately describe its complex pathological processes. Recently, a new definition has been published, stating that sepsis refers to a life-threatening, multiple-organ failure syndrome, caused by dysregulated responses to infection (21, 22). It is generally believed that immunological abnormalities are the pathological basis of sepsis (23), which is tightly associated with microvascular injury, abnormal coagulation, hemodynamic instability, multiple organ damage and other conditions (24). The immunological abnormities exhibit distinct disease stage-specific characteristics during sepsis: hyperinflammation at the initial stage and immunosuppression at the late stage (25). A diagram illustrating this process is shown in Figure 1.

After invading the body, pathogens will encounter the first line of defense composed of innate immune cells, activating PAMP (pathogen-associated molecular pattern)- or DAMP (damage-associated molecular pattern)-associated signaling pathways in these cells (26, 27). Once activated, these cells generate large amounts of inflammatory cytokines, such as IL-1β, IL-6, IL-12, TNF-α and IFN-γ

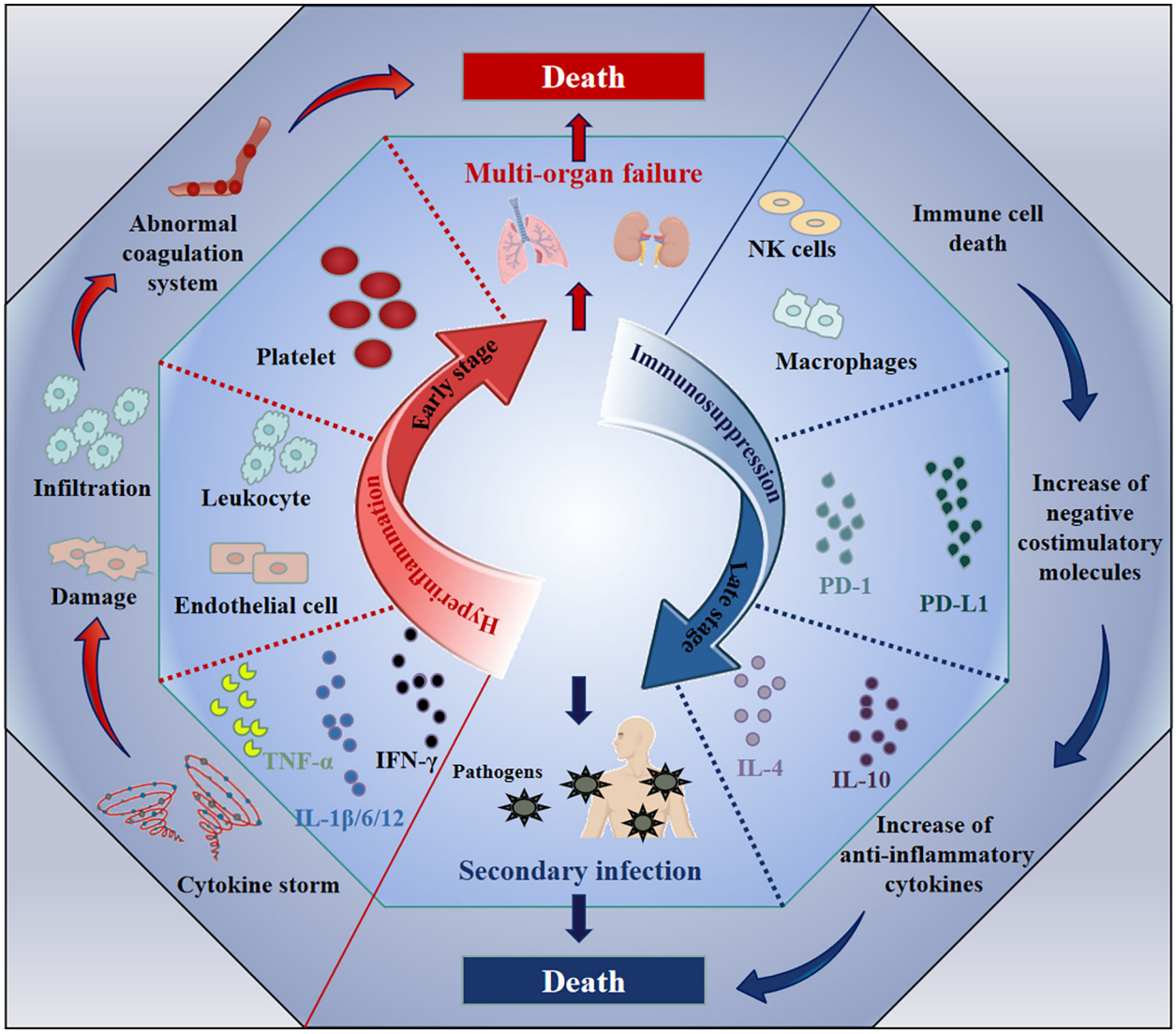

FIGURE 1
The immune changes during the pathological process of sepsis. The immunological abnormity exhibits two distinct stages accompanying with the sepsis development: hyperinflammation and immunosuppression. During the hyperinflammatory phase at early, the increase of pro-inflammatory cytokines (e.g., IL-1β/6/12, IFN-γ, and TNF-α) leads to cytokine storm, resulting in the vascular system damage (e.g., endothelial cell damage), the abnormal coagulation, finally multi-organ failure and death. Subsequently, the death of immune cells, the increase of negative costimulatory molecules (e.g., PD-1 and PD-L1) or anti-inflammatory cytokines (e.g., IL-4 and IL-10) induces immunosuppression, which leads to uncontrolled secondary infection and death.

(28, 29). These host responses are not limited to the infectious focus. The cytokines may trigger additional immune cells at distant sites to secrete inflammatory cytokines, and this cascading amplification reaction may finally result in systemically uncontrolled over-inflammation, which is termed a "cytokine storm" (30, 31). The massively increased cytokine levels may potentially enhance the elimination of pathogens by innate immune cells. However, they also lead to a series of pathological changes, such as endothelial cell damage, leukocyte infiltration, abnormal activation of the coagulation system and other abnormalities, resulting in multi-organ failure and even death (32–34). Consequently, the direct cause of death is not the invasive pathogens themselves, but the over-activated immune reactions. Therefore, the focus of clinical treatment at this inflammatory stage of sepsis is on ameliorating the uncontrolled inflammation (35).

The hyperinflammation at the early stage of sepsis will lead to immunosuppression during the late stage of sepsis: on the one hand, the cytokine storm directly induces cell death in various immune cells; on the other hand, the functions of some effector cells will be exhausted after their excessive activation (36, 37). Moreover, upregulation of some negative costimulatory molecules and anti-inflammatory cytokines has also been observed during this stage, and includes programmed cell death 1 (PD-1) (38), programmed cell death ligand 1 (PD-L1) (39), T-cell immunoglobulin and mucin domain-containing protein-3 (TIM-3) (40), T cell Ig and ITIM domain (TIGIT) (41), IL-4 (36), IL-10 (42, 43) and TGF-β (44, 45). These factors are mainly related to exhaustion of immune cells or inhibition of their effector functions (38, 46–48). As a result, the body presents with a continuously immunosuppressive state, nearly losing its capacity to clear pathogens (49). This will cause an extremely high risk for secondary infections, such as those mediated by opportunistic pathogens or iatrogenic infections caused by interventional therapy, which eventually leads to death of sepsis patients (50). For example, Huang et al. observed that the expression of TIM-3 on CD4 T cells in patients with sepsis-induced immunosuppression was significantly elevated, which impaired anti-infective responses and positively correlated with mortality (51). Hou et al. also found that, in a lipopolysaccharide (LPS)-induced murine sepsis model, TIM-3 expression on NK cells negatively regulated the production of IFN-γ, which caused death (40). Therefore, reestablishing immune functions is critical to reduce mortality risk of sepsis patients during the late immunosuppressive stage (52, 53).

3 NK cells play a role in antimicrobial responses

NK cells, a group of large granular lymphocytes derived from the bone marrow, are essential components of the innate immune response and can directly kill tumors and other target cells without prior activation (54–56). In humans, about 5-15% of lymphocytes are defined as NK cells in peripheral blood, and tissue-specific subpopulations are found in the spleen, liver, and lung (57–61). Generally, human NK cells can be divided into two subpopulations by the expression of CD56 and CD16 on the cell membrane (62, 63). About 90% of all NK cells in human peripheral blood are $CD56^{dim}CD16^{bright}$, whereas only 10% are $CD56^{bright}CD16^{-/dim}$ (64). Distinct human NK cell subpopulations found in different tissues significantly differ in cytotoxicity and cytokine secreting capacity (65, 66). The two main subpopulations possess distinct functions: $CD56^{dim}CD16^{bright}$ NK cells exhibit higher cytotoxicity and express increased levels of killer immunoglobulin-like receptors (KIR) or CD57 receptors; $CD56^{bright}CD16^{-/dim}$ NK cells can secrete more cytokines and possess greater proliferative capacity (67, 68).

NK cells can be activated in several ways. Most importantly, the balance between signals from the inhibitory or activating receptors expressed on the cell surface plays a critical role in regulating their responses (69, 70). The activating receptors mainly include NCRs (NKp30, NKp44, and NKp46), KIR-2Ds, KIR-3Ds, NKG2D, CD226, 2B4, and NKG2C, whereas the inhibitory receptors mainly include NKG2A, TIGIT, KIR-2DL, and KIR-3DL (71). The biased expression of these receptors or their ligands calibrates the activation status of NK cells. For example, a clinical study reported that, in human immunodeficiency virus (HIV)-infected patients, a subpopulation of human NK cells that expresses NKG2C but not NKG2A has a stronger ability to secrete IFN-γ compared with other NK cells (72). Another typical way of NK activation is *via* their pathogen recognition receptors (PRRs), which bind with PAMPs on bacteria (73). For example, a previous study reported that high-mobility group box-1 (HMGB-1) up-regulated the levels of TLR-2/4, which belongs to the group of classical PRRs (74), on murine NK cells, leading to their activation in rotavirus-induced murine biliary atresia (75). Additionally, NK cells can also be activated by several cytokines, including type 1 interferon, IL-2, IL-12, IL-15, IL-18, IL-21, and IL-27 (76–80). For instance, IL-12 binding to IL-12Rβ1/2 stimulates NK cells through signal transducer and activator of transcription 4 (STAT4) phosphorylation, leading to abundant IFN-γ and TNF-α production (81).

During infection, activated NK cells perform their activity mainly in two ways: cytotoxicity and immune regulation. First, NK cells can directly lyse bacteria-infected cells with their cytotoxicity: on the one hand, they can induce target cell apoptosis depending on the binding of FAS-L to FAS death receptors (82); on the other hand, they directly kill targets by secreting cytotoxic proteins, such as perforin, granzyme and α-defensins (83–85). Specifically, some studies have reported that these cytotoxic proteins could disrupt the membrane of some bacteria, such as *Mycobacterium, Salmonella typhimurium*, *Bacillus anthracis*, *Escherichia coli*, and *Staphylococcus aureus* (86–89), thus causing their death. In addition to cytotoxicity, activated NK cells also secrete several cytokines to undertake the roles of immune regulation (90). IFN-γ, which is the major cytokine released by NK cells, was reported to play a critical role in fighting microbial infections (91). It modulates the activation of other immune cells, such as macrophages or dendritic cells, enabling them to perform comprehensive anti-bacterial responses (92, 93). Moreover, IL-32, previously named as NK cell transcript 4 (NK4), can be produced by NK cells when activated by IL-2 (94, 95). It also stimulates inflammatory responses by inducing monocytes or macrophages to secrete various cytokines, including TNF-α, IL-1β, IL-6 or IL-8 (96). Thus, IL-32 has been

reported to exacerbate sepsis in the cecal-ligation and puncture (CLP) mouse model, *via* propagating vascular inflammation (97).

In addition to their positive regulatory roles, NK cells also possess the ability to limit antimicrobial responses. A recent study uncovered that NK cell-derived IFN-γ worsened macrophage phagocytosis of zymosan in mice and increased the susceptibility to secondary *Candida* infection during post-sepsis immunosuppression (98). However, whether this phenomenon exists in sepsis caused by other pathogens needs further study. Furthermore, activated NK cells also secrete IL-10, which is a well-known immunosuppressive cytokine (99–101). In fact, NK cells are the main source of IL-10 in systemic infection caused by some pathogens, such as *Yersinia pestis*, *Listeria monocytogenes* or *Toxoplasma gondii* (99). Interestingly, the NK cell-derived IL-10 appears to play dual roles in different types of infections. For example, in *Listeria monocytogenes* infection, the NK cell-derived IL-10 shows detrimental effects on host resistance against the invasive pathogen (102), whereas it can protect the host from murine cytomegalovirus infection or CLP-induced sepsis by reducing systemic inflammation (103, 104). The authors consider that the beneficial or detrimental roles of IL-10 might depend on whether the major cause of host death is pathogen overload or excessive inflammation during infection.

Summarily, the patterns of NK cell activation and their roles in antimicrobial responses are illustrated in Figure 2.

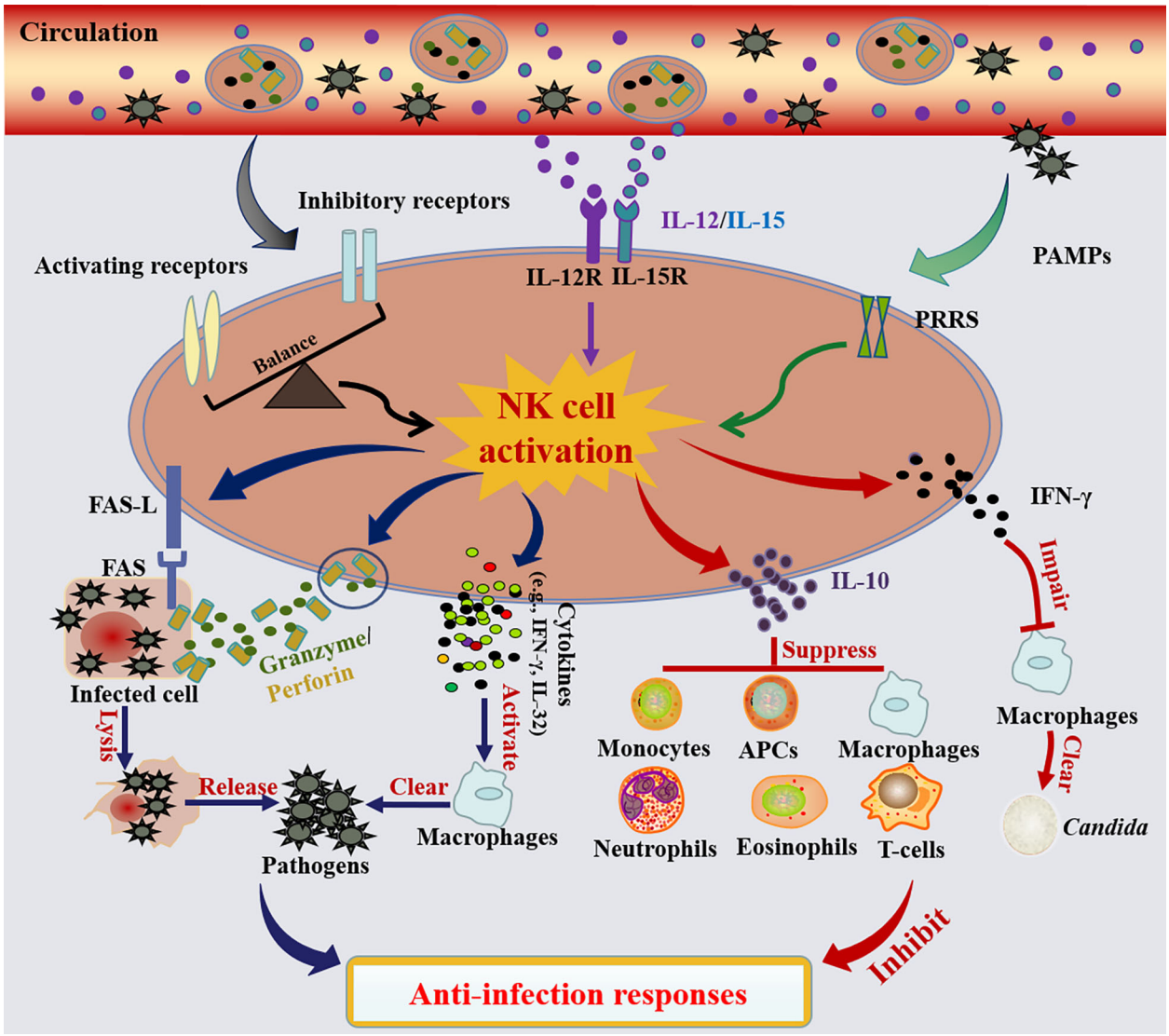

FIGURE 2
NK cell activation and their roles in the anti-infection responses. NK cells are mainly activated in three ways: 1) The activation of NK cells is governed by a balance between signals delivered through activated and inhibitory receptors. When the activating signal dominates, NK cells will be activated, and vice versa. 2) Activation of NK cells can also be achieved by stimulation with cytokines (e.g., IL-12 and IL-15). 3) NK cells are activated by pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs). Activated NK cells lysis infected cells and release pathogens *via* death receptor ligand/death receptor (e.g., FAS-L/FAS) and secreting cytotoxic proteins (e.g., perforin and granzyme). Meanwhile, activated NK cells promote the activation of macrophage-mediated microbial killing by the secretion of cytokines (e.g., IFN-γ, IL-32). In contrast, activated NK cells also possess the ability to limit the anti-infection responses. On one hand, NK cell-derived IFN-γ especially worsened macrophage phagocytosis of zymosan.; on the other hand, the activated NK cells also secrete IL-10, which can generally inhibit the anti-infection responses of monocytes, antigen-presenting cells (APCs), macrophages, neutrophils, eosinophils or T cells.

4 NK cells act as risk factors in sepsis

Accumulating studies have shown that NK cells play a contributing role in the inflammatory responses caused by infection (105, 106). In this context, they are considered a risk factor for aggravating the septic process during the hyperinflammation stage (107). At the early stage of sepsis, NK cells will be activated through the ways discussed above, secreting abundant cytokines, such as IFN-γ, TNF-α or IL-32, which can trigger dramatic responses in macrophages or dendritic cells (54, 96). Mutually, the activated macrophages and dendritic cells secrete IL-2, IL-12 or IL-18 to subsequently further activate NK cells, forming a positive feedback loop (108, 109). This loop amplifies the pro-inflammatory responses, resulting in a cytokine storm and finally causing multiple organ failure (54). In addition, the cytotoxic proteins secreted from activated NK cells, including perforin and granzyme, are also reported to directly mediate tissue necrosis and damage (54) (Figure 3). Therefore, several studies have shown that antagonizing murine NK cells during sepsis significantly ameliorates multiorgan damage caused by inflammation and enhanced tolerance in mice. For example, in sepsis mouse models caused by CLP surgery, *Streptococcus pneumoniae*, *Escherichia coli* or *Streptococcus pyogenes* infection, NK cell clearance using anti-asialoGM1 and anti-NK1.1 antibodies can reduce systemic inflammation, stabilize acid-base balance in the circulation, improve organ damage, reverse physiological disorders and prolong overall survival (110–116). Moreover, in a murine polytrauma model, which is a major instigator of sepsis, murine NK cell depletion also attenuated inflammatory responses and improved the outcomes (117).

IL-15 is an essential cytokine to maintain NK cell development and maturation, which can also strongly activate NK cells at high concentrations (118). It has been reported that excessive IL-15 stimulation leads to pathological inflammatory responses similar to sepsis, resulting in the death of mice due to massive NK cell proliferation and IFN-γ production (119). Furthermore, IL-15 knockout mice, characterized by NK cell loss, also showed

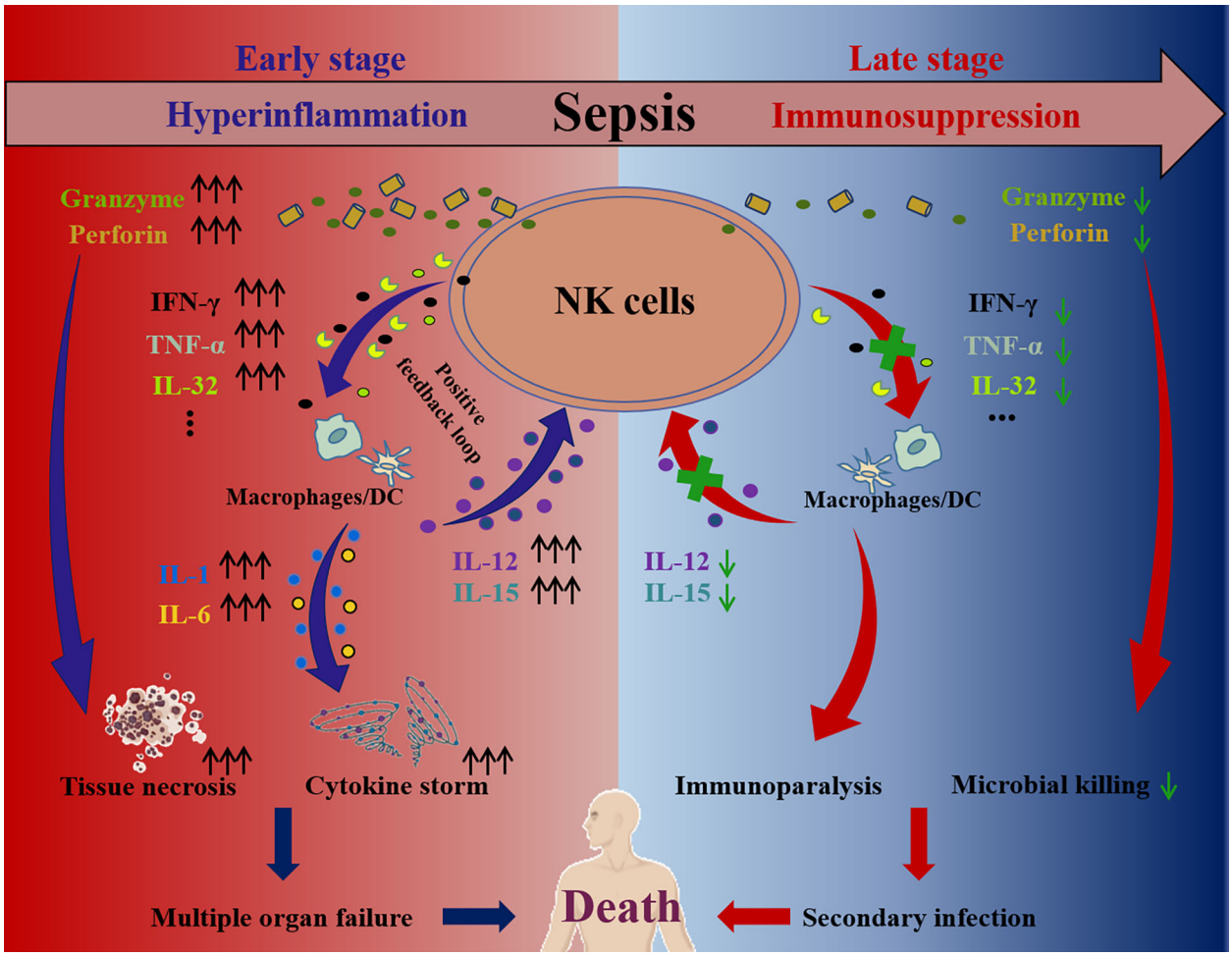

FIGURE 3
The pathological roles of NK cell at the hyperinflammation and immunosuppression stage of sepsis. During sepsis hyperinflammation, NK cells activation is dysregulated and NK cells secrete abundant cytokines, including IFN-γ, TNF-α, IL-32 and so on. These cytokines subsequently facilitate secretion of more cytokines (e.g., IL-12, IL-15, IL-1, IL-6, and so on) by dendritic cells and macrophages, establishing a positive feedback loop and amplifying cytokine storm. Furthermore, cytotoxic proteins (e.g., perforin, granzyme) secreted by NK cells are increased and cause tissue necrosis. As a result, the cytokine storm and tissue necrosis eventually lead to multiple organ failure and death. In contrast, the secretions of cytokines (e.g., IFN-γ, TNF-α, IL-32, and so on) and cytotoxic proteins (e.g., perforin, granzyme) of NK cells are impaired at the immunosuppression stage of sepsis, which contributes to the immunoparalysis, causing secondary infection and even death.

tolerance to sepsis due to CLP surgery (120). When bacterial infection occurs, NK cells may rapidly migrate to the infection site and promote inflammation (121, 122). It has been reported that murine NK cells expressing CXCR3 can rapidly migrate to the abdominal cavity within 4-6 h following severe abdominal infection (123). These CXCR3-positive NK cells are similar to the human $CD56^{bright}$ subpopulation in their ability to secrete more proinflammatory cytokines and express more activation makers (124). Blocking CXCR3 or its ligand, CXCL10, can significantly reduce inflammation during sepsis in mice and increase their survival rate (125). In addition to the organ damage caused by massive inflammatory cytokine secretion, NK cell-mediated cytotoxicity is also detrimental in sepsis. For example, mice deficient in perforin or in granzymes A/M exhibit increased tolerance to sepsis caused by LPS (126).

Additionally, significant changes in the number, phenotypes, and functions of NK cells in sepsis patients have been observed in several clinical studies. David Andaluz-Ojeda et al. showed that NK cell levels were significantly increased in patients who died from sepsis and the cell counts at day 1 were independently associated with increased risk of death at 28 days (hazard ratio = 3.34, 95% CI = 1.29 to 8.64; $P = 0.013$). Analysis of survival curves provided evidence that human NK cell levels at day 1 (> 83 cells/mm^3) were associated with early mortality (127). Palo et al. also found that sepsis patients with the highest NK cell numbers exhibit the lowest survival probability (128).

In all, during the hyperinflammation stage, the disturbance of inflammatory factors leads to abnormal NK cell activation, which can trigger a cytokine storm through a positive feedback loop, resulting in severe organ damage (92, 109). Thus, neutralizing or inhibiting NK cell-derived pro-inflammatory cytokines (e.g., IFN-γ) or cytotoxic proteins (e.g., perforin, granzyme) can alleviate systemic inflammatory responses and protect against organ damage. Furthermore, using anti-inflammatory cytokines, such as IL-10, to treat sepsis is also worth considering. We have summarized the evidence showing the detrimental roles of NK cells from both animal and human sepsis in Table 1. These findings implicate NK cells as risk factors during sepsis.

5 The protective roles of NK cells in sepsis

Conversely, some other studies have provided evidence for a protective role of NK cells in a variety of microbial infections. For instance, murine NK cells are essential in coordinating host responses against sepsis caused by *Staphylococcus aureus* infection (129, 130). This may be due to their interactions with the anti-inflammatory mechanisms of the host. Moreover, once the ability of NK cells to secrete IFN-γ is impaired, progressive immune disorders might be induced. There is evidence showing that neutralization of IL-10 with antibodies in mice improves the ability of NK cells to secrete IFN-γ, resulting in improved survival (131). Notably, in the *Citrobacter rodentium* infection model, murine NK cells not only directly lyse the bacteria but also recruit other intrinsic immune cells and activate their antibacterial functions by secreting cytokines (132). Similarly, during *Pseudomonas aeruginosa* infection, NK cells can recruit neutrophils to the lungs, alleviating infection and improving animal survival (133). In mice infected with pulmonary nontuberculous mycobacteria, the bacterial load and mortality rate are increased by NK cell clearance (134). Interestingly, it has also been reported that IL-15 treatment after CLP surgery can reduce immune cell apoptosis, improve immune disorders, and increase mouse survival (135, 136).

A protective role of NK cells in sepsis has also been documented in several clinical studies. Some researchers reported a significant increase in the number of human peripheral blood NK cells, their expression of active biomarkers, and their ability to secrete granzyme A/B, IFN-γ or IL-12P40 (117, 137–139), which were considered to provide a survival benefit for septic patients. Bourboulis et al. showed that sepsis patients with increased levels of NK cells (>20% of all

TABLE 1 Summary of the detrimental roles of NK cells in sepsis.

Disease	Animal/ Human	Supporting evidence	Reference
CLP	Animal	Using anti-asialoGM1 and anti-NK1.1 antibodies to clear NK cells *in vivo* enhanced tolerance in mice	(110–113)
E. coli infection	Animal	NK cell-depleted and NK cell-deficient mice exhibited 80% survival after *E. coli* infection, whereas control mice all died within 12 h.	(114)
S. pyogenes infection	Animal	NK cell-deficient mutant mice were more resistant to *S. pyogenes* than control mice	(115)
S. pneumonia infection	Animal	NK depletion by antibodies reduced systemic inflammation, stabilized acid-base balance in circulation, and significantly improved the survival of mice	(116)
Murine polytrauma	Animal	Depleting NK cells resulted in attenuated inflammatory responses and an overall improvement in outcome	(117)
CLP	Animal	IL-15-deficient mice (lacking NK cells) exhibited improved survival, attenuated hypothermia, and reduced proinflammatory cytokine production during sepsis	(120)
Patients within the first 1 d, 3 d, 10 d of sepsis (50 patients)	Human	Analysis of survival curves provided evidence that NK cell levels at day 1 (> 83 cells/mm^3) were associated with early mortality	(127)
Patients with sepsis during the first 28 d in the ICU (52 patients)	Human	Patients with the highest NK cell number may have the lowest probability to survive	(128)

CLP, Cecal-ligation and puncture; E. coli, Escherichia coli; S. pyogenes, Streptococcus pyogenes; S. pneumonia, Streptococcus pneumoniae.

lymphocytes) survived longer than those patients with lower levels of NK cells (< or =20% of all lymphocytes) (140). Boomer et al. reported that NK cells in peripheral blood of sepsis patients were significantly reduced within 24 h, which may predispose some patients to nosocomial infections and poor outcomes (141). Consistently, Holub et al. found that human NK cells were decreased within the first 48 h of sepsis, especially in patients with Gram-negative bacterial infection, resulting in increased risk of septic complications (142). Moreover, single-cell RNA-sequencing (scRNA-seq) analysis revealed that various cytotoxic genes of NK cells were downregulated in patients with late sepsis (n=4), which might be associated with the re-occurrence of severe infections (143).

Under the conditions described in this section, replenishing subjects with functional NK cells may hinder the immunosuppressive stage of sepsis. Furthermore, blocking inhibitory receptors, activating NK cells by cytokines (e.g., IL-15, IL-2) or neutralizing suppressive cytokines (e.g., IL-4, IL-10) may also be beneficial. In summary, the evidence supporting the protective roles of NK cells in both animal and clinical studies are shown in Table 2.

Taken together, the roles of NK cells in sepsis remain controversial. Furthermore, animal and clinical studies have revealed dual roles of NK cell activity on sepsis progression. The impact on disease mainly depends on the pathological stage and the initial infection focus. Although the functional changes of NK cells and their influence on pathological progresses have been explored in previous studies, they mainly focused on the early stages after sepsis. During the sepsis process lasting several months from occurrence to recovery, the impact of continuous changes in NK cell numbers and characteristics remains unclear.

6 NK cells in COVID-19 infection

In late 2019, coronavirus disease 2019 (COVID-19) emerged and rapidly spread throughout the world (144, 145). As of December 2022, the COVID-19 pandemic has resulted in approximately 641,915,931 confirmed cases, including 6,622,760 deaths worldwide (https://covid19.who.int/). A meta-analysis revealed that the overall pooled sepsis prevalence estimates among 218,184 COVID-19 patients, irrespective of ICU or non-ICU admission, were 51.6% (95% CI, 47.6-55.5, I^2 = 100%) (146). Sepsis was one of the major causes of death for COVID-19 patients. During acute COVID-19 infection, the number of the $CD56^{bright}$ and $CD56^{dim}$ human NK cells dropped dramatically in the circulation (147, 148). However, this drop was likely related to the homing of human NK cells from the circulation to the lung because NK cells were increased in bronchoalveolar lavage (BAL) (149, 150). Moreover, a clinical trial discovered that a high frequency of NK cells was significantly associated with asymptomatic COVID-19 infection (151). In addition to lower circulating counts, NK cell dysfunction was also observed. NK cell hyperactivation driven by IL-6, IL-15 and IL-18 has been considered as one of the features of COVID-19 (152–154). Furthermore, Maucourant et al. used high-dimensional flow cytometry to reveal that NK cells in COVID-19 patients were at a higher activation state containing high levels of cytotoxic proteins, such as perforin (155). However, prolonged hyperactivation usually leads to impaired NK cell function. Yao et al. reported that genes involved in NK cell cytotoxicity were suppressed in severely ill COVID-19 patients (156). Moreover, some studies also reported that NK cell activity was impaired *via* over expression of the inhibitory receptor NKG2A in COVID-19 patients (157, 158).

Due to their lower circulating counts and dysfunction, NK cell adoptive transfer or reconstitution could be a possible treatment for COVID-19 patients. In fact, some innovate clinical trials using human NK cells to treat COVID-19 patients are active (ClinicalTrials.gov# NCT04280224, NCT04578210). Additionally, a clinical trial to determine the safety and efficacy of NK cells derived from human placental hematopoietic stem cells in patients with moderate COVID-19 is also ongoing (ClinicalTrials.gov# NCT04365101). Finally, an NKG2D chimeric antigen receptor (CAR)-NK cell-based trial may provide a safe and effective cell therapy for COVID-19 (ClinicalTrials.gov# NCT04324996). These studies are summarized in Table 3.

TABLE 2 Summary of the protective roles of NK cells in sepsis.

Disease	Animal/ Human	Supporting evidence	Reference
S. aureus infection	Animal	NK cell-depleted mice (using anti-NK1.1 antibodies) developed more frequent and severe arthritis	(129, 130)
C. rodentium infection	Animal	Depletion of NK cells led to higher bacterial load and developed disseminated systemic infection, associated with reduced immune cell recruitment and lower cytokines	(132)
P. aeruginosa infection	Animal	NK cells can recruit neutrophils to the lungs, alleviate infection and improve the survival of mice	(133)
NTM infection	Animal	NK1.1 cell depletion increased bacterial load and mortality in mouse model	(134)
Patients within 12 h of the advent of severe sepsis (49 patients)	Human	An increase in circulating NK cells increased the survival rate of patients	(140)
Patients within 24 h of the onset of sepsis (24 patients)	Human	The number of NK cells in the blood of patients was decreased, which may be necessary for predisposing some patients to nosocomial infection and poor outcome	(141)
Patients within 48 h of sepsis (40 patients)	Human	NK cells numbers steadily decreased within 48 hours after admission, associated with an increased risk of septic complications	(142)
Patients with sepsis during 14-21 d (4 patients)	Human	Various cytotoxic genes of NK cells were downregulated in patients with late sepsis, which might be associated with the re-occurrence of severe infections	(143)

S. aureus, Staphylococcus aureus; C. rodentium, Citrobacter rodentium; P. aeruginosa, Pseudomonas aeruginosa; NTM, Nontuberculous mycobacteria.-

TABLE 3 Summary of the clinical trials on NK cell-based immunotherapy.

Disease type	Patient number	Cell source	Supporting evidence	Phase	Reference or identifier
COVID-19	30	–	–	I (recruiting)	NCT04280224
COVID-19	58	Allogeneic	–	I/II (recruiting)	NCT04578210
COVID-19	86	Human placental hematopoietic stem cell	–	I/II (Active, not recruiting)	NCT04365101
COVID-19	90	CAR	–	I/II (recruiting)	NCT04324996
AML	21	Haploidentical	All patients but 1 had absolute neutrophil and platelet count recovery within 45 d after NK cell infusion	II (completed)	(159)
AML	10	UCB	*In vivo*, hematopoietic stem and progenitor cell-NK cell maturation was observed, indicated by the rapid acquisition of CD16 and most activating receptors	–	(160)
NHL	16	Haploidentical	Three responding patients with extensive bulky disease had robust tumor regressions	II (completed)	(161)
Neuroblastoma	35	Haploidentical	Ten of thirty-five patients had complete or partial responses and had improved progression free survival	I (completed)	(162)
MM	8	Allogeneic	After fresh NK cell infusion, dramatic *in vivo* expansion was observed and circulating NK cells retained the ability to kill myeloma cells	–	(163)
NHL and CLL	11	CAR	8 patients had an objective response, including 7 patients who had a complete response	I/II (Active, not recruiting)	(164)
Ovarian carcinomas	12	UCB	–	I (recruiting)	NCT03539406
Hematological cancer	37	iPSCs	–	I (Active, not recruiting)	NCT03841110
B cell lymphoma	234	iPSCs	–	I (recruiting)	NCT04023071
Glioblastoma	42	CAR	–	I (recruiting)	NCT03383978
HIV	9	Haploidentical	–	I (completed)	NCT03899480
HIV	4	Haploidentical	–	I (completed)	NCT03346499

Identifier from ClinicalTrials.gov. COVID-19, Coronavirus disease 2019; AML, Acute myeloid leukemia; NHL, Non-Hodgkin lymphoma; MM, Multiple myeloma; CLL, Chronic lymphocytic leukemia; HIV, Human immunodeficiency virus; CAR, Chimeric antigen receptors; UCB, Umbilical cord blood; iPSCs, Induced pluripotent stem cells.

7 The prospects of NK cell-based immunotherapy for sepsis

Recently, NK cells have gained great attention in the field of immunotherapy, especially in cancer treatment. The anti-tumor activities of infused NK cells have been demonstrated widely in mouse models of glioblastoma, ovarian cancer, and metastatic colorectal cancer (165–167). For example, Veluchamy et al. showed that adoptive transfer of NK cells into mice with metastatic colorectal cancer inhibited tumor growth *in vivo* and prolonged survival time (168). There has an explosion of NK cell-based cancer immunotherapies in clinical trials on acute myeloid leukemia (AML), non-Hodgkin lymphoma (NHL), neuroblastoma, multiple myeloma (MM) and other cancers (159–164). In addition, a few clinical trials using NK cells to treat patients with ovarian carcinomas, hematological cancer, B cell lymphoma, and glioblastoma are ongoing (ClinicalTrials.gov# NCT03539406, NCT03841110, NCT04023071, NCT03383978). We have summarized these completed and ongoing clinical trials in Table 3. Recently, a variety of NK cell-based immunotherapies were developed to treat viral infections such as COVID-19 (as discussed above) and HIV (ClinicalTrials.gov# NCT03899480, NCT03346499). Although these treatments have not yet achieved the same degree of success as clinical T cell-based therapies, the abundant pre-clinical or clinical studies with NK cell-based immunotherapies have led to increasing enthusiasm in exploring their potential to treat other diseases, including sepsis.

A variety of tissue sources for deriving NK cells for immunotherapy have been developed, including autologous and

allogeneic NK cells (169). Autologous NK cell infusion using the patient's own blood as a source was the first focus in adoptive NK cell therapy, which is associated with low risk of graft-versus-host disease (169). However, this approach usually leads to exhausted NK cell functions (170). Furthermore, patients must receive an extensive preparative treatment regimen before infusion, which may cause serious negative side effects (171). For allogeneic NK cells, the requirement for a healthy donor as source of NK cells and expanding them to clinically relevant doses is the most critical step (172). Therefore, umbilical cord blood (UCB) (173) and induced pluripotent stem cells (iPSCs) have been considered as optimal sources (174). UCB NK cells are younger and more proliferative (175), can be manufactured at multiple doses (176), and possess high cytotoxicity to lyse target cells (177). However, UCB NK cells are relatively unstable due to common delays in blood collection and heterogeneity of leukocytes from different donors (169). Stem cells represent a potentially unlimited source of NK cells for adoptive immunotherapy, and iPSCs provide a universal cell source (174). NK cells derived from iPSCs can be genetically modified and expanded to a homogenous population on a large scale (178). Furthermore, NK cells derived from iPSCs display increased cytotoxicity and greater antitumor activity than UCB NK cells in models of leukemia (179). However, more efficient strategies to generate NK cells from iPSCs are still needed.

As discussed above, NK cells significantly impact the pathological progression of sepsis. We postulate that NK cell-based immunotherapies may be developed as an excellent therapeutic option for sepsis, for the following reasons: 1. The adoptive transfer of NK cells has been proven safe due to their short lifespan and the low risk of triggering graft-versus-host reactions (180, 181); 2. NK cells can kill targets without sensitization; therefore, developing NK cells as "off-the-shelf" products has recently attracted great attention in the field (182), which can overcome the challenging problem of the narrow time window available for sepsis treatment; 3. The pathological process of sepsis is characterized by distinct stages of hyperinflammation and immunosuppression, and NK cells also have dual roles in immune regulation. Therefore, we may envisage an "off-the-shelf" NK cell product developed from editable iPSC-NK cells, which can sense its immune microenvironment to program opposing activities: in a hyperinflammatory environment, these NK cells may be programmed to mainly exert anti-inflammatory properties, whereas in an immunosuppressive environment, they are programmed to promote immune activation. Although few studies on NK cell-based immunotherapies for sepsis have been performed, inspired by explorations on cancer and viral infection and with the expanded knowledge on mechanisms of NK cell responses in sepsis, we can make the bold prediction that the future of NK cell-based immunotherapy for sepsis is bright.

In conclusion, developing NK cell-targeted immunotherapeutic strategies for sepsis highly depends on the disease state. A dynamic and more comprehensive understanding of the pathological process of sepsis will be critically important. Therefore, we consider using high-throughput sequencing technologies to dynamically monitor NK cell alterations during the early, middle, and late stages of sepsis essential for an accurate and deep understanding of NK cells in sepsis. Hopefully, with the growing understanding about NK cells in sepsis, safer and more efficient immunotherapies for sepsis can be developed.

Author contributions

The work presented was performed in collaboration by all authors. FW and YC designed and wrote the manuscript. DH revised the manuscript. LG improved the language. HL devised the concept and revised the paper. 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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Controversial role of ILC3s in intestinal diseases: A novelty perspective on immunotherapy

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ILC3s have been identified as crucial immune regulators that play a role in maintaining host homeostasis and modulating the antitumor response. Emerging evidence supports the idea that LTi cells play an important role in initiating lymphoid tissue development, while other ILC3s can promote host defense and orchestrate adaptive immunity, mainly through the secretion of specific cytokines and crosstalk with other immune cells or tissues. Additionally, dysregulation of ILC3-mediated overexpression of cytokines, changes in subset abundance, and conversion toward other ILC subsets are closely linked with the occurrence of tumors and inflammatory diseases. Regulation of ILC3 cytokines, ILC conversion and LTi-induced TLSs may be a novel strategy for treating tumors and intestinal or extraintestinal inflammatory diseases. Herein, we discuss the development of ILCs, the biology of ILC3s, ILC plasticity, the correlation of ILC3s and adaptive immunity, crosstalk with the intestinal microenvironment, controversial roles of ILC3s in intestinal diseases and potential applications for treatment.

KEYWORDS

innate lymphocyte cell, plasticity, secondary lymphoid organ, tertiary lymphoid tissue, transcription factor, intestine homeostasis, inflammatory disease, cancer

Abbreviations: ILCs, innate lymphoid cells; LTi, lymphoid tissue inducer; DC, dendritic cell; RORγt, Retinoic acid receptor-related orphan receptor γ T; retinoic acid, RA; IBD, inflammatory bowel disease; CRC, colorectal cancer; RBP-Jκ, recombining binding protein suppressor of hairless κ; SCA-1, stem cell antigen 1; ILCP, ILC progenitors; LTiP, LTi progenitors; TLS, tertiary lymphoid structure; TF, transcription factor; SLO, secondary lymphoid structure; HEVs, high endothelial venules; LTo, lymphoid tissue organizer; CP, Cryptopatche; ILF, isolated lymphoid follicle; PP, Peyer's Patche; GPR183, G-protein-coupled receptor 183, AMD antimicrobial peptides; ISL, intestinal stem cell; SFB, segmented filamentous bacteria; SCFAs short chain fatty acids; UC, ulcerative colitis; CD, Crohn's disease; GPCRs, G-protein-coupled receptors; TNF, anti-tumor necrosis factor; XRE, canonical xenobiotic responsive; HIF, hypoxia-inducible transcription factors; BATF, Basic leucine zipper ATF-like transcription factor.

1 Introduction

Innate lymphoid cells (ILCs) are lymphoid-derived innate cells that play a critical role in host defense and can be divided into the following four subsets: NK cells, ILC1s, ILC2s, and ILC3s (including lymphoid tissue inducer (LTi) or LTi-like cells) based on their functions, cytokine profile, and transcription factor (TF) expression during ILC development (1, 2).

ILC3s include three distinct lineages, NCR+ ILC3s (NKp46+ in mice and NKp44+ in humans), NCR- ILC3s and LTi/LTi-like cells; ILC3s primarily reside in the mucosa of the gastrointestinal tract, where they mediate the development of lymphoid tissue and mucosal protection (3–5). LTi cells can express the ILC3-specific TF RORγt and produce ILC3-specific cytokines, but they develop from different progenitor lymphatic tissue inducer precursors, while all other ILCs develop from precursors of innate lymphoid cells (6). LTi cells develop embryonically and initiate the formation of secondary lymphoid organs via LTβR signaling, while the development of LTi-like cells is postnatal and cannot induce the formation of secondary lymphoid organs (7). ILC3s are crucial in response to bacterial infection in the gut, especially for Citrobacter rodentium (8, 9). Once gut immune cells sense bacterial antigens, tissue-resident dendritic cells (DCs) and mononuclear cells produce numerous IL-23 and IL-1β-stimulating ILC3s to produce IL-17 and IL-22 to maintain intestinal homeostasis (4, 10–12). Furthermore, commensal flora influences the functional characteristics of intestinal NKp46+ cells. The levels of NKp46+ RORγt+ ILC3s are significantly decreased in germ-free mice, indicating that microenvironmental factors mediate these distinct effector cells in the gut, and commensal organisms influence gut immunity via a variety of sophisticated methods (13, 14). The development and functions of NCR+ ILC3s are largely dependent on RORγt and IL-7Rα (15–17). In addition, Notch acted on NCR- precursors. The Notch intracellular domain translocates into the nucleus, where it binds to recombining binding protein suppressor of hairless κ (RBP-Jκ), eliciting the expression of RORγt, AHR, and T-bet; thus, Notch is also an important signal for the generation of the NCR+ ILC3 population (18). ILC3s and LTi cells are the first line of defense in the response against pathogens. Thus, the deficit or overactivation of their functions will result in gastrointestinal disorders such as bacterial infection, inflammatory bowel disease (IBD), and colorectal cancer (CRC) (19).

Recent studies have found that ILC3s and LTi cells are emerging as an essential innate lymphocyte population for intestinal infection and respond distinctly to different intestinal diseases (20). The balance of ILC3s and LTi cells ensures host homeostasis, and their regulation may contribute to the alleviation of both tumor and inflammatory diseases. Here, we focus on recent encouraging findings in the field of ILC3s and highlight the biological mechanisms of ILC3s in intestinal diseases.

2 Main transcription factors mediating ILC3s and LTi/LTi-like cells

2.1 AHR

ILC3s express the specific TF aryl hydrocarbon receptor (AHR) (16, 21). AHR is a cytosolic sensor of small polycyclic aromatic compounds and can regulate Notch signaling in the formation of isolated lymphoid follicles (ILFs). It has been observed that AHR overexpression in NK cells could increase Notch2 expression, which suggests that AHR could modulate the Notch pathway (22, 23). TCDD, an AHR ligand, has also been found to induce the expression of Notch transcripts in the gut through an AHR-dependent pathway. AHR-/- mice exhibit significant RAR-related orphan receptor γt+ (RORγt) ILC deficiency, leading to a decrease in IL-22 production and poor protection against intestinal bacterial infections (21). Similarly, in a mouse model that lacks the expression of RBP-Jk, which regulates the output of Notch signaling, the frequency of RORγt+ ILCs was notably reduced, supporting the idea that Notch signaling regulates RORγt+ ILCs. Interestingly, increased apoptosis in RORγt+ ILCs is observed in the intestine of adult AHR-/- mice but not in that of AHR-/- fetal mice (21).

Similarly, AHR-/- mice lack postnatally imprinted Cryptopatche (CP) and ILFs but not embryonically imprinted Peyer's Patche (PP), indicating that RORγt+ LTi cells play an important role in the generation of postnatal intestinal lymphoid tissues and that heterogeneity in cell types plays a role in the organogenesis of lymphoid tissue. The effects of AHR in inducing lymphoid organogenesis are supposed to be related to its role in facilitating LTi cell development, as LTi cells can recruit stromal cells and other lymphoid cells to form the lymphoid structure. Moreover, the receptor tyrosine kinase c-kit has also been identified as a downstream target of AHR. AHR interacts with the canonical xenobiotic responsive (XRE) element on c-kit, inducing the transcription of c-kit. Recent studies have proven that c-kit can also regulate the frequency of postnatal intestinal RORγt+ ILCs and lymphoid organogenesis. In mice that express a receptor with impaired kinase activity, RORγt+ ILC frequency diminished considerably, and the number of CPs and ILFs decreased as well (24). These results suggest that AHR is important for the maintenance and activity of postnatal RORγt- ILCs, thereby mediating lymphoid organogenesis.

In addition, AHR activated by its ligands may result in Ncr1 fate-mapped ILC3s expressing higher levels of CD117 and IL-22, leading to better protection against pathogens (25). Compared to wild-type ILCs, AHR-deficient ILCs produce less IL-22, which is closely associated with a high bacteria load (26). It has also been found that AHR ligands that drive ILC3s are mostly endogenous ligands, such as Kynurenine (tryptophan catabolite), and not natural diet ligands (21, 27). These results suggest that intestinal commensal bacteria that participate in the synthesis of endogenous AHR ligands could play an important role in the AHR-Notch pathway and influence the generation of ILCs and postnatal lymphoid tissue.

2.2 ROR

RORγt, with 495 amino acids encoded by the Rorc gene (28), is mainly expressed in TH17 cells and thymocytes (28). RORγt is the key TF for ILC3s and LTi cells, regulating their development (29). The formation of ILC3s in RORγt-knockout mice was completely suspended, suggesting the paramount role of RORγt in ILC3 formation, but the precise regulatory mechanisms are still unclear (30). Recent studies have shown that ILCs do not require RORγt and RORα, another crucial ROR TF, for survival, but the continuous expression of RORγt and RORα is closely associated with their metabolism, proliferation and functions (31). When RORγt and RORα expression is lacking, the expression of crucial metabolism regulators such as Arg-1 diminishes notably, and thus, the cells lose their phenotype and functions. Meanwhile, RORγt-RORα- T-bet+ NCR+ ILC3s also convert to ILC1s (31). The expression of RORγt is a potential signature to recognize IL-17- and IL-22-producing cells in both adaptive and innate immune responses. RORγt is crucial in controlling ILC3s producing IL-17 and IL-22 and promoting the development of LTi cells (12). LTi cells constitutively express RORγt, and IL-23 can upregulate the expression of RORγt (12, 21). In addition, current studies have confirmed that one of the most important functions of RORγt is mediating the differentiation of proinflammatory TH17 cells (28, 32–34). Thus, RORγt is a promising therapeutic target in treating gut inflammation and chronic autoimmune diseases (34). In the context of temporary intestinal infection with Citrobacter rodentium, inhibition of RORγt in mice reduced cytokine production from TH17 cells but not ILCs, selectively preserving innate immunity (34). Withers et al. found that the transient inhibition of RORγt led to remarkably favorable results in mouse models of intestinal inflammation and suggested that the inhibition of RORγt is an effective strategy during intestinal inflammation. It has been identified that ILCs expressing RORγt, Thy1 and stem cell antigen 1 (SCA-1) accumulate in the inflamed gut, which is triggered by IL-23, but RORγt- mice fail to develop innate colitis under IL-23 stimulation. Thus, RORγt, a TF of IL-23, has a functional role in IL-23-induced innate colitis (35).

2.3 T-bet

T-bet inhibits the transcription of Rorc, downregulating the expression of RORγt (36). It has been confirmed that ex-ILC3s, ILC3-to-ILC1 transitional subsets, upregulate the expression of T-bet, which indicates that the balance between RORγt and T-bet plays an important role in ILC3-ILC1 equilibrium. Fiancette et al. observed that T-bet-/- RORγt-/- ILC3s failed to convert to ILC1s, but a group of ILCs with unknown functions. They also failed to produce IL-22 in response to IL-23 (31). This finding suggests that in addition to RORγt, T-bet is indispensable for ILC3-to-ILC1 conversion. Similarly, Stehle et al. found that T-bet deficiency could reverse the suppressive effect on lymphoid organogenesis caused by RORγt deficiency in mouse model, and T-bet-RORγt- innate lymphoid progenitors (ILCPs), instead of ILC3s, restored the intestinal barrier via the secretion of IL-22 (37). Thus, the low expression of T-bet in ILC3s ensures the equilibrium of ILC subgroups and the homeostasis of host immunity.

3 Connections and reversible plasticity between ILC3s and other subtypes of ILCs

ILCPs can differentiate into ILC1, ILC2 and ILC3 subtypes, and the development of those ILCs depends on TFs, such as RORγt, T-bet and GATA3. All ILC subgroups reserve other lineage-specific genes and can be activated under stimulation. The activation of ILC subset-associated signature cytokine loci allows ILC plasticity and expression of specific cytokines that were initially expressed by other ILC subgroups (38) (Figure 1).

3.1 Conversion between ILC1s and ILC3s

In the presence of IL-23, IL-12 and IL-1β, which are derived from tissue-resident myeloid cells (3), CD127+ ILC1s differentiate into ILC3s in a manner dependent on the TF RORγt. This process can be accelerated in the presence of retinoic acid (RA) secreted by DCs (30, 39). In contrast, DC-derived IL-12 could promote ILC3 differentiation to CD127+ ILC1 cells in vitro (39). Reversible conversion between CD127+ ILC1s and ILC3s is a process reliant on RORγt, RORα, T-bet, and other cytokines they are exposed to (31). In response to bacterial infections or IL-1β and IL-12 (40), NCR+ ILC3s convert into IFNγ-producing ILC1s, following RORγt downregulation and upregulation of T-bet (39). Similarly, Muraoka et al. found that C. jejuni infection-induced IL-12, IL-15, and IL-18 could upregulate T-bet expression and downregulate RORγt expression and therefore promote ILC3-to-ILC1 conversion and inflammation progression. In addition, hypoxia-inducible

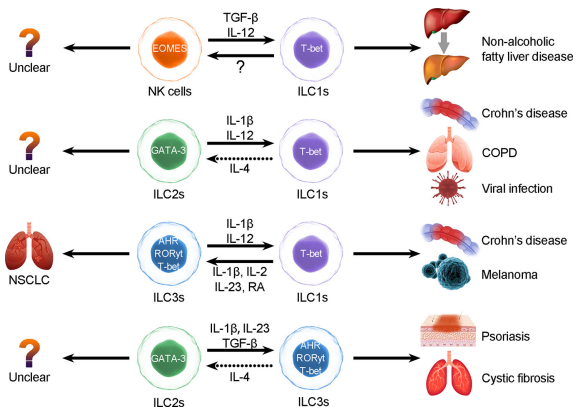

FIGURE 1
ILC plasticity. ILCs can be converted to each other through the induction of cytokines, but the interconversion may induce diseases. The types of ILC conversion are divided into the following four types: 1) conversion between NK cells and ILC1s; 2) conversion between ILC2s and ILC1s; 3) conversion between ILC3s and ILC1s; and 4) conversion between ILC2s and ILC3s.

transcription factor (HIF) 1α could upregulate the expression of T-bet and support the conversion to ILC1s (41). It has also been observed that ILC3s isolated from RORα-/- RORγt-/- mice acquire more potent ILC1 types than those isolated from RORγt-/- mice. T-bet-associated regulon activity was particularly predicted in RORα-/- RORγt-/- ILC3s, while it was predicted in only one cluster of RORγt-/- ILC3s (31). These results suggest that RORγt deficiency initiates ILC3-to-ILC1 conversion and that T-bet-associated regulon activity is fully activated in the absence of RORα, which fully realizes conversion toward the ILC1 phenotype.

3.2 Conversion between ILC2s and ILC3s

Recent studies identified that the Notch transcriptional complex binds to the Rorc gene locus and promotes RORγt expression, conferring ILC3-like functions to ILC2s (42). Notch-induced ILC2s can produce IL-17 (ILC3-characteristic cytokine) through Gata3 expression and increased RORγt expression (42). Therefore, Notch may be a crucial driver in triggering ILC conversion. TGF-β signaling also elicits ILC2s to differentiate into IL-17-producing ILC3-like cells following the expression of IL-23R (43, 44). Ligands of Toll-like receptor 2 activate ILC3s to produce IL-5 and IL-13, which indicates that ILC3s could convert into ILC2s and that the conversion between ILC3s and ILC2s is bidirectional (44).

3.3 Conversion between ILC2s and ILC1s

Under homeostatic conditions, the key TF GATA3 binds to the Ifng-controlling element and restricts IFN-γ production and ILC2-to-ILC1 conversion (45). ILC2s can also differentiate into ILC1s. Under the stimulation of IL-1β and IL-12, ILC2s enhanced the expression of ILC1-related genes and acquired an ILC1 phenotype with decreased expression of the TF GATA-3 (46). IL-1β could induce this process. In contrast, only IL-12 stimulation could not lead to conversion, which suggests that IL-12 may regulate downstream signaling of the conversion process. IL-1β induces IL-12 receptor B2 and upregulates the expression of ST2 and IL-17 receptor B, which are IL-33 and IL-25 receptor components on ILC2s (45). Exposure to IL-4 could reverse this process (47). However, IL-4 could not induce ILC1-to-ILC2 conversion. ILC2s downregulate the expression of ILC2-specific GATA-3 and IL-33R (ST3) in the presence of cigarette smoke and are gradually converted into IFN-γ-producing IL-12R+ IL-18R+ ILC1s (44). The conversion of ILC2s to ILC1-like cells can be reversed by IL-4; however, it has not been proven that ILC1s can convert to ILC2s (43, 44).

3.4 Conversion between NK cells and ILC1s

It has been observed that NK-to-ILC1 conversion could be induced by TGF-β and IL-12 in a mouse model, while subsets with mixed NK and ILC1 features have been discovered in humans (48). The TF TGF-β supports NK-to-ILC1 conversion by upregulating the expression of T-bet while repressing EOMES. Another TF, SMAD4, binds to TGF-β receptor 1, suppressing the TGF-β signaling pathway. In the SMAD4-deficient mouse model, NK cells respond more strongly to TGF-β than those in normal mice, and the abundance of ILC1s is higher than that in the control group (49). However, the ILC1-to-NK conversion is still unclear.

In short, we suggest that the plasticity of ILCs is closely related to transcriptional regulation and that conversions are highly related to distinct pathological processes (50). In intestinal diseases (40), IL-12 elicits ILC1s to switch into ILC3s, whereas IL-1 plus IL-12 collaboratively induces ILC2s to convert into ILC1s in respiratory diseases, and overactivation of the IL-17 and IL-22 pathways results in escalation of NCR+ ILC3s in patients with psoriasis (51–53). In patients with COPD, the increase in ILC1s appears to be associated with poor prognosis (44). Blockade of conversion-promoting cytokines such as IL-1β and IL-12 or activation of IL-4 may help to reverse ILC2-to-ILC1 conversion and alleviate chronic inflammation. Thus, converting ILCs may result in autoimmunity, inflammation, and carcinoma (42). Therefore, further exploration of conversion between ILCs is necessary, as the conversions between distinct ILC subgroups may be useful biomarkers or predictable signs (Figure 1).

4 ILC3s in initiating the secondary lymphoid organ

ILC-driven TLSs are similar to secondary lymphoid organs (54–56). The formation of TLSs is under the continuous stimulation of ongoing chronic inflammation (54). Local activation of T cells and B cells in TLSs results in faster immune responses and better efficacy (57). LTi cells express integrin α4β7 that interacts with MAdCAM-1 on high endothelial venules (HEVs), which allows them to migrate toward lymph niches (future lymphoid organ sites) such as the intestine, fetal spleen and thymus (58–60); the migration of LTi cells leads to the expression of adhesion molecules and chemokines that are involved in lymphoid organogenesis through the lymphotoxin-α/β receptor (LTαR/LTβR) pathway (15, 61, 62). In addition, TNF expressed by LTi cells binds to TNFR1 on LTo cells and can facilitate SLO formation synergistically with the LTβR/LTαR pathway, activate the noncanonical NF-κB pathway to produce chemokines and adhesion molecules and enhance LTβR engagement, thereby forming a positive feedback loop (63). Consequently, LTi cells stimulate lymphoid tissue organizer (LTo) cells to secrete CXCL13, CCL19, and CCL21 through the LTβR pathway and consequently recruit hematopoietic cells via the expression of CXCR5 and IL-7R (64). The activated LTo cells could differentiate into fibroblastic cells, marginal reticular cells and follicular DCs, providing a reticular structure for migrated cells. In addition, activated LTo cells in turn recruit LTi cells via the expression of CXCL12, CXCL13, CCL19 and CCL21. The crosstalk between LTo and LTi cells and the increased LTα1β2 expression induced by B cells and LTi cells via CXCR5 signals ensures sustained LTo-LTi stimulation, which forms positive feedback loops (64, 65). Those recruited T cells, B cells, DC cells, etc., then form T or B-cell areas. Growth factors released by LTo cells, such as

VEGF, FGF and HGF, contribute to the formation of HEVs and lymphatic vessels, which consequently ensures mature SLO formation (54) (Figure 2).

In mouse intestines, secondary lymphoid organ CPs, approximately 1000 cell clusters, are mainly composed of LTi-like cells, DCs. Stromal cell populations are situated at the bottom of intestinal crypt structures (21, 58). In addition, as a site where B cells are recruited, CPs can develop into ILFs, which resemble TLSs in structure (66), and accumulate more lymphocytes and stromal cells. Consequently, highly organized ILFs develop into mature lymphoid structures after bacterial colonization in the gut (58). LTi cells located in lymphoid niches trigger LTo cells to secrete CXCL13, CCL19 and CCL21 to recruit B cells, T cells and DCs to form ILFs via LTi cell-stromal gathering (16, 58, 67). The number and size of CPs and ILFs are variable, depending on the microbiome (68), while the number and size of location PPs are fixed embryonically. Moreover, the development of ILFs partially depends on Notch signaling induced by AHR, but when Notch signaling is lost, LTi cells, CPs and ILFs are less impaired than AHR deficits, indicating that there are different signaling pathways that have similar functions to Notch (69). However, LTi cells in mature mice failed to initiate the formation of SLOs; the specific regulatory mechanism is still unclear, but current studies suggest that these may be related to changes in biomarkers on LTi cells during different life stages.

Oxysterols are ligands for LTi-expressed G-protein-coupled receptor 183 (GPR183), which has been verified to regulate ILC3s since GPR183 can modulate immune cell migration (70). GPR183-LTi cells cause formation deficiency in CPs and ILFs, and in the Ch25h-lacking mouse model, the same phenotype was also observed. 7a,25-OHC (GPR183 ligand) produced by fibroblastic stromal cells attracts GPR183+ LTi cells to CP formation sites. This process stimulates the crosstalk of LTα1β2+ ILC3s and LTβR+ stromal cells, which facilitates GPR183+ B-cell recruitment to form ILFs (71). When lacking the GPR183 pathway, microbiota, CXCL13 and CCL20 could activate B-cell recruitment to form B-cell follicles and ILFs in the small intestine (72). Thus, the 7a,25-OHC-GPR183 pathway is crucial for lymphoid tissue formation in the colon, but this pathway is only an alternative in the small intestine (71–73).

LTi-induced TLS formation is linked with better survival and better prognosis. Consistently, a recent study found that in NSCLC, NCR+ ILC3s, similar to LTi cells, could induce the expression of VCAM-1 and ICAM-1 on MSCs and lead to the formation of lymphoid tissue (74). In addition, LTi cells or LTi-like ILCs also promote the formation of TLSs in extraintestinal tissues. B cells experienced more active effector differentiation, clonal proliferation and isotype switching, and T cells also expressed more activation markers in TLSs. Increasing evidence has shown that LTi-drived TLSs contribute to a favorable prognosis in extraintestinal cancers, such as lung cancer, pancreatic cancer and melanoma (54, 75). Thus, inducing the formation of TLSs may be a promising strategy for both intestinal and extraintestinal tumors.

5 Roles of different subtypes of ILC3s in maintaining intestinal homeostasis

ILC3s mainly reside in intestinal mucous, participate in innate responses and protect against pathogens (2). ILC3s are a large RORγt+ ILC population that can be divided into the following three distinct lineages: LTi cells, NCR+ ILC3s and NCR- ILC3s (30). NCR + ILC3s can produce IL-17, IL-22 and GM-CSF, and mouse Nkp46 + ILC3s also express IFN-γ (76). The pathogen defense role of ILC3s predominantly relies on IL-22, and this function can be enhanced by RA (3, 19). NCR- ILC3s primarily express IL-17, while NCR+ ILC3s mainly produce IL-22. In the presence of IL-1β plus IL-23, mouse NCR- ILC3s could develop into NCR+ ILC3s in vitro (30). LTi cells facilitate the formation of lymphoid tissue at the fetal stage. It is difficult to identify LTi cells and NCR+ ILC3s based on biomarkers, but they have different development paths. In the

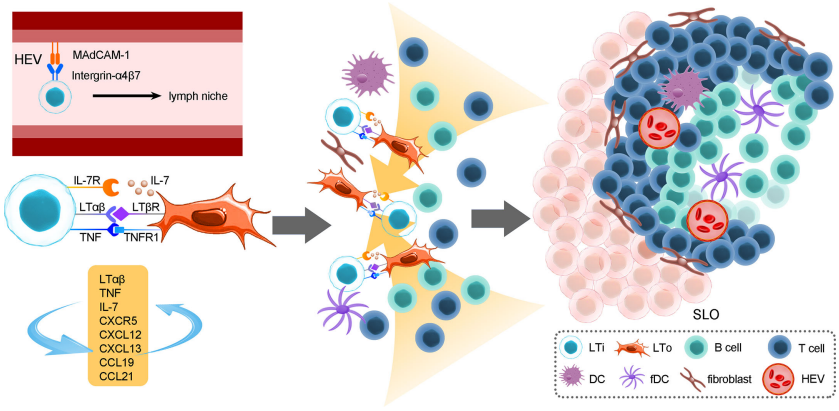

FIGURE 2
The roles of LTi cells in the development of secondary lymphoid organs. The formation of SLOs in the mouse intestine strictly depends on LTi cells. SLO development is highly reliant on the crosstalk between LTi cells and LTo cells and consequently immune cell recruitment, reticular scaffold formation and HEV or lymphoid vessel formation. In addition, positive feedback loops between LTi and LTo cells sustain sufficient activation signals for the development of SLOs. The location of PPs is fixed embryonically, and their formation cannot be induced by bacteria after birth, while CP and ILF formation is more flexible; the initiation of CPs or ILFs can occur both prenatally and postnatally. In addition, the unevenly colonized ILFs indicate that the gut microenvironment, which includes microbes and dietary metabolites, could exert a crucial role in ILF development.

adult stage, LTi-like cells are similar to LTi cells in gene expression, but they cannot initiate secondary lymphoid organ formation (7).

5.1 ILC3s

ILC3s play an important role in protecting against bacteria through the secretion of IL-22. GPR183, an ILC3-expressed chemotactic receptor, modulates the accumulation of ILC3s, which is required for the production of IL-22 (69, 77). Chu et al. confirmed that IL-22-producing ILC3s were reduced in GPR183-/- mice, suggesting that the GPR183 pathway promoted IL-22 production by inducing the accumulation of IL-22-producing ILC3s, and no other ILC3 subsets (77). IL-22 can induce the release of antimicrobial peptides (AMPs), establishing a gradient of bactericidal activity (78), facilitating antimicrobial defense and maintaining the epithelial barrier by promoting epithelial cell proliferation (79–81). Additionally, IL-22 has been proven to alleviate gut inflammation by promoting the production of mucus and then improving the epithelial barrier (82). It was observed that IL-22-deficient mouse models infected with Citrobacter rodentium developed UC (8, 19, 83). In addition, ILC3-derived IL-22 supports Lgr5+ intestinal stem cells (ISLs) to protect against inflammation (84). IL-22 acts directly on Lgr5+ ISLs, inducing phosphorylation of STAT3, which is vital for IL-22-dependent epithelial regeneration, especially after tissue damage (85). Moreover, IL-22-promoting fucosylation of intestinal carbohydrates favors commensal bacteria colonization but not pathogenic bacteria colonization because most of them cannot utilize carbohydrate fucose as an energy resource (86). Thus, IL-22 could prevent intestinal inflammation by promoting beneficial commensal bacterial colonization (43).

ILC3-derived IL-17 and GM-CSF also mediate pathogen defense, but their efficacy is not as potent as that of IL-22. NCR- ILC3-derived IL-17 expressed ROS and α-defensin to recruit neutrophils, thereby enhancing the epithelial barrier (30). GM-CSF can induce the generation of IL-10 and RA, inducing the formation of oral tolerance via activation of DCs and macrophages (30). Therefore, NCR+ ILC3s could protect homeostasis in the intestine through the efficacy of cytokines they secrete. IL-22 is a key ILC3-derived cytokine that protects the integrity of the epithelial barrier, regulates the gut microbiota and defends against pathogens. However, ILC3s also play an important role in extraintestinal host defense. Similar to ILC3 protection against intestinal bacteria, ILC3s also induce immune defense in lung diseases, especially Mycobacterium tuberculosis. Upon host infection with M.tuberculosis, ILC3s upregulate proinflammatory-expressing genes and thereby recruit macrophages and neutrophils to fight against the infection (87).

5.2 LTi/LTi-like cells

Both NCR+ ILC3s and LTi cells could produce IL-22 but in different niches of intestinal lymphoid structures/lamina propria. Deficiency of the ILC3 response results in expanding segmented filamentous bacteria (SFB). It increases the incidence of colitis (88–90). Nevertheless, the expansion of SFB and homeostasis dysbiosis were not observed in specific NCR+ ILC3-deficient mice, suggesting that LTi cells may have particular functions in ILC3-induced antimicrobial immunity (9). LTi cells, the first identified ILC subgroup, produce IL-22, IL-17A and IL-17F, and they develop from lymphatic tissue inducer precursors that differ from other ILCs (7). Although the lineage development of LTi cells is different from that of NCR+ ILC3s and all other ILC subtypes, both subtypes of ILC3s play an important role in mucosal protection (7, 12, 91). CCR6+ LTi cells locate in the intestine embryonically, develop distinctly from other ILC populations and, more importantly, promote lymphoid tissue development in the presence of lymphotoxin-β and TNF during embryogenesis (30, 71, 92).

CCR6+ LTi cells, the majority of ILC3s in lymphoid organs, internalize antigens and present antigens to CD4+ T cells, activating the production of T-cell-dependent antibodies (7). In the presence of IL-1β, LTi/LTi-like cells in lymphoid organs express CD80 and CD86 and produce IL-2, TNF-α and IFN-γ to fully activate T cells, and a specific discussion about the effects of LTi/LTi-like cells in antigen presentation and regulation of T cells will be presented in Section 5.1 (93).

In the fetal stage, LTi cells play a critical role in secondary organogenesis (94). As we mentioned above, LTo cells (95, 96), as a specialized stromal cell group, produce CXCL13, which recruits LTi cells to form the initial hematopoietic cell cluster (65, 95, 96).

6 ILC3s interaction with adaptive immune cells

6.1 T-cell

ILC3s could convert to MHC-II+ ILC3s via Basic leucine zipper ATF-like transcription factor (BATF)-induced enhanced chromatin accessibility of MHC-II antigen processing and presentation genes (97). MHC-II-expressing ILC3s present antigens to CD4+ T cells and result in suppression of cellular immunity and humoral immunity in the intestine (98). It has been demonstrated that MHC-II+ CCR6+ ILC3s could downregulate CD4+ T-cell abundance and TH17 cells while upregulating regulatory T cells (Tregs) through antigen presentation in the intestine (99). MHC-II+ CCR6+ ILC3-induced antigen presentation could result in the upregulation of Nur77 and Bim, which have been verified to be associated with negative selection in the thymus (99). Under infection with SFB and H. hepaticus, TH17 cells were expanded, and the differentiation of Tregs was impaired significantly in an MHC-II-deficient mouse model (100). This indicates that MHC-II+ ILC3s are necessary to protect the intestinal microenvironment by suppressing inflammatory T-cell expansion.

However, splenic ILC3s upregulate the expression of surface MHC-II molecules, activate CD4+ T cells and upregulate the expression of the costimulatory molecules CD80 and CD86 under stimulation with IL-1β (101). It has also been reported that the abundance of TH17 cells and IgG titers are significantly reduced in MHC-II-deficient ILC3 mouse spleens (93). These inconsistent

results also showed that the function of ILC3s is associated with different microenvironments. In addition, the microbiota triggers the production of IL-23 and consequently downregulates MHC-II+ ILC3s under steady conditions, while virus-induced IFN-γ can promote the expression of MHC-II and induce the proliferation of CD4+ T cells under viral infection (101). Thus, alteration of the microenvironment could be an effector that regulates the MHC-II+ ILC3-driven T-cell response.

IL-2 has been reported to exert protective effects that could play a critical role in the generation and function of CD4+ T cells. Recently, Zhou et al. found that the transcription level of IL-2 in ILC3s is much higher than that in IL-2-producing CD4+ T cells through RNA sequencing of the small intestine. IL-2 transcription in ILC3s could be specifically induced by macrophage-derived IL-1β. Consistent with a mouse model, patients with Crohn's disease have diminished IL-2+ ILC3s in the intestine but no remarkable difference in other IL-2-producing cells compared to healthy controls (102). However, MHC-II+ CCR6+ ILC3s could induce IL-2 withdrawal through their combination with IL-2 and initiate the TCR-induced apoptotic program, as the IL-2 requirement of T cells is intrinsic, but T-cell apoptosis could be reduced when given additional IL-2 (99). These results showed that a deficit in the IL-1β-ILC3-IL-2 axis could lead to changes in the abundance of CD4+ T cells and Tregs and impaired immune regulation in the intestine. Moreover, LTi-like cells induce T-memory and T-independent antibody responses by expressing APRIL, BAFF, CD30L and OX40L (88). Memory CD4+ T cells are RORγt dependent. A marrow chimeric mouse model showed that LTi cells are crucial RORγt-expressing cells, supporting memory CD4+ T-cell survival in the absence of antigen stimulation (88).

6.2 B-cell

ILC3s could mediate IgA responses with or without interacting with T cells. Human tonsillar NKp44p ILC3s secrete the B-cell activation factor BAFF, indicating that ILC3s support B-cell activation and survival in mucosal tissues to facilitate the production of IgA antibodies (89). In addition, CCR6+ LTi cells induce secondary lymphoid structure formation and contribute to the accumulation of B cells, which is required to synthesize T-independent IgA (90). Within PPs and ILFs generated by LTi cells, B cells can also interact with CD40L+ CD4+ T cells and then convert into IgA+ plasma cells, promoting intestinal immunity (66, 90). Strikingly, one recent study proved that ILC3s could suppress the generation of IgA+ B cells to protect against both commensal and pathogenic bacteria by presenting antigens to T follicular helper cells (Tfhs) and suppressing the Tfh response, except in PPs or the intestinal lamina propria (98). Tfh cells, a distinct lineage of CD4+ T cells, play an important role in assisting B-cell responses (103). In mice that lack ILC3-intrinsic MHC-II expression (MHCIIΔILC3), the Tfh response increased in mesenteric lymph nodes, and IgA+ B cells notably increased in the colon. A number of studies have reported that mucosal bacteria such as Helicobacter and Mucispirillum help to build up mucosal defense in the early stages of life (104). However, mucosal bacteria fail to colonize colon niches due to dysregulation of the IgA response in the absence of ILC3s (98). Thus, ILC3s could mediate intestinal bacterial colonization to maintain homeostasis by regulating the IgA response. Therefore, further studies examining the connections between LTi cells and B-cell immunity for the important role of LTi cells in adaptive immunity may be helpful for the treatment of cancer, autoimmune diseases and inflammatory diseases.

7 Crosstalk between ILC3s, dietary metabolites and the gut microbiota alters the intestinal microenvironment

Trillions of microbes colonize the intestine; the crosstalk between commensal microbes and ILC3s residing in the gut plays a vital role in regulating the intestinal microenvironment and intestinal health (105). Current studies have found that dietary metabolites can indirectly modulate the intestinal microenvironment by altering the gut microbiota. In this review, we present the idea that the diet could mediate the intestinal microenvironment through crosstalk between the gut microbiota and ILC3s. Dietary metabolites impact the structure and activity of intestinal microbes and then regulate intestinal immunity. Recent studies have proposed that dietary metabolites modulate the functions of ILC3s through TFs and thereby stimulate specific transcription programs (71). A ketogenic diet, characterized by low carbohydrate and high fat, could alleviate colitis by reducing ILC3s by altering the gut microbiome. In addition, a ketogenic diet increased the abundance of intestinal bacteria such as Akkermansia and butyric acid-producing Roseburia, which are conducive to the maintenance of intestinal health. It has been confirmed that the microbiota plays an important role in the physiology of ILC3s and that a ketogenic diet-altered intestinal microbiota alleviates inflammation by reducing the frequency of ILC3s (106). Consistently, the endogenous Trp catabolite kynurenine can also modulate ILC3s by activating the intestinal microbiota (27). Kynurenine activates the AHR-Notch pathway and consequently promotes ILC3 production of IL-22 (107). ILC3-regulated immune responses allow colonization of commensal microbiota while providing resistance to C. albicans. These results suggest that dietary metabolites could activate the ILC3-induced mucosal innate response, which mainly relied on IL-22, with the participation of gut microbiota. However, it is still unclear which signals from the microbiota specifically induce IL-22 production.

Vitamin A, an important nutrient, is enriched in fruits, vegetables, dairy products and so on. The metabolites of vitamin A, RA, play a vital role in fetal LTi cell development and lymphoid tissue formation (108). ID2+ RORγt+ CD4+ LTi cells differentiated from ID2+ RORγt+ CD4- LTi cells initiate the formation of SLOs (109). Blockade of the RA signal resulted in a decrease in ID2+ RORγt+ CD4- LTi cells and SLO density, while the frequency of ID2+ RORγt+ CD4+ LTi cells increased after RA stimulation of lymph node cells (109). In addition, a diet lacking RA hinders ILC3 proliferation and the development of a secondary lymphoid organ (110). Moreover, mice fed a vitamin A-deprived diet had a notable

decrease in ILC3s and ILC3-derived cytokines IL-22 and IL-17, while ILC2s and ILC2-derived cytokines such as IL-4, IL-5 and IL-13 were increased, suggesting that vitamin A may be related to the equilibrium between ILC2s and ILC3s (111). Additionally, RA secreted by DCs can accelerate the differentiation of ILC1s into ILC3s (30, 39).

Short-chain fatty acids (SCFAs), metabolites of dietary fibers (DF), are mainly produced by microbial fermentation (112). As the most abundant microbial metabolites in the intestine, SCFAs, combined with G-protein-coupled receptors (GPCRs), could support antibody production, promote T-cell production of IL-10 and stimulate ILC2s and ILC3s (105). It has been observed that the abundance of ILC3s in mice fed high DF (high SCFAs) was much higher than that in mice fed low DF (high SCFAs), while ILC1s in mice fed high DF were much higher; the expression of IL-17A, IL-22 and Ffar2 (GPCR expressed by ILCs) in mice fed low DF was obviously lower than that in mice fed high DF (113). SCFAs activate the STAT3, STAT5, mTOR and PI3K pathways to support ILC proliferation following Ffar2 activation. In addition, Chun et al. found that Ffar2 could also regulate the expression of ILC3 apoptotic or survival factors (105). Notable decreases in gut pathogens and inflammation remission in the high DF group showed the positive role of SCFAs in improving enteric immunity against intestinal infection. Taken together, these results show that dietary metabolites could modulate ILC3-induced intestinal responses through the activation of ILC3s or the regulation of ILC3 conversion. In addition, as we mentioned above, 7a,25-OHC binding to GPR183 could also contribute to host defense by supporting SLO formation (71).

Considering the crosstalk between dietary metabolites that exerts an eminent effect on ILC3s, understanding the mechanism of crosstalk between enteric microbes and ILC3s through diet metabolism helps to alleviate the progression of intestinal inflammatory diseases (Figure 3).

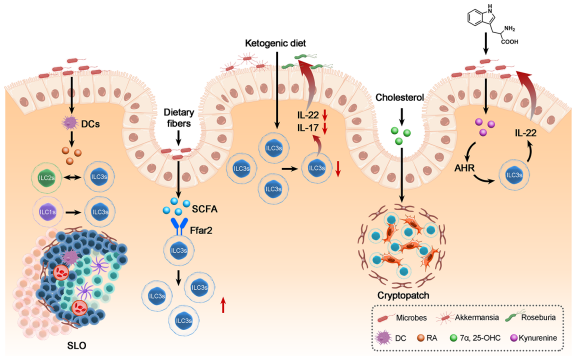

FIGURE 3
The crosstalk between ILC3s, dietary metabolites and the gut microbiota in maintenance of intestinal homeostasis. Dietary metabolites derived from microbes can regulate the biology of ILC3s: Including modulates the frequency of ILC3s, the conversion between ILC3 and ILC1 or ILC2 and the development of SLO, thereby affecting the balance of gut immunity and thus the biology of gut microbes and the homeostasis of intestine.

8 ILC3s in intestinal disease: Foes or friends?

8.1 Dysbiosis of ILC3s contributes to the development of IBD

IBD, characterized by chronical gut inflammation, mainly consists of ulcerative colitis (UC) and Crohn's disease (CD) (114). Recent studies found that ILC3s support the mucosal homeostasis of intestine but the dysregulation of ILC3s population would result in the formation and progression of IBD (30). Increased IFN-γ-producing ILC1s were also found in inflammatory intestine from IBD patients (19). It has been proven that in gut inflammatory tissue, the frequency of ILC3s was decreased in patients with IBD, but the number of ILC1s was increased, which suggests that the conversion of ILC3s to ILC1s could also contribute to the deterioration of IBD (115). Moreover, the reduction in ILC3s are also closely linked with the severity of IBD (115). The increased frequency of ILC2s is also related to the progression of IBD (19, 30). ILC2s upregulate the plasticity toward ILC1s and ILC2s separated from mucosa of CD patients also showed the IFN-γ producing capability, which significantly contributes to intestinal inflammation. In addition, ILC2-derived IL-33 was found to be increased in colitis mice model. IL-33 therapy and ILC2 removal was identified to be helpful for inflammation alleviation (116).

The GPR183 pathway is closely related to IBD (69). Colonic inflammation could activate GPR183 pathway by increasing 7a,25-OHC production. In CD40-AB treated mice model, the amount of 7a,25-OHC in colon would increase, in response to inflammation (71). Overexpressed 7a,25-OHC excessively activates GPR183, promoting ILC3s migration toward 7a,25-OHC and thus contributing to ILC3 population dysregulation which further induces IL-22 overexpression (69). ILC3-derived IL-22 can help to maintain the homeostasis in intestine but overexpression of IL-22 would recruit excessive neutrophil cells to produce proinflammatory cytokines and thus lead to quick and swift enhancement of epithelial barrier permeability (30). However, DCs stimulated by IL-25 downregulate IL-22 production, suggesting a complicated regulatory mechanism of the DCs-ILC3 axis (11).

Vonarbourg et al. found that NKR+ RORγt+ LTi cells release IL-22 to activate the epithelial barrier, but NKR+ RORγt- LTi cells (ex-ILC1s) could produce IFN-γ, which can induce the progression of colitis (117–119). Therefore, the RORγt gradient could mediate the functions of NKR+ RORγt+ LTi cells. T-bet and RORγt act reversely and negatively regulate each other (120, 121). Chronic colitis triggers the release of IL-12, which promotes NKR+ RORγt+ ILC3 conversion into IFN-γ-producing ILC1s. Accumulation of ILC1s in inflammatory tissue and overexpression of IFN-γ results in epithelial barrier damage and aggravation of intestinal inflammation (40).

8.2 Promising immunotherapy in IBD treatment

Current medical treatment of IBD mainly focused on anti-inflammation drugs. The usage of them could alleviate inflammation but cause a variety of side effects. Therefore, it is urgent to study new drugs with better safety and higher efficiency. Over the past decade, ILC3s have been recognized to highly correlate with IBD pathogenesis, and thus drugs targeting ILC3-regulation factors may provide a novel strategy for IBD treatment (122).

TNF could result in intestinal epithelium cell death and chronic inflammation in intestine. Blocking of TNF is widely used in IBD treatment. Recent studies have found that ILC3s could protect intestinal epithelium from TNF-related apoptosis through production of heparin-binding epidermal growth factor–like growth factor (HB-EGF). IL-1β induces ILC3 to produce PGE2. Then, PGE2 significantly stimulates ILC3s to produce HB-EGF. The abundance of HB-EGF+ ILC3s was found to be decreased in inflammatory intestine tissue (123).

Hueber et al. found that the IL-17A inhibitor, secukinumab, showed worse efficacy than placebo. CD persistent disease activity was observed in patients treated with secukinumab, leading to inflammation aggravation and severe adverse events in patients with apparent inflammation (124). Compared with the satisfying efficacy of secukinumab in psoriasis, unpleasant outcomes in CD treatment may be related to different immune microenvironment in these diseases. IL-17A participates as an important factor in innate intestinal protection (19); therefore, inhibition of IL-17A may leads to homeostasis dysbiosis (20). Clinical remission can be observed in patients with CD who were treated with ustekinumab, an antibody of the shared p40 unit of IL-12 and IL-23 (30, 125). Ustekinumab treatment partially restored the balance of ILC subsets with a decrease in ILC1s and an increase in ILC3s (126). However, p40 antibody only attenuates CD in the first two month since ustekinumab not only inhibits IL-12-induced ILC3-ILC1 conversion but also suppresses IL-23-induced IL-22 production, blocking the crucial anti-inflammation responses (127). Thus, an antibody, specifically blocking IL-12 may show better efficacy in IBD treatment than an anti-p40 antibody.

Unlike the favorable prognosis in CRC, the increased TLS in the DSS-colitis mouse model is linked with inflammation progression. Previous studies have demonstrated that in response to dysregulation of intestinal commensal bacteria and immune tolerance loss, TLS develops during chronic inflammation. TLS formation is an important characteristic of UC, which is more likely to develop into extraintestinal inflammation than CD (128). Unlike the protective role in CAC, it has been found that intestinal TLSs can produce antibodies aberrantly under immune dysregulation and lead to IBD progression. Thus, site-directed delivery of LTβR inhibitors may be a more viable modality for the treatment of IBD with less harm to other lymphoid structures. However, how to utilize the antimicrobial defense function of TLSs and avoid dysbiosis-related disease progression and the detailed regulatory mechanism of TLSs in IBD remain unclear.

As we mentioned above, MHC-II+ ILC3s could downregulate the frequency of CD4+ T cells and other inflammatory cells to maintain intestinal homeostasis. Recent studies have observed that MHC-II expression was obviously reduced on ILC3s from pediatric patients with IBD. Thus, MHC-II+ may be a promising target for IBD treatment considering their crucial role in inducing apoptosis of CD4+ T cells (99).

Considering the complicated regulatory mechanism of intestinal immune and biological functions of cytokines in IBD pathogenesis, it is hard to choose appropriate inhibition therapy in CD treatment. Hence, further study of ILC3s mechanisms in IBD is necessary.

8.3 Roles of ILC3s in colorectal cancer

IBD is likely to develop to CRC since IBD often results in chronic inflammation in gut mucosa (19). ILC3s was identified as possessing both pro- and antitumor properties (129). The frequency of ILC3s decreased while that of ILC1s increased in CRC tissue compared to benign adjacent tissue, which is consistent with IBD. CCR6+ LTi cells could restrict the TH17 response and gut inflammation via MHC-II to limit the invasion and progression of CRC (130). However, the level of CCR6+ MHC-II+ LTi cells was lower in CRC tissue (131). The ratio of CD4+ T cells to MHC-II-expressing LTi cells notably increased in CRC, which indicates that the interaction between T cells and CCR6+ MHC-II+ LTi cells in CRC tissue is interrupted due to the decrease in MHC-II+ LTi cells (131). Consistently, transforming growth factor-β (TGF-β) in the tumor microenvironment suppressed the upregulation of HLA-DR, CD80 and CD86, and consequently inhibited antigen-presentation, leading to diminished T-cell responses (132).

In contrast, many studies identified that ILC3-specfic IL-22 contribute to progression and metastasis of intestinal tumors (19). In a bacteria-driven CRC mice model, ILC3s and IL-22 are closely linked with the progression of metastatic CRC (133). Soluble IL-22 binding protein derived from DCs can neutralized IL-22 and then suppress cancer progression by preventing the binding between IL-22 and IL-22R (134). Huber et al. found that in an IBD mouse model, IL-22BP-/- mice were more likely to develop CRC (135). IL-22BP is highly expressed in the normal microenvironment and downregulated when intestinal tissue are damaged (135). IL-22 could promote gut tissue repair and epithelial cell proliferation during intestinal damage, but uncontrolled IL-22 production would result in tumorigenesis (136).

Microbial dysbiosis contributes to the pathogenesis of CRC through regulation of ILC3s (137). Previous studies confirmed that the accumulation of IL-22 was postnatal. IL-22 frequency gradually increased after birth. Moreover, it has been shown that the complexity of the intestinal microbiome is consistent with IL-22 production (138). C. albicans could stimulate macrophage IL-7 production, elicited ILC3s to produce IL-22, and consequently promote CRC formation. IL-7 combined with IL-23 and IL-1β has a synergistic effect on IL-22 secretion and leads to CRC progression (138, 139). However, IL-1β could also support the capacity of ILC3s to produce CXCL10 and high expression of CXCL10 is associated with better antitumor responses (129). These results may suggest that the controversial role of cytokines

may be attributed to the difference in the intensity of signaling and the tumor microenvironment.

8.4 Novelty perspective on treatments of colorectal cancer

Similar to IBD, the excessive expression of IL-22 and dysregulation of ILC3s also contribute to the development of CRC. It has been confirmed that treatments targeting ILC3 regulating factors also have encouraging efficacy in patients with CRC. LTi-driven TLS was closely linked with favorable prognosis in CRC patients (140). Consistently, high TLS was identified as a sensitive marker of more prolonged survival in clinical trails (141). It has been confirmed that ILFs recruited lymphocytes to the tumor microenvironment (TME), facilitating the antitumor immune responses (73). Vaccination treatment using engineered CCL21-expressing DCs increase the formation of TLS in melanoma through the recruitment of T cells. The crosstalk of immune cells sited in TLS enhanced the antitumor response and contributed to the regression (142).

Moreover, recent studies has reported that under the stimulation of IL-1β, ILC3-like cells upregulated the expression of MHC-II (HLA-DA), CD70, CD80 and CD89, while TGF-β could suppress this process and the expression levels of MHC-II and CD89 were comparable to that in professional antigen-presenting cells (APC). Enhanced antigen-presenting function could facilitate cytomegalovirus specific memory CD4+ T cells. These results suggest ILC3-like cells could respond to specific cytokines, increase antigen-presenting properties and consequently regulate memory CD4+ T-cell responses. Thus, whether we could enhance CD4+ T cells response in CAC treatment by using cytokines that mediate the antigen-presenting role of ILC3-like cells may be a new perspective for CAC treatment (132). Thus, we suggest that treatment using engineered chemokine delivery cells that activate TLS in CRC and facilitating antigen-presenting to prompt T-cell responses in tumor milieu may contribute to the treatment of CRC.

9 Conclusion

While many regulatory mechanisms of Group 3 ILCs in maintaining intestinal homeostasis and promoting or alleviating autoimmune diseases have been identified, several transcriptional pathways and functions of specific cytokines are still unclear. Previous studies have reported the following findings: 1. dysregulation and conversion of ILC3s could accelerate the progression of tumor and autoimmune disease while ILC3s supports the immunity against pathogens and help maintain gut homeostasis. 2. ILC3s play a protective role by producing cytokines, inducing TLS formation and supporting the adaptive immune response. 3. Autoimmune diseases or tumors account for ILC3s decrease and the conversion of ILC3s to other ILCs subsets; this could also lead to disease progression and hence positive-feedback loops. 4. The crosstalk between ILC3s and microbes plays crucial role in the immune environment *via* diet metabolites produced by microbes. Here, we point to 5 critical questions about how to utilize ILC3s in controlling autoimmune diseases and tumors. 1. Could we block the expression of specific TF like T-bet to restrict ILC3s conversion to ILC2s or ILC1s to alleviate disease progression? 2. What signals or pathways ensure that LTi cells support SLO in the fetal stage and could we induce SLO formation and enhance the intestine's immune system? 3. Could we promote anti-pathogen immunity through a specific diet? Further understanding of ILC3s regulatory mechanism and the crosstalk of ILC3s and other ILC subsets, adaptive immune cells, lymphoid tissues and microbes will have a place in future inflammatory disease and tumor treatment. 4. Can all ILC conversion be reversed and how can ILC plasticity be regulated to alleviate inflammation or tumor progression? 5. Why does TLS exert a different role in IBD and cancer, and what is the detailed regulatory mechanism of IgA production and host defense functions in TLS? Thus, further study of ILC regulatory mechanism may help to understand the roles of ILC in cancer and inflammatory diseases and provide a novel perspective for innovative and targeted therapies.

Author contributions

YZ: literature search, study selection, draw figure, manuscript writing. ZM:literature search, manuscript writing, revision of manuscript. PR: study design, revision of manuscript. XF, JW, HT and JL: revision of 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

Abbreviation

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Die Kämpfe únd schlächten—the struggles and battles of innate-like effector T lymphocytes with microbes

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The large majority of lymphocytes belong to the adaptive immune system, which are made up of B2 B cells and the αβ T cells; these are the effectors in an adaptive immune response. A multitudinous group of lymphoid lineage cells does not fit the conventional lymphocyte paradigm; it is the unconventional lymphocytes. Unconventional lymphocytes—here called innate/innate-like lymphocytes, include those that express rearranged antigen receptor genes and those that do not. Even though the innate/innate-like lymphocytes express rearranged, adaptive antigen-specific receptors, they behave like innate immune cells, which allows them to integrate sensory signals from the innate immune system and relay that umwelt to downstream innate and adaptive effector responses. Here, we review natural killer T cells and mucosal-associated invariant T cells—two prototypic innate-like T lymphocytes, which sense their local environment and relay that umwelt to downstream innate and adaptive effector cells to actuate an appropriate host response that confers immunity to infectious agents.

KEYWORDS

NKT (natural killer T) cell, MAIT (mucosal-associated invariant T) cell, innate-like effector lymphocyte, symbionts, pathobiont

Introduction: 'For a secret offence, a secret revenge'

This subtitle 'For a secret offence, a secret revenge' (see Box 1) exemplifies the metaphorical descriptions of *fin-de-siècle*—turn of the 19th century, scientific discoveries written for the benefit of the general public; this style, quite common then and in the early 20th (3, 5), remains in textbooks and lectures in pathology, microbiology, and immunology. By that time, many—Antony van Leeuwenhoek (6), Robert Hooke (7), Theodor Schwann (8), and Matthias Schleiden (9), had independently peered down the microscope, developing the 'cell theory'—the cell as the fundamental unit of life. Now

BOX 1 *Fin-de-siècle*—a turn-of-the-19th-century metaphorical description of the defense system as a warring system of the body that restores balance when tipped over by an infection.

The subtitle 'For a secret offence, a secret revenge' owes to the title of one of the fables in '*Vacation Stories: Five Science Fiction Tales*' written by the 1906 Nobel Laurate Santiago Ramón y Cajal, published originally in the Spanish language assuming the alias of 'Dr. Bacteria'. These fables were written for Cajal's scientific friends. Famed for the 'neuron doctrine' and precise and beautiful drawings of the nervous system (1), Cajal is less known for his artistic and literary works because much of these cultural contributions were poorly recorded and archived. Cajal "*wrote a collection of twelve fables or semi-philosophical, pseudoscientific tales that* [*I*]" he "*never dared take to press, both for the oddness of their ideas and the laxity and carelessness of their style* (2)." Fortunately, the collection of five science fiction works have survived Cajal and time in 'Vacation Stories'; the remaining seven "*sleep the slumber … far deeper than the so-called* sleep of slumber[.]" not as "*failed artistic works*" as Cajal's Preface would make the reader to believe (2) but rather because those manuscripts were never found (3, 4).

entered Rudolf Virchow (10) who espoused '*omnis cellula e cellula*'—every living cell derives from another cell, the melodic phrase coined by François-Vincent Raspail (11)—from observations of *leukocythemia*—leukemic cells in the blood of a 50-year-old woman and formed the cellular basis of disease (10, 12). Robert Koch and Louis Jean Pasteur independently developed the microbial basis of infectious disease (13), and Élie Metchnikoff (previously Ilya Ilyich Mechnikov) whose astute observations of cells swarming toward the splinter prick in the starfish larva and their attempts to eat it, voraciously gnawing at it—that is termed phagocytosis, birthed cellular immunology (5, 14, 15), while from the opposing and warring Paul Ehrlich school originated humoral immunity (15–17).

Viewed against this historic backdrop, 'for a secret offence, a secret revenge' refers to the body's elegant defense system working against agents that cause infectious diseases—the battles raged between immune cells and bacteria. The immune system is generally described as a warring system that oftentimes wins battles yet may lose a war: the morbidity and mortality caused by severe acute respiratory syndrome coronavirus 2 infection is a sorry reminder of the perils of the warring immune system. While it is a warring system indeed, it does not attack indiscriminately. The immune system has learnt over eons to coexist with billions and zillions of bacteria and other microbes in a symbiotic habit.

Amid *kämpfe únd schláchten* with microbes and other forms of external (irritants and allergens) and internal (mutant cells and metabolic toxicants) dangers, in complex multicellular metazoans arose a sensing-and-actuating system—the immune system. In vertebrates, the initial response to aforementioned dangers is actuated by the older innate immune system. In vertebrates, the innate immune system, which arose in early metazoan faunas—the simple invertebrates, is made entirely of the myeloid lineage of hematopoietic cells such as macrophages, dendritic cells and mast cells in tissues and by monocytes, neutrophils, basophils, and eosinophils patrolling the blood and, on demand, tissues as well. As the innate immune system responds to danger, it alerts the adaptive immune system, which kicks into full gear should the innate immune response not restore the host's altered *milieu intérieur* (homeostasis) to its original state—or close to it. The adaptive system is slow in acting and is made entirely of lymphoid lineage cells. These cells sense alterations in the homeostatic state with the use of antigen-specific receptors encoded by somatically rearranged gene segments, clonally expressed by B and T lymphocytes—the B-cell receptor (BCR) and αβ T-cell receptor (TCR). Such B and T lymphocytes together constitute the conventional lymphocytes. The clonal expression of BCR and TCR requires the priming of the adaptive immune system by either immunization with antigen or natural infection for the clonal expansion of the low-frequency antigen-specific lymphocytes to clear infections and to protect against infectious diseases. This requirement for priming distinguishes the adaptive immune system from the innate, which reacts quickly, without the need for prepriming.

Circa 1973, a non-B, non-T—the 'null' killer lymphocyte, which could kill tumor cells without prior priming of the immune system, was discovered. Now called natural killer (NK) cells, their discovery alerted to lymphocytes that behave like the cells of the innate immune system and featured the quiet annunciation of unconventional lymphocytes (18). Next, a decade later, the start of the year 1983 unveiled with the discovery of B lymphocyte subsets: one that secreted natural antibodies (B1a) and the other that produced antibodies to bacterial polysaccharides and T lymphocyte–independent antigens (B1b) in addition to the conventional B2 B cells of the adaptive immune system (19). Then in ca. 1986 came the discovery of γδ T cells, which express the γδ TCR genes—a kin to the αβ TCR (20). The ensuing decades announced the discovery of many more unconventional lymphocytes (Figure 1): e.g., natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, mouse CD8αα intraepithelial T lymphocytes, mouse H-2M3-restricted T cells, mouse/human H-2Qa1/HLA-E-restricted T cells, and human group 1 CD1-restricted T cells as well as lymphoid tissue inducer cells and innate lymphoid cells [reviewed in refs (21, 22).]. This collection of unconventional T lymphocytes we here call innate/innate-like effector lymphocytes.

The multitudinous innate/innate-like effector lymphocytes share several common features. In addition to being of lymphoid origin, they act quickly as they display a memory phenotype similar to antigen-experienced conventional lymphocytes yet, unalike conventional lymphocytes, retain no memories of past pathogen encounters. After development, innate/innate-like effector lymphocytes become home to secondary lymphoid and/or nonlymphoid tissues. They are stationed at barrier sites where the microbial consortia are known to congregate (19, 23, 24). As discussed below in the "Hygiene Hypothesis" section, products from these consortia facilitate the development and/or maturation

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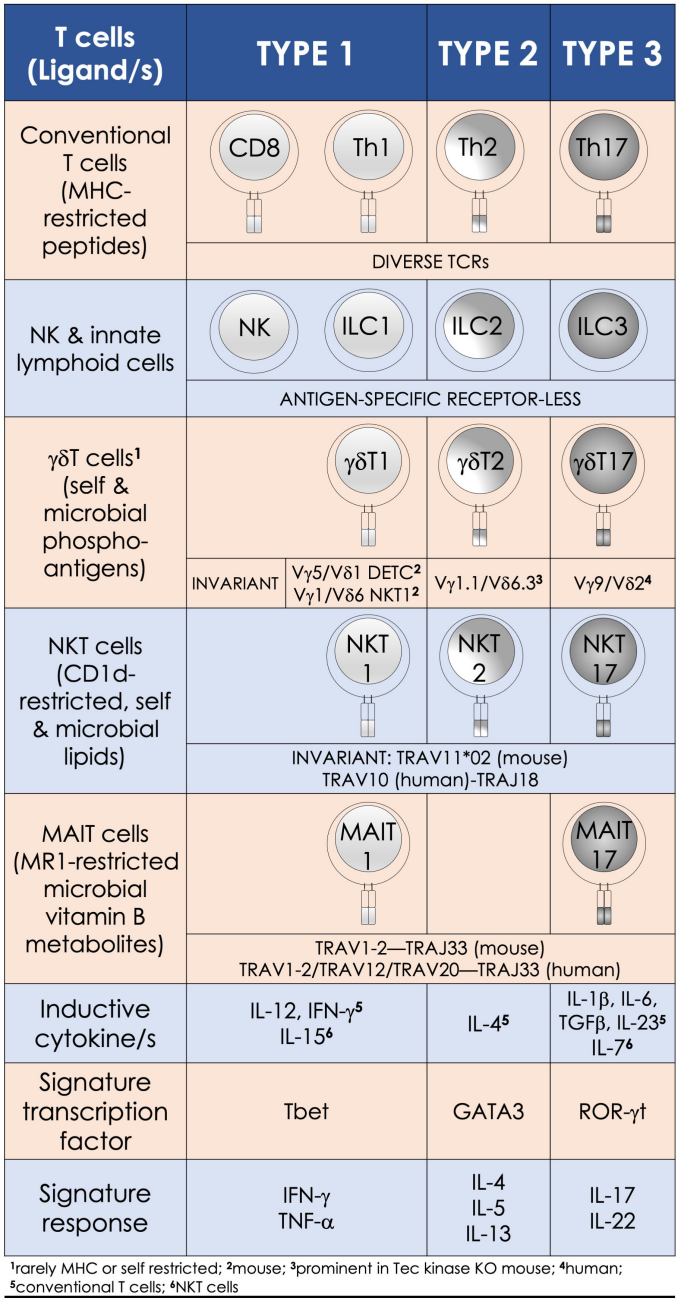

FIGURE 1
Innate-like effector lymphocyte functions mirror type 1, type 2, and type 3 effector cells. Natural killer T (NKT), mucosal-associated invariant T (MAIT), and γδT cells are characterized by semi-invariant T-cell receptor (TCR) expression by contrast to conventional T cells express a diverse TCR (IMGT nomenclature) repertoire. By contrast, innate lymphoid cells and NK cells do not express rearranged antigen receptors. Type 1 effectors include the cytotoxic NK and $CD8^+$ T cells and T helper (Th) 1 cells, as well as NKT1, MAIT1, and γδT1 cells. They require IL-12 for induction, which is bolstered by IFN-γ. T-bet and the related eomesodermin transcription factors control the differentiation of type 1 effector cells, which are essential for immunity against intracellular pathogens. Type 2 effector cells include Th2, NKT2, and γδT2 cells. These cells are activated by IL-4 and require GATA3 for their effector differentiation. Their physiologic functions—e.g., parasite expulsion, and pathologic—e.g., airway hypersensitivity, are mediated by IL-4, IL-5, and IL-13 secretions. RORγt—the lineage specific transcription factor program type 3 effectors, which include Th17 and NKT17, MAIT17, and γδT17 cells. Lineage-specific inducive factors include IL-6, TGF-β, IL-1β, IL-23, and IL-7. Type 3 effector cells secrete IL-17 and IL-22 upon activation, by which they mediate tissue repair and confer immunity to extracellular bacteria and fungi.

of a subset of innate/innate-like effector lymphocytes (25–29). Those innate-like lymphocytes that express rearranged BCRs or αβ/γδ TCRs recognize their cognate ligands by germ-line encoded portions of the antigen-specific receptors using an innate recognition logic (30–34). Innate/innate-like lymphocytes react to self- and nonself-ligands: Some recognize H-2Qa1/HLA-E-restricted self and/or microbial peptides, H-2M3-restricted *N*-formylated mitochondrial/microbial peptides, group I and group

II CD1-restricted lipids—e.g., αβ and γδ T cells and NKT cells, or major histocompatibility complex (MHC)–related 1 (MR1)-restricted metabolites—e.g., MAIT cells. Others recognize ligands directly without the need for MHC/non-MHC restricted presentation—e.g., intact proteins, small molecules/phosphometabolites—e.g., γδ T cells, or phospholipids—e.g., B1a cells and γδ T cells. Further, inflammatory cytokines alone—e.g., type I interferons (IFNs) or interleukin (IL)-12 and IL-18 by themselves—without the need for antigenic or agonistic ligands, can activate innate-like T lymphocytes. Innate/innate-like effector lymphocytes are quick responders; they can act as quickly as cells of innate immune system or faster [reviewed in refs (21, 22)]. This feature in several innate/innate-like effector lymphocytes is ingrained during development by a genome regulatory network under the control of a promyelocytic leukemia zinc finger transcription factor (encoded by *Zbtb16*; reviewed in ref (35, 36)]. Activated innate/innate-like effector lymphocytes secrete a wide variety of cytokines and chemokines with which they can steer downstream type I, II, and III immune responses (Figure 2). Thereby, they integrate sensory output/s received from the innate immune system to provide context to downstream innate and adaptive immune responses (35, 42). Here we review how NKT cells and MAIT cells—two prototypic innate-like T lymphocytes, sense their local environment and relay that umwelt to downstream innate and adaptive effector cells to actuate an appropriate response that confers protection from infectious diseases.

Natural killer T and mucosal-associated invariant T cells—two peas in a pod

There are multiple types of NKT and MAIT cells that are distinguished by their αβ TCR usage and, consequently, the ligands they recognize (21). The focus in this review is on NKT and MAIT cells that express an invariant TCR α-chain: semi-invariant NKT cells begotten from the rearrangement of TRAV11*02 (mouse Vα14i) or TRAV10 (human Vα24i) to TRAJ18 and MAIT cells from TRAV1-2 (mouse and human Vα9i and human TRAV12/TRAV20) to TRAJ33 rearrangement [reviewed in refs (35, 43–45)]. A curious feature of these rearrangements is not only the conserved TRAV to TRAJ usage but also that this rearrangement results in conserved residues that make up the CDR3α (complementarity determining region 3α) loop of the TCR α-chain. Furthermore, invariant Vα14i α-chain pairs with TRBV13-2*01 (Vβ8.2), TRBV29*02 (Vβ7), or TRBV1 (Vβ2) β-chain to form a functional mouse semi-invariant NKT cell TCR. Additionally, the Vα24i α-

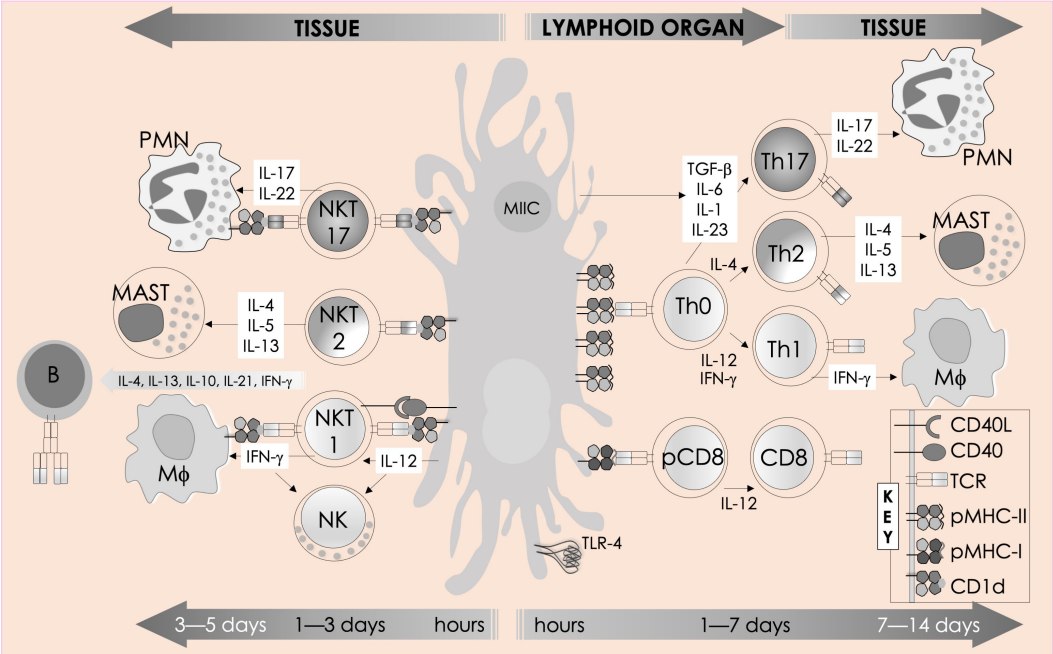

FIGURE 2
Immune functions of mouse NKT cells. NKT cell activation is initiated by semi-invariant NKT cell receptor interactions with cognate antigen and bolstered by costimulatory interactions between CD28 and CD40 and their cognate ligands CD80/86 (B7.1/7.2) and CD40L, respectively. The resulting activated NKT cells crosstalk with members of the innate and the adaptive immune systems by deploying cytokine and chemokine messengers. Upon activation *in vivo*, NKT cells rapidly secrete a variety of cytokines and chemokines, which influence the polarization of $CD4^+$ T cells toward Th1 or Th2 cells as well as the differentiation of precursor $CD8^+$ T cells to effector lymphocytes, and B cells to antibody-secreting plasma cells. Some of these mediators facilitate the recruitment, activation, and differentiation of macrophages and DCs, which results in the production of interleukin (IL)-12 and possibly other factors. IL-12, in turn, stimulates NK cells to secrete IFN-γ. Thus, activated NKT cells have the potential to enhance as well as temper the immune response. This schematic rendition is an adaptation of past reviews (35, 37–41) and works cited in the text.

chain pairs with the mouse TRBV13-2*01 orthologue—TRBV25-1 (Vβ11) to form a functional human semi–invariant NKT cell TCR. Akin to the semi-invariant NKT cells, MAIT cells pair with a limited set of β-chains to form a functional MAIT cell TCR. The conserved nature of the functional NKT and MAIT cell TCRs allow them to recognize their respective ligands—CD1d+lipid/s and MR1 +vitamin metabolites, respectively, by means of conserved interactions—i.e., with an innate-like recognition logic (reviewed elsewhere: refs (29, 30, 34)].

In a similar vein, the pig semi-invariant NKT cells use the pTRAV10 TCR Vα gene segment, which is highly homologous to segments encoding human TRAV10, mouse TRAV11, and rat TRAV14S1—the canonical Vα segments used by the semi-invariant NKT cells in these species. The best alignments for pTRAJ18*01 were TRAJ18, TRAJ18, and TRAJ18, the Jα18 gene segments used by the human, rat, and mouse invariant α-chain, respectively. pTRBV25 is most similar to human TRBV25-1 (Vβ11), mouse TRBV13-2*01 (Vβ8.2), and rat Vβ8.2—the canonical Vβ segments used by the semi-invariant NKT cells in these species (46).

NKT cell functions are controlled by a variety of lipid agonists presented by CD1d molecules. These agonists include glycosphingolipids such as α-galactosylceramide (αGalCer) and α-glucosyldiacylglycerols and related compounds—both of host/self and microbial origins (see Table 1 and references therein). MAIT cell functions are controlled by metabolites in the riboflavin biosynthesis pathway when presented by MR1 (43, 44, 59–62). One such MAIT cell agonist is a derivative of vitamin B2 metabolite 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), which is synthesized by both symbiotic and pathogenic bacteria (43, 44, 59, 60). Consequently, infections with bacteria- harboring mutations in the *rib* gene/s prevent MAIT cell activation, which in some infections can prove fatal (62).

By the last *fin-de-siècle*, the roles for NKT cells were implicated in steering immune responses to pathogens: to bacteria—*Salmonella choleraesuis*, *Listeria monocytogenes*, *Mycobacterium bovis*, and *M. tuberculosis*; to viruses—hepatitis B virus and lymphocytic choriomeningitis virus; to parasites—*Plasmodium* spp., *Leishmania major*, and *Schistosoma mansonii*; and to worms—*Nippostrongylus brasiliensis* [refs (63–80); see also Supplemental Table 1]. How NKT cells were activated by these pathogens was not understood. At that time, the only known NKT cell agonist was αGalCer (49, 81, 82). αGalCer (KRN7000) was isolated from the marine sponge—*Agelas mauritianus*, whose potent antitumor activity is mediated by NKT cells (47–49, 83) (see Box 2). In the ensuing two decades, much has been learnt about how NKT and MAIT cells control immune responses to infections with bacteria and viruses, many of which do not biosynthesize agonistic ligands. There are three distinct ways to activate NKT and MAIT cells (Figure 3): the first is termed TCR agonist–dependent direct activation. In this mode, the presentation of the agonist αGalCer by CD1d or 5-OP-RU by MR1 activates NKT or MAIT cells, respectively (Tables 1–3), to initiate their effector function/s (reviewed in ref (35). The second mode is termed TCR agonist–dependent and cytokine-assisted activation. Weak ligands—e.g., α-galacturonosylceramide (αGalUCer) biosynthesized by *Sphingomonas* spp (50, 51, 87, 111), αGalCer-like asperamide B by *Aspergillus fumigatus* (52), α-glycosyldiacylglycerols from *Borrelia burgdorferi* and *Streptococcus pneumoniae* (54, 55), or self αGalCer or isogloboside 3 (iGb3) induced by certain bacterial infections or sterile inflammation [ref (37, 56, 158–160); for structures, see Table 1]—that poorly activate NKT or MAIT cells require an immune push. That push is provided by inflammatory cytokines produced by the activation of DCs—e.g., IL-1β, IL-12, IL-18, or type I IFNs (96, 103, 116, 131, 155, 161). Hence, the context of infection can influence the activation of NKT and MAIT cells. The third mode of activation occurs in a manner independent of TCR stimulation but is reliant on cytokine/s alone. This mode of NKT and MAIT cell activation is termed TCR-independent inflammatory cytokine–induced activation. Bacteria that do not biosynthesize agonistic lipids but contain microbial pattern recognition receptor ligands such as lipopolysaccharide result in a TCR-independent inflammatory cytokine response from myeloid cells. These inflammatory cytokines can activate NKT cells. This mode of NKT and MAIT cell activation plays a protective role during infectious diseases, especially caused by virus infections (156, 157, 162–166).

Once activated, NKT cells produce a variety of cytokines and chemokines that steer downstream innate and adaptive immune responses. This response includes type I, II, and III cytokines, which are secreted by NKT1, NKT2, and NKT17 cells, respectively. Corresponding MAIT1 and MAIT17 cells and attendant cytokine responses are similarly described. The three subsets emerge under the transcriptional activity of factors similar to those established in conventional $CD4^+$ T cells (Figures 1, 2). Broadly, akin to conventional $CD4^+$ T cells, NKT and MAIT cells play roles in immunity to infections and tumors and in autoimmune and allergic reactions. These features of NKT and MAIT cells are reviewed in detail elsewhere (35, 38). In addition to the three NKT cell subsets, NKT10 cells—which secrete IL-10—play regulatory functions in conjunction with T regulatory cells. NKTfh cells—which provide cognate and noncognate help to conventional B cells to secrete antibodies—may control immunity to human pathogens such as *Borrelia hermsii*, *S. pneumoniae*, and *P. falciparum* (167–169). These features of NKT and MAIT cells are reviewed in detail elsewhere (35),

Human NKT cell responses are as diverse as the mouse NKT cells (170). Two functional subsets were recognized that were segregated by the lack of CD4 or CD8 coreceptor expression (NKT1) or by CD4 expression (NKT2). Human NKT1 cells produce IFN-γ and TNF-α and, when activated under the influence of inflammatory cytokines, upregulate NKG2D and perforin expression priming them for cytotoxic response against infected cells and cancer cells (171, 172). Akin to mouse, the human NKT2 subset, which produces IL-4 and IL-13 and their accumulation in the lungs, may underlie the pathology in chronic asthmatic patients (173). Activated human NKT cells also produce IL-17 (170), which may reflect the existence of an NKT17 subset in humans. Further, NKT17 and MAIT17 subsets are present in higher frequency when compared to NKT1 and MAIT1 subsets in liver perfusates, which produce IL-17 and IFN-γ, respectively (174). Human NKT and MAIT subsets have some semblance to mouse NKT and MAIT subsets, but further studies are necessary to understand how similar they are in the two species.

The evolutionary origins of NKT and MAIT cell subsets have not been traced yet. Both NKT and MAIT cells arose as eutherian

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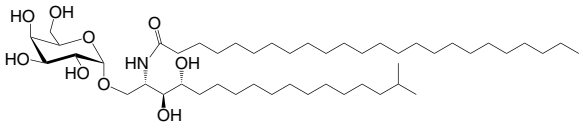
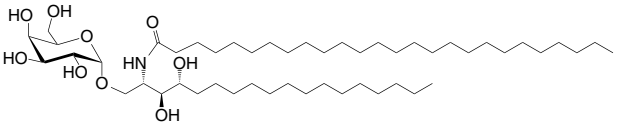
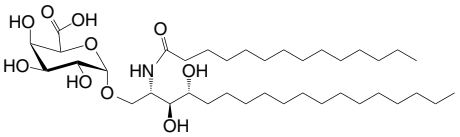
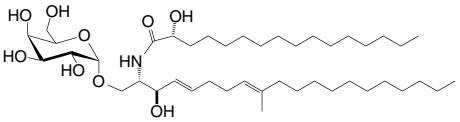
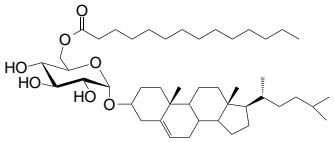
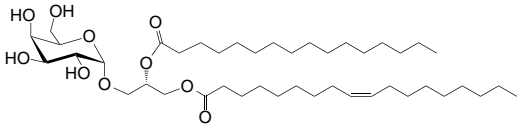
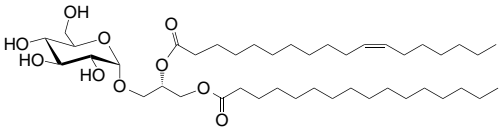
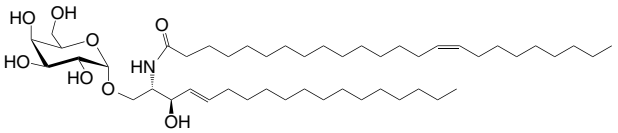
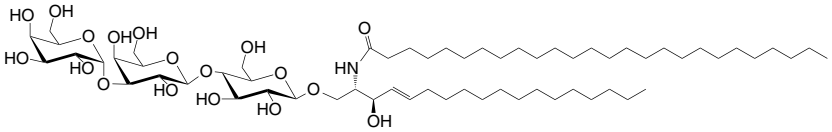
TABLE 1 Natural, synthetic, microbial, and self natural killer T (NKT) cell agonists: structures and properties.

Lipid (class[1]) origin	Chain length[2]	Structure	Agonist activity [3,4]	Ref.
Natural and synthetic				
Agel 9b (GSL) The sponge *Agelas mauritianus*	C17 (C_{16}-Me) phyto C24		Antitumor	(47, 48)
KRN7000 αGalCer (GSL) synthetic analogue of Agel 9b	C18-phyto C26		Very strong; robust IFN-γ, IL-4, and other cytokines	(49)
Microbial				
αGalUCer (GSL)	C18-phyto C14		Weak; *Sphingomonas* spp.	(50, 51)
Asp B (GSL) *Aspergillus fumigatus*	C20:2-C_9 Me C16-C_2 OH		Weak	(52)
Acyl-αGlcChol *Helicobacter pylori*	C14		Strong; binds a small NKT cell subset (mo)	(53)
αGalDAG (GGL) *Borrelia burgdorferi*	*sn1*-C18:1 *sn2*-C16		Weak (mo)-to-none (hu)	(54)
αGlcDAG (GGL) *Streptococcus pneumoniae*	*sn1*-C18:1 *sn2*-C16		Weak	(55)
Self—mammalian cells				
αGalCer (GSL)	C18 C24:1		IFN-γ, IL-4	(56)
iGb3 (GSL)	C18-C24		Weak (mo)-to-none (hu)	(57)

[1]Agel, agelasphin; Asp B, asperamide B; Chol, cholesterol; DAG, diacylglycerol; GalCer, galactosylceramide; GalUCer, galacturonosylceramide; GlcCer, glucosylceramide; *sn*, stereo nomenclature for glycerolipids; GGL, glycoglycerolipid; GSL, glycosphingolipid.
[2]sphingosine/phytosphingosine chain length indicated first and *N*-acyl chain length second,
[3]agonist strength based on Ref (58).
[4]relative potencies in comparison to αGalCer; mo, mouse; hu, human.

BOX 2 A tale of α-galactosylceramides and its biosynthesis.

αGalCer/KRN7000 was first isolated from the marine sponge—*Agelas mauritianus*. As mammalian symbionts—e.g., *Bacteroides fragilis*, biosynthesize αGalCer-related compounds (26, 28, 84), it remains open whether the αGalCer was isolated from *A. mauritianus* or was derived from bacteria living in a symbiotic relationship with those sponges (85, 86). Bacteroidetes and α-Proteobacteria are the residents of sponges, members of which are known to biosynthesize α-anomeric glycosphingolipids that activate NKT cells (26, 28, 50, 51, 87). Of note, however, αGalCer was isolated from an *Agelas*-related marine sponge species—*Axinella corrugata* whose symbionts include α-Proteobacteria (88, 89). Nonetheless, current evidence suggests that the *A. corrugata* αGalCer was derived from the sponge itself and not its symbionts (88, 90). Resolving the source of αGalCer can yield insights into the biosynthesis of αGalCer in mammals (56). One possible route to the biosynthesis of αGalCer and αGlcCer might be the CGT1 (β-galactosylceramide synthase) and CGS (β-glucosylceramide synthase) themselves, which may have an α-linkage retention property. The two hexosylceramide synthases use α-linked uridyldiphosphate-charged sugar donors to form β-linked monohexosylceramides by catalyzing α to β mutarotation prior to the condensation reaction. The potential presence of αGlcCer/αGalCer in the absence of α-hexosylceramide synthase genes within mouse and human genomes poses a quandary, however (56, 91). Biochemical evidence suggests that hexosylceramide synthases may contain α-linkage retention activity, which retains the α-linkage of the charged sugar donor to generate α-linked monohexosylceramides (92–95). This α-anomer retaining activity may explain the synthesis of α-anomeric glycosphingolipids in sponges and mammals, and, potentially, in bacterial species discussed in the text that biosynthesize such lipids.

innovations approximately 125 million years ago in an ancestor after the therian mammals split to metatherians and eutherians—the true placental mammals (35, 175, 176). Among mammals other than the mouse and human, the development and function of NKT cells in pigs—*Sus scrofa* (var. *domesticus*)—are intensely studied.

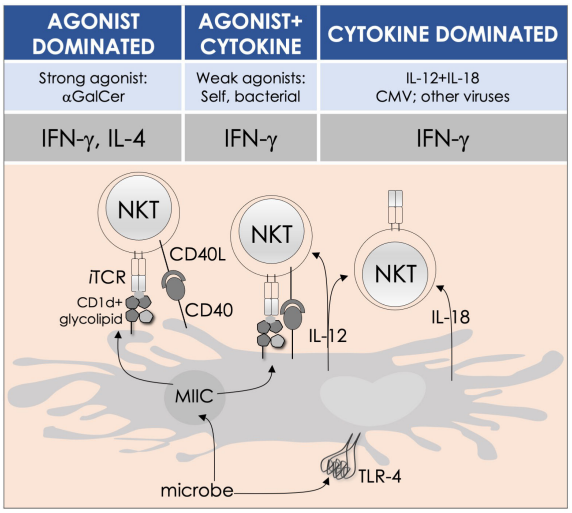


FIGURE 3
Modes of NKT and MAIT cell activation by microbes. Potent agonists—such as αGalCer, directly activate NKT cells, without the need for a second signal, in a TCR signaling–dominated fashion (left panel). Alternatively, microbes containing TLR ligands such as LPS activate NKT cells by inducing IL-12 production by DCs, which amplifies weak responses elicited upon the recognition of CD1d bound with self-glycolipids by the NKT cell TCR. Several endogenous lipid agonists have been identified and characterized (see Table 1). Some microbes—such as *Sphingomonas capsulata* and *Borrelia burgdorferi*—synthesize α-anomeric glycolipids for their cell walls. These glycolipids, when presented by CD1d, weakly activate NKT cells directly. In the presence of a second signal—generally a proinflammatory cytokine such as IL-12, such weak agonists strongly activate NKT cells (middle panel). By contrast, the mode of MAIT cell activation appears to be agonist concentration dependent: microbes that produce high levels of 5-OP-RU—a product of *ribD*-controlled catalytic activity, directly activate MAIT cells, while those that produce low levels of 5-OP-RU require a cytokine boost. Unlike conventional T cells, cytokines alone can activate both NKT and MAIT cells. Such cytokines, which include a combination of IL-12 and IL-18, activate NKT cells in a TCR-independent manner (right panel). This diagram renders the different strategies for NKT cell activation; they apply to MAIT cells as well. Similarities and differences, if any, are described in the text. Adapted from past reviews (35, 37, 38, 41) and works cited in the text.

Pig NKT cell subsets were recently described using the single-cell RNA sequencing analysis of more than 11,000 differentiating thymic NKT cells (177). The vast majority of porcine NKT thymocytes resemble mouse NKT2 cells. Surprisingly, these pig NKT2-like cells do not differentiate into NKT1 or NKT17 subsets. Instead, some develop into a population enriched for interferon-stimulated genes that simultaneously maintain an NKT2-like gene profile, as well as two very rare subsets, designated iNKT-swine (sw) 1 and iNKT-sw2. iNKT-sw1 and iNKT-sw2 cells are most similar to two minor populations of innate-like CD8αα T cells present in pig thymocytes, sharing the expression of *FCGR3A*, *ZNF683*, *NKG7*, and MHC class II–encoding genes. They also downregulate tissue emigration genes, suggesting that both are long-term thymus residents. Similar thymus-resident populations of MAIT cells, γδ T cells, and CD8αα T cells have been described before and have been speculated to modulate thymocyte differentiation to respond to peripheral perturbations, such as infection (24, 174, 178, 179). Interestingly, iNKT-sw2 cells are enriched for *CD244* and *CXCR6*, which are upregulated on a newly discovered population of NKT cells found in mice and humans that are highly cytotoxic and protect mice from melanoma metastasis and influenza infection (180).

Although peripheral pig NKT cells can be stimulated nonspecifically to secrete IFN-γ and IL-17 (181, 182), thymus-resident pig NKT cells appear to produce little if any IFN-γ, IL-4, or IL-17 under steady-state conditions (177). One explanation for the surprisingly undifferentiated state of pig NKT thymocytes is that they emerge from the thymus in a functionally immature state and undergo further differentiation in the periphery. Since human NKT thymocytes do not also produce IFN-γ or IL-4 under steady-state conditions, it is possible that the diversity of NKT thymocyte subsets observed in mice is unusual and that it is more normal for species with the NKT-CD1d system to express fewer and/or less differentiated NKT thymocytes.

In comparison to NKT cells, relatively little is known about porcine MAIT cells. However, MAIT cell TRAV1-TRAJ33 TCRα sequences have been cloned from pig blood and tissues and found to pair with a limited number of TCR β-chains (183). It was further shown that pig MAIT cells can be $CD4^{POS}CD8^{POS}$, $CD4^{POS}CD8^{NEG}$, and $CD4^{NEG}CD8^{POS}$ T cells and express transcripts for the MAIT cell–associated surface molecules IL-18Rα, IL-7Rα, CCR9, CCR5, and/or CXCR6 and the transcription factors PLZF and T-bet or RORγt.

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TABLE 2 Role of NKT cells in microbial infection and immunity.

Microbe	Activation mechanism/s (antigen)[1]	NKT cell role[2]	Model	Infection route	Reference (s)
Gram-positive bacteria					
S. pneumoniae	CD1d-dependent self and nonself (αGalDAG) + IL-12	Protective	Jα18$^{-/-}$, CD1d$^{-/-}$	i.n., i.t.	(55, 96, 97)
S. aureus	Non-self (lysyl-PG)	Not protective	Jα18$^{-/-}$, CD1d$^{-/-}$	i.v.	(98, 99)
L. monocytogenes	Self + IL-12	Protective Detrimental	CD1d$^{-/-}$	i.v.	(99–101)
Gram-negative bacteria					
P. aeruginosa	CD1d-dependent (unknown)	Protective	CD1d$^{-/-}$	i.n.	(102)
		Not protective	Jα18$^{-/-}$, CD1d$^{-/-}$	i.t.	
S. typhimurium	CD1d-dependent self (iGb3)	Not protective	CD1d$^{-/-}$	p.o.	(51, 99, 103, 104)
H. pylori	CD1d-dependent nonself (αCgT)	Protective	Jα18$^{-/-}$	p.o.	(105)
C. trachomatis (muridarum)	CD1d-dependent nonself (GLXA)	Detrimental Not protective	CD1d$^{-/-}$	i.n. intravaginal	(106–108)
C. pneumonia	CD1d-dependent self and nonself (unknown)	Protective	Jα18$^{-/-}$, CD1d$^{-/-}$	i.n.	(109)
L. pnemophilla	Cytokine dependent, IL-12	Detrimental	Jα18$^{-/-}$	i.t.	(106–108)
Francisella tularensis subspp. *tularensis SchuS4*	CD1d dependent (unknown)	Detrimental	CD1d$^{-/-}$[4]	i.n.	(110)
Ft subspp. *holarctica* live vaccine strain	CD1d dependent (unknown)	Detrimental	CD1d$^{-/-}$	i.n.	(110)
F. novicida	CD1d dependent (unknown)	Not protective	CD1d$^{-/-}$	s.c., i.d.	(110)
α-Proteobacteria					
Sphingomonas spp.	CD1d dependent nonself (αGlcACer) + IL-12	Protective (low dose) Detrimental (high dose)	Jα18$^{-/-}$, CD1d$^{-/-}$	i.v.	(50, 51, 96, 111)
N. aromaticivorans	CD1d-dependent nonself (αGalUCer)	Primary biliary cirrhosis	CD1d$^{-/-}$	i.v.	(112)
Spirochetes					
B. burgdorferi	CD1d-dependent, nonself (αGalDAG) + IL-12	Protective	[4]CD1d$^{-/-}$	i.d.	(54, 96, 113, 114)
Mycobacteria					
M. tuberculosis	CD1d-dependent self	Not protective Protective[3]	CD1d$^{-/-}$ Cell transfer	i.v. aerosol	(72, 115)
Fungi					
A. fumigatus	CD1d-dependent non-self (asperamide-B) and self + IL-12	Detrimental (AHR)[3] Protective (early)	CD1d$^{-/-}$	i.n. i.t.	(52, 116)
C. neoformans	CD1d-dependent self	Protective	CD1d$^{-/-}$	i.t.	(117)
Parasites					
P. berghei	ND	Detrimental	CD1d$^{-/-}$	i.d.	(118)
P. yoelii	CD1d dependent	Protective	CD1d$^{-/-}$	i.v.	(119)
T. gondii	ND	Protective Detrimental[3]	Jα18$^{-/-}$, CD1d$^{-/-}$ Jα18$^{-/-}$, CD1d$^{-/-}$; Vα14^{tg}	p.o.	(120, 121)

(Continued)

TABLE 2 Continued

Microbe	Activation mechanism/s (antigen)[1]	NKT cell role[2]	Model	Infection route	Reference (s)
L. donovani	CD1d dependent, lipophosphoglycan	Protective	CD1d$^{-/-}$	i.v.	(122)
E. histolytica	CD1d dependent, foreign antigen (EhLPPG)	Protective	CD1d$^{-/-}$	i.h.	(123)
Viruses					
HSV-1	CD1d dependent, nonself (glycoprotein B and US3)	Protective Not protective[3]	Jα18$^{-/-}$, CD1d$^{-/-}$	Scarification	(124–126)
HSV-2	ND	Protective	CD1d$^{-/-}$	Intravaginal	(127)
Sendai virus	ND	Detrimental	Jα18$^{-/-}$, CD1d$^{-/-}$	i.n.	(128)
RSV	CD1d dependent, self	Protective	CD1d$^{-/-}$	i.n.	(129, 130)
Influenza virus H1N1 and H3N2	ND	Protective	Jα18$^{-/-}$, CD1d$^{-/-}$	i.n.	(131–135)
HBV	ND	Protective	Jα18$^{-/-}$, CD1d$^{-/-}$	i.v.	(136)

[1]see Table 1 for the structures of NKT cell agonists.
[2]differential outcomes in the different studies may have arisen from the use of different microbial/parasite strains.
[3]the outcome of infection in Jα18$^{-/-}$ mouse model may require additional validation as the deletion of this TRAJ gene segment by homologous recombination had resulted in the deletion of additional TRAJ gene segments, including TRAJ33—the gene segment essential for the construction of MAIT cell TCR α-chain; additional TRAJ gene segment losses severely constricted the TCR repertoire of conventional T cells as well [see ref (137)].
[4]BALB/c background of mouse strains used in these studies; others were in C57BL/6 background.
AHR, airway hyperreactivity; GLXA, chlamydial glycolipid exoantigen; ND, not determined; i.d., intradermal; i.h., intrahepatic; i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; i.t., intratracheal; p.o., per oral; s.c. subcutaneous.

Collectively, current evidence indicate that pig NKT and MAIT cells have characteristics similar to their human and mouse counterparts. Nonetheless, several key lineage-defining differences in mouse and pig NKT cell subsets point toward the acquisition of species-specific innate/innate-like T cell adaptations, perhaps for different pathogens or may reflect the different niches in which the two species evolved and the symbiotic microbes they live with. Hence, the species-specific developmental aspects should be considered, especially in the light of ecology and evolution, when assessing the suitability of mice and pigs as biomedical models for innate/innate-like T cell research.

The hygiene hypothesis: yes, you may pick your nose and eat it

This subtitle was motivated by a burgeoning field of rhinotillexis—yes, nose picking, a new area of scientific enquiry. Beneath this otherwise aversive and socially inept and unacceptable behavior, yet innate to primates, may lie a means to the periodic reinforcement of disease tolerance [see Box 3; ref (184)].

The pervasive presence of microbes, flourishing at every nook and cranny of the earth and on the surfaces and the insides of metazoans, make them a formidable friend and foe. Hence, on being birthed unto a dirty world, to gain fitness, metazoans found ways to befriend and tame microbes, especially the beneficial, and ward off unfriendly ones over eons of evolution. Symbiosis emerged, lending to fitness in both directions—in the metazoan hosts and their microbial partners. So much so, symbiosis has led to the coevolution of the hosts with their microbiota, or vice versa, to the point of codependence, wherein the immune system evolved to manage the microbial consortium from going 'wild' and, reciprocally, the diversity of the consortium and its biosynthetic products control the immune system from going 'rouge'. Thus, the hygiene hypothesis postulates that early life exposure to a full range of diverse microbes (and worms) promotes the development and maturation of an immune system—which reacts in a balanced measure to prevent disease whether incited by external (infections and allergens) or internal (autoinflammation) agencies of inflammation (188).

For example, under sterile, germ-free conditions, the immune system of the laboratory mouse develops and matures poorly, rendering them susceptible to infectious diseases and autoimmune disorders such as colitis (189–196). Conversely, the equilibration of the gut microbiome of the laboratory mouse to that of the 'dirty' pet store mouse by cohousing the two, altered, in the former, the immune cell composition at the barrier sites, resistance to infection, and T-cell differentiation in response to virus infection (197). A similar equilibration of the gut microbiome of a laboratory mouse raised under germ-free conditions by the transfer of the gut microbiota from a feral relative of the laboratory mouse and its maintenance over several generations by breeding increased disease tolerance and fitness. Inflammatory responses in such mice to a lethal influenza virus challenge was highly tempered and so was mutagen- and inflammation-induced tumorigenesis (198). All of these altered immune features acquired by the laboratory mouse reflected those of the pet store or feral mouse and those of the adult human (197, 198). The ability to approximate the human immune system in the laboratory mouse by the transfer of the microbiome indigenous of a feral mouse may facilitate and enhance preclinical vaccine development and testing (198–201). Furthermore, the role of the microbiota in the maturation of T cells may explain the intriguing finding that, at steady state—in the absence of an infection—DC emigrees from the barrier epithelium of nonlymphoid tissues stochastically prime and program resting,

TABLE 3 Role of mucosal-associated invariant T cells in microbial infection and immunity.

Microbe	Activation mechanism/s	MAIT cell role[1]	Model	Infection route	Reference (s)
Gram-positive bacteria					
C. difficile	MR1 and cytokine dependent	Detrimental	Human PBMC	*in vitro*	(138)
S. pneumoniae	MR1 dependent, SAgs	Detrimental	C57BL/6, CAST : EiJ	*in vitro*	(139)
	MR1 dependent, Spn polysaccharide	Protective	Human PBMCs	*in vitro*	(140)
	MR1 (SAgs) and cytokine dependent IL-12 and IL-18	Detrimental	Human PBMCs	*in vitro*	(141)
S. aureus	MR1 dependent, SAgs	Detrimental	C57BL/6, CAST : EiJ	*in vitro*	(139)
Gram-negative bacteria					
K. pneumoniae	ND	Protective	$MR1^{-/-}$	i.p.	(142)
P. aeruginosa	ND	Protective	Human PBMCs	*in vitro*	(143)
L. longbeachae	MR1 dependent	Protective	$MR1^{-/-}$	i.n.	(144)
H. pylori	MR1 dependent	Detrimental	$MR1^{-/-}$	p.o.	(145, 146)
E. coli	MR1 dependent	Protective	$V\alpha19^{tg}$, $MR1^{-/-}$	i.p., i.v.	(147)
S. enterica serovar *Typhi*	MR1 dependent	Detrimental	Human PBMCs	*in vitro*	(148)
S. enterica serovar *paratyphi A*	MR1 dependent	Protective	Human PBMCs	*in vitro*	(149)
S. typhimurium	MR1 dependent	Protective	Human PBMCs	*in vitro*	(33)
F. tularensis subspp. *holarctica LVS*	ND	Protective	$MR1^{-/-}$	i.v.	(150)
	MR1- and cytokine- dependent IL-12p40	Protective	$MR1^{-/-}$	i.n.	(151)
Mycobacteria					
M. abscessus	MR1 dependent	Protective	$V\alpha19^{tg}$, $MR1^{-/-}$	i.p., i.v.	(147)
M. tuberculosis	MR1 dependent, riboflavin derivatives	Protective	C57BL/6, Cast;EiJ	i.n.	(152)
Viruses					
Dengue virus	Cytokine dependent: IL-12 and IL-18	Protective	Human PBMCs	*in vitro*	(153)
Zika virus	Cytokine dependent: IL-12 and IL-18	Protective	Human PBMCs	*in vitro*	(153)
HIV-1	Cytokine dependent: IL-12 and IL-18	Protective	Human PBMCs	*in vitro*	(154)
Influenza A	MR1 and cytokine dependent	Protective	Human PBMCs and LDMCs	*in vitro*	(155)
	Cytokine dependent: IL-18	Protective	Human PBMCs	*in vitro*	(156)
Influenza virus H1N1	Cytokine dependent: IL-12 and IL-18	Protective	$MR1^{-/-}$	i.n.	(157)

[1]differential outcomes in the different studies may have arisen from the use of different microbial/parasite strains.
ND, not determined; i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; p.o., per oral.

naïve $CD8^+$ T cells within the local draining lymph nodes for tissue residency (202).

After development in the thymus, NKT and MAIT cells emigrate and home to lymphoid and nonlymphoid tissues, presumably to patrol and maintain the integrity of the tissue borders. The NKT and MAIT cell content at these borders varies by tissues and the mouse strain. Their tissue distribution and functions are best studied in the mouse; only a bit is known of

BOX 3 Rhinotillexis—a new, burgeoning field of scientific enquiry.

It is so new and burgeoning that the National Public Radio felt compelled to interview Dr. Anne-Claire Fabre—a pioneer in the field at the Naturhistorisches Museum in Bern, Switzerland, on the matter (npr.org/2022/11/15/1136423436/researchers-dig-into-why-nose-picking-is-a-common-behavior). It is so new that the word rhinotillexis is neither in the Oxford English Dictionary nor the Merriam-Webster American English Dictionary yet but has appeared in Wikipedia, the free encyclopedia, however. Unless careful, excessive rhinotillexis may cause self-induced ethmoidectomy, especially if one suffers from rhinotillexomania (see en.wikipedia.org/wiki/Nose-picking). Rhinotillexis is not peculiar of repulsive men or their man cubs, but it is a primate thing [(184) and references therein]. Self-vaccination, per oral distribution of nasal microflora, and dental hygiene are a few proposed immunologic attributes of rhinotillexis (185–187).

their distribution in the human body (24, 172, 203–205). In mice, thymic NKT cell development, after commitment to this lineage and positive selection, progresses from stage 0 to stage 1 to stage 3—the mature NK1.1^{POS} NKT cells, known to consist largely of NKT1 cells. Of these, CD24^{NEG} CCR7^{POS} stage 1/2 NKT cells emigrate from the thymus and seed both the lymphoid and non-lymphoid tissues, where they undergo further maturation, largely driven by the local cytokine milieu (206–208), and perhaps the microbiota.

Verily, the early life exposure of NKT and MAIT cells to the host microbiota has profound, lifelong effect/s on these innate-like lymphocytes (25, 27, 161). Their development itself is dependent on positive selection by agonistic ligands—αGalCer in the case of NKT cells and 5-OP-RU in the case of MAIT cells [reviewed in refs (44, 203), and references therein]. The origins of these agonists are less clearly defined. Because CD4^{POS}CD8^{POS} thymocytes activate Vα14i NKT cell hybridomas, it is thought that an NKT cell agonist/s may be of self origin. Thus, β-galactosylceramide synthase (CGT)-deficient thymocytes foster NKT cell development; hence, βGalCer or its derivatives are less likely the thymic NKT cell agonist. Conversely, β-glucosylceramide synthase (CGT-1)–deficient thymocytes poorly activate Vα14i NKT cell hybridomas and conditional CGT1-deficient thymocytes to not promote NKT cell development (57, 209). As βGlcCer itself does not activate Vα14i NKT cell hybridomas, a βGlcCer derivative—iGb3 or a self αGlcCer (Table 1)—is a potential NKT cell–activating self-agonist. While iGb3 synthase deficiency does not alter NKT cell development and function and no known mammalian enzyme/s synthesize α-anomeric glucosylceramide or galactosylceramide, how these agonists are biosynthesized is unclear [see Box 2 for details, see ref (35)]. Alternatively, as several gut symbionts common to many mammals biosynthesize α-anomeric glycosylceramides, their transport by lipid transfer proteins such as apolipoprotein E (210) could potentially deliver the agonist/s to the thymus. This is less likely because NKT cells develop in germ-free mice, but they are not without defects (25, 27, 211).

In a similar vein, mammalian cells do not biosynthesize vitamin B2, whose precursor is a precursor to the MAIT cell agonist 5-OP-RU (33, 59, 147, 212), but rather acquire it from symbionts (161, 203, 213). Consequently, MAIT cells develop poorly in germ-free mice bred under sterile conditions (205, 214). By contrast, NKT cells develop in such mice as noted above. It appears as though NKT cells and MAIT cells compete for niche such that, mice, which have more NKT cells than humans, have a low frequency of MAIT cells. Reciprocally, humans have a high frequency of MAIT cells but are low in NKT cell frequency (205, 214).

NKT cell numbers in the intestinal mucosa are controlled by the neonatal colonization of bacterial symbionts. NKT cells accumulate in significant numbers within the intestinal mucosa, lungs, and liver but not the thymus or spleen of germ-free mice (25, 27). The increased NKT cell number observed in germ-free mouse intestinal mucosa perhaps owes to increased levels of CXCL16—the ligand of CXCR6, the levels of which are controlled by the gut microbiota (25, 215). Moreover, NKT cells developing in germ-free mice do not mature and are hyporesponsive to the glycolipid agonist αGalCer (27). Colonization with NKT cell agonist–bearing bacteria—e.g., *Sphingomonas yanoikuyae*, during early life but not in adulthood restored NKT cell maturation and normoresponsiveness to αGalCer (27). Nevertheless, αGalCer compounds synthesized by different bacterial symbionts—e.g., *Bacteriodes fragilis* and *S. yanoikuyae* (see Table 1), appear to exert differential effects on developing NKT cells (26, 28, 84); why this is awaits resolution.

Early-life microbial ecology has implications for health. Thus, consistent with increased NKT cell frequency in the gut and lungs, germ-free mice are overly sensitive to oxazolone/dextran sodium sulfate–induced inflammatory colitis and airway hypersensitivity (25, 27, 215). This disease phenotype is reversed by early-life exposure to *B. fragilis*–derived glycosphingolipid(s) (28). Whether the normal development and functions of human NKT cells require interactions with the gut microbiota awaits discovery. So also, whether the microbiota—known to vary between individuals of different genetic, ethnic, and geographic backgrounds (216)—controls human peripheral NKT cell frequency, which varies tremendously between individuals—from undetectable to 5%—remains unknown.

Unlike the gut, which hosts swarms of thousands of microbial species, it is generally assumed that the internal organs not exposed to the outside—such as the liver, heart, and brain—are sterile, devoid of resident microbes. Counter to this assumption, a recent study found mouse and human liver hosts its own, unique microbial consortium distinct from the gut as it was enriched in Proteobacteria (217). This microbiome was seeded from the gut microbiota in a selective manner that depended on the sex of the mouse and the local environment. Moreover, the local immune response was dependent on the liver microbiome, which was influenced by *Bacteroidetes* species. The hepatic microbiome controlled antigen-presenting cell maturation and adaptive immunity through the mediation of NKT cells (217). *Bacteroidetes* species biosynthesize αGalCer (26, 28, 84), which activate NKT cells to secrete CCL5 chemokine, in turn, recruiting immune cells to the liver and their activation, expansion, and function (217). Hence, local tissue microbiomes influence local immunity in an NKT cell—dependent mechanism.

NKT cell homeostasis described above requires intestinal microbial lipid presentation by CD11c^{+} DCs and macrophages (218). Reciprocally, NKT cells appear to control the bacterial composition of the gut microbiota. Consequently, dysbiosis and disruption in intestinal homeostasis ensue in mice deficient in NKT cells—CD1d$^{-/-}$ (218–221) or Jα18$^{-/-}$ (222–224) mice —or mice that lack CD1d expression by DCs, which thereby are unable to present intestinal lipids to activate local (218), intestinal mucosal NKT cells. This dysbiosis and altered intestinal homeostasis are consistent with alterations in the IgA repertoire (223, 225) and the induction and function of regulatory T cells within the gut (192, 222), which are observed in these mice as well (218, 223).

By contrast to the above reports, a recent study found that there are no differences in the composition of the gut microbial consortium in CD1d$^{-/-}$ mice (226). Similarly, no differences were observed in the consortium in Vα14 transgenic mice, which carry high numbers of NKT cells—largely the IL-4 producing NKT2 subset (227, 228). While NKT cell activation by peroral delivery of αGalCer minimally, yet consistently, altered the diversity of the consortium, this effect was only transient. However, the shift in

microbiota composition was comparable to the natural drift found in the colony. Critically, this report noted that the natural drift in the microbial composition of individual vivarium over time and, perhaps, the differences in the microbial composition between vivaria, but not NKT cells, had significant influence on the composition of the mouse gut microbial consortium even at steady state (226). Because this is a report from a single center, whether mouse and human NKT cells have an impact on the microbial consortium of the gut will require a concerted, multicenter study.

Mouse and human skin abound with MAIT cells. MAIT cell frequency varies between individuals (229). MAIT cell frequency is similar in genetically identical mice housed in the same cage but varied between those housed in distinct cages. This suggested that the microbiota may have a role in determining the frequency. Studies in germ-free mice revealed that MAIT cells depended on early-life exposure to gut microbial consortium (45, 161, 203, 213). Hence, germ-free mice failed to develop MAIT cells that localize to barrier tissues—such as the skin, when exposed to microbes later in life.

The development of mouse MAIT cells in the thymus is dependent on the presentation of a by-product of riboflavin biosynthesis—5-OP-RU (33, 44, 59, 147, 203, 212). Even though flavonoids are essential, mammalian cells are riboflavin auxotrophs. They depend on external sources of riboflavin, which is biosynthesized by several bacteria and fungi—both symbionts and pathobionts, as well as plants. The microbial origin of riboflavin and biosynthetic metabolites explains the intimate dependence of MAIT cell development on the gut microbiota. 5-OP-RU is biosynthesized in a *ribD*-dependent manner by the gut, and potentially the skin as well, transported to the thymus, and made available to MR1-expressing cells for assembly and display at the cell surface (213). The mechanism by which 5-OP-RU is transported to the thymus and how cells capture it to make available in the ER lumen for assembly with MR1 are poorly, if at all, understood (203).

Thymic MAIT cell emigrees home to barrier tissues. Their numbers at the barrier tissues depend on the local concentration of microbial derivatives, which is emulated by the painting of skin with varying concentrations of 5-OP-RU (161, 213). In the skin, they surveil the dermal—epidermal interface. Cutaneous-resident cells are the MAIT17 subset; their homeostasis is IL-23 dependent, and they respond to skin commensals upon MR1-ligand recognition in an IL-1- and IL-18-dependent manner. These MAIT17 cells are genetically programmed for tissue repair and, hence, contribute to normal skin physiology (161). Given the intimacies of NKT and MAIT cells with the symbiotic consortium, one might wonder what roles innate-like effector lymphocytes might have in precipitating *erythema toxicum neonatorum*—which is perhaps an innate immune response to skin microbiont/s that may have penetrated the newborn infant (230).

When van Leeuwenhoek peered down his microscope, curious what might live on his teeth, and perhaps his gums, little did he know he would find many 'little animals'. In his *Letter 39* to the Royal Society, he claimed,

"*For my part I judge, from myself (howbeit I clean my mouth …), that all people living in our United Netherlands are not as many as the living animals that I carry in my own mouth this very day: for I noticed one of my back teeth, up against the gum, was coated with the said matter for about the width of a horse-hair, where, to all appearance, it had not been scoured by the salt for a few days; and there were such an enormous number of living animalcules here, that I imagined I could see a good number of 'em in a quantity of this material that was no bigger than a hundredth part of a sand-grain*" (from a collection of surviving van Leeuwenhoek letters, translated and compiled in ref (6). [see letter 39: *Phil. Trans.* XIV (231) 568, 1684)].

What those 'little animals' or 'animalcules' on man's teeth meant remained cloaked for over two centuries. Elie Metchnikoff had a hunch to which, later in his career and life, he laid, to an obsession, much attention to prolong his life, in futility notwithstanding (232). The foregoing advances, which awaited next-generation 'omics' technologies and platforms, vindicates Metchnikoff's hunch on beneficial and harmful gut microbes and lends support to the physiologic functions of early-life exposure to a diverse array of microbes—and, hence, the hygiene hypothesis.

Kämpfe únd schlächten of natural killer T and mucosal-associated invariant T cells with pathogens

NKT cells and MAIT cells perform specialized roles during infections to confer immunity to the host as they struggle (kampf) with and battle (schlacht) pathogens (see Tables 2, 3 and Supplementary Tables 1, 2). While both possess the phenotype of activated T cells, their induction differs from conventional T cells in that they can be triggered during pathogen infections through invariant receptors and cytokine signals in much the same fashion as innate cells. This results in the rapid secretion of multiple cytokines that are released with similar kinetics to innate cell-derived cytokines—i.e., minutes to hours after stimulation. Accordingly, NKT and MAIT cells can influence the behavior of cells in the innate branch of the immune response while also shaping downstream adaptive immune responses. Over the past decades, it has become clear that the innate properties of NKT and MAIT cells are shared by a wide variety of MHC class I–like restricted innate-like αβ T cells with invariant TCRs that are widespread among jawed vertebrates [reviewed in Ref (233)]. These types of lymphocytes are specialized to allow the recognition of common or particular pathogens with relatively few T cells (231). A good example is *Xenopus laevis* (African clawed frog) tadpoles, which are able to survive in antigen-rich waters using 15,000–20,000 T cells exhibiting limited TCR diversity (234).

As regard the role of NKT cells in immunity, mice deficient in CD1d or TRAJ18 that lack invariant NKT cells have shown that these cells play nonredundant roles in several models of infectious disease (235); NKT cell–deficient mice are more susceptible to several bacteria species (Table 2 and Supplemental Table 1), including *S. pneumoniae* (97, 236), *Borrelia burgdorferi* (113), *Sphingomonas* spp. (50, 51), *Pseudomonas* spp. (102), *Chlamydia pneumoniae* (109), and *M. tuberculosis* (73). They also exhibit

greater susceptibility to fungal infections with *Cryptococcus neoformans* (117) and *Aspergillus fumigatus* (116); viral infections with herpes simplex virus (124, 237), hepatitis B virus (80, 136), and influenza A virus (131, 132, 238); and protozoan parasite infections with *Plasmodium* spp (76) and *L. donovani* (239). A wide array of microbes and microbial products can stimulate NKT cells, either by direct TCR activation, cytokine-mediated activation, or a combination of both and induce them to express activation markers and cytokines, which have diverse effects on other immune cells and the course of an infection (see Tables 1, 2 and references therein). Indeed, microbially activated NKT cells typically secrete a narrower range of cytokines than αGalCer-stimulated NKT cells, which are usually predominated by IFN-γ. This is consistent with the paradigm that the microbial activation of NKT cells is mediated, to a large extent, through innate cytokines such as IL-12 and IL-18, with weak or no TCR stimulation (240). In some infections, NKT17 cells play a significant role. NKT17 cells in a granulocyte–monocyte colony-stimulating factor (CSF2)–dependent manner plays a protective role against *S. pneumoniae* infection of mouse lungs (236). While *Csf2*-deficient NKT cells are impaired in αGalCer-induced cytokine secretion and the transactivation of downstream innate and adaptive immune responses (241), anti-CSF2 blocking experiments confirm the role of NKT17 cell–derived CSF2 in immunity against *S. pneumoniae* (236). Moreover, NKT cells activated by microbes do not usually undergo systemic expansion *in vivo* even when they contain NKT cell antigens. However, NKT cells have been found to congregate at the sites of infection in mice infected with lymphocytic choriomeningitis virus (79), malaria parasites (119), and *C. neoformans* (117). They have also been shown to expand in the lungs and draining lymph nodes of pigs infected with influenza and in the peripheral blood, draining lymph nodes, and lungs of pigs infected with African swine fever virus (182). An intriguing aspect of NKT cell biology is that these cells are programmed to undergo apoptosis and/or become functionally anergic after stimulation (242–244). This reduces the risk of a cytokine storm or chronic inflammation arising from the large efflux of proinflammatory cytokines that activated NKT cells produce. Usually, the degree of NKT cell deletion/dysfunction corresponds with the strength of activation, with some microbes such as the lymphocytic choriomeningitis virus capable of rendering NKT cells anergic for up to 3 months after infection (79, 245). Nevertheless, the overactivation of NKT cells does occur in some mouse models of infection, especially in tissues where NKT cells are found at high concentrations, such as the liver in mice (80, 246, 247).

Among the lessons learnt from studying NKT cells in mice is that genetic background can strongly influence the immunomodulatory activities of NKT cells. For example, the same αGalCer analog treatment protocols cause divergent effects on disease between different mouse strains in the mouse models of autoimmune diabetes (248), experimental autoimmune encephalomyelitis (249), collagen-induced arthritis (250, 251), and systemic lupus erythematosus (252). Such differing outcomes are probably related to the diverse concentrations and functional phenotypes of NKT cells that exist among inbred mouse strains. For instance, in a survey of 38 inbred mouse strains, NKT cells as a percentage of αβ T cells ranged from 3.2% to 0.01% in peripheral blood, 4.12% to 0.02% in the spleen, and 9.39% to 0.02% in the thymus (253). The proportion of $CD4^+$ to $CD4^-CD8^-$ double-negative NKT cells showed similar profound strain variation. Functional differences have been ascribed to these subsets, with the $CD4^+$ subset exerting immunological tolerance in several disease models.

Humans present comparable levels of heterogeneity in NKT cell frequency and cytokine secretion profiles (171, 172, 254–258), which may result in distinct NKT cell responses to microbial infections that vary between individuals. However, whether NKT cells play nonredundant roles in human infectious diseases is largely unknown. Infection with the human immunodeficiency virus, dengue virus, and *M. tuberculosis* have been linked to reduced NKT cell responses to subsequent αGalCer stimulation (259–261). While these results suggest that at least some of the findings from mouse NKT cell studies apply to human infections, there is little evidence to indicate that humans with unusually high or low NKT cell concentrations or effector responses have altered susceptibility to microbial infections. Moreover, assessing this relationship is complicated by the fact that circulating NKT cells are often a poor reflection of NKT cells in organs and tissues (253, 254). In due course, questions about the translatability of mouse model studies may be partly addressed using CD1d knockout pigs as pig and human immune systems share many similarities, and pigs can be infected with a wide range of human pathogens (262–266).

MAIT cells are activated by microbial species that have an intact riboflavin pathway (Table 3). Accordingly, mice deficient in MAIT cells have an impaired ability to clear 5-OP-RU-producing bacteria, such as *Francisella tularensis* (151, 267), *M. bovis* bacillus *Calmette*-Guérin (268), *M. abscesses* (147), and *Legionella longbeachae* (144). Furthermore, TRAV1-TRAJ33 TCR-transgenic mice that express high concentrations of MAIT cells are more resistant to disease in a mouse model of *M. tuberculosis* infection (147). The mechanisms underlying MAIT cell antimicrobial immunity are not fully understood (see Supplemental Table 2). However, MAIT cells can lyse infected cells through perforin and granzymes (269, 270). They also secrete a variety of effector cytokines, such as IFN-γ, TNF-α, GM-CSF, and IL-17, which potentiate bacterial killing through myeloid cell activation (44, 196, 203, 205, 271, 272).

In addition to TCR-mediated activation, MAIT cells can respond to microbial infections through a variety of cytokine receptors that these cells express, including receptors for IL-1, IL-7, IL-12, IL-15, IL-18, and IL-23 (203, 271). This capacity for TCR-independent stimulation enables MAIT cells to participate in immune responses against viruses that do not produce 5-A-RU derivatives. For instance, in a mouse model of lethal influenza virus infection, MR1-deficient mice had a significantly higher mortality rate than MR1-intact mice (157). Similar results have been reported for both CD1d and TRAJ18 knockout mice demonstrating that NKT cells also play a nonredundant role in influenza virus infections (131, 132, 238). However, while NKT cells were found to be important for inhibiting virus replication, MR1-deficient mice had a similar virus load to MR1-intact mice. Moreover, TCR-dependent stimulation was found to be indispensable and dispensable for NKT cells and MAIT cells, respectively, to control

influenza virus infections (132, 157). These results suggest that there exists significant overlap as well as cell type–specific differences in the antiviral activity of NKT cells and MAIT cells.

The role of MAIT cells in human antimicrobial responses remains largely uncertain. However, their high abundance in humans suggests that they may play a more prominent role in host defense and tissue homeostasis than they do in mice. MAIT cell deficiencies have not been directly associated with susceptibility to a particular pathogen in humans. Nevertheless, the frequency of MAIT cells has been found to decrease in the blood of humans infected with various types of bacteria. In some cases, this was accompanied by an increase in MAIT cell frequency at the site of infection (203, 272), suggesting that circulating MAIT cells migrate from circulation to the infection site.

In addition to their contribution to antimicrobial immunity, MAIT cells play a role in wound healing, including repairing host tissues damaged by immune cells during pathogen clearance (203, 272). Activated MAIT cells express a variety of tissue repair factors, including TGF-α, amphiregulin, vascular endothelial growth factor A, IL-5, IL-13, and IL-22 (155, 273, 274). MAIT cells in barrier tissues of the lung and skin are particularly enriched for tissue repair genes, and MAIT cell–mediated wound healing has been demonstrated in punch biopsy and *Staphylococcus epidermis* infection models of skin damage (161). Together, these findings indicate that MAIT cells play Janus-like opposing roles during infection, on the one hand promoting cytotoxic and proinflammatory responses that destroy infected cells while also restoring tissue integrity after the resolution of the infection.

Stymied by microbial stealth

Unsurprisingly, pathogens have devised ways to stymie CD1d-restricted antigen presentation. Most evade intracellular CD1d trafficking. For example, the modulator of immune recognition (MIR)-1 and MIR-2 proteins of Kaposi sarcoma–associated herpesvirus (KSHV) are ubiquitin ligases. The two KSHV proteins ubiquitinylate the cytoplasmic tail of human CD1d, forcing the endocytosis of surface CD1d and, thereby, reducing cell-surface CD1d expression (275). The human immunodeficiency virus 1-encoded Nef protein mirrors the effects of MIR-1 and MIR-2 proteins to reduce CD1d expression perhaps by increased endocytosis coupled with the inhibition of the return transport of CD1d to the cell surface (276, 277). Similarly, in herpes simplex virus 1 (HSV-1)–infected cells, CD1d molecules accumulate in the MHC class II–enriched compartment due to a defect in CD1d recycling from endosomal compartments back to the cell surface (278). HSV-1 also inhibits the upregulation of cell surface MR1 *via* the US3 gene product to evade MAIT cell recognition (279). Vaccinia virus and vesicular stomatitis virus also abrogate CD1d antigen presentation, likely by impeding the intracellular trafficking of CD1d molecules induced by mitogen-activated protein kinase signaling (280). Some bacteria have also devised strategies to evade CD1d-restricted antigen presentation. Notably, the infection of monocytes by the human pathogen *M. tuberculosis* results in reduced CD1d mRNA expression, indicating the transcriptional control of *Cd1d* expression by a mycobacterial product (281).

While pathogens evade NKT cell activation by way of interference with intracellular CD1d trafficking and, thereby, antigen presentation, pathogens induce MAIT cell dysfunction to evade MAIT cell response. To that end, patients with *S. pneumoniae*–induced sepsis show significantly reduced but more active and dysfunctional MAIT cell responses compared to healthy donors or paired 90-day samples (139). The hyperactive MAIT cells stir up a pathological cytokine storm thought to be responsible for mortality (141). Furthermore, the hyperactive MAIT cell response poorly induces the differentiation of inflammatory monocytes to dendritic cells during pulmonary infection (139). Similarly, studies of *C. difficile* pathology indicate that these bacteria potently activate MAIT cells in a combined TCR- and cytokine-dependent manner inducing a pathological cytokine storm. The resultant runaway inflammation perhaps enables *C. difficile* to overcome cellular barriers to potentiate *C. difficile*–induced antibiotic-associated colitis (138). In a similar vein, gastric *H. pylori* infections elicit a hyperactive MAIT cell response, promoting an increased recruitment of inflammatory immune cells to the gastric mucosa exacerbating *H. pylori* gastritis (145). Thus, while some pathogens evade NKT cell recognition, the effects on MAIT cells focus on inducing MAIT cell hyperactivation and dysfunction as a means of potentiating bacterial pathogenicity.

Sic parvis magna—greatness from small things come

Some 50 years ago, Ivan Riott and John Playfair and their respective groups, independently and a year or so apart, described a small subset of lymphocytes that were neither B nor T cells yet killed tumor cells without prior priming. While no small discovery in and of itself, it was a small beginning considering the numerous unconventional lymphocytes that were discovered in the ensuing decades. Unbeknownst, the discovery of NK cells had silently annunciated the existence of a grander system of cells whose constituents played critical roles in immunity to infectious diseases and cancer, as well as in precipitating autoimmune disorders and allergic reactions. Multitudinous, they are yet cluster together by several common phenotypic and functional features. Their purpose is to process and integrate signals received from the innate immune response to convey that umwelt to downstream innate and adaptive effector responses. In this manner, they appear to function in between, at the edges of the innate and adaptive immune systems. Hence, innate/innate-like effector lymphocytes are called in-betweeners—or, alternatively, Latinate edge, and a 'limbic immune system' arises, perchance. In this proposal for a triumvirate immune system, we do not insinuate that the 'limbic immune system' is an evolutionary transition between the innate and adaptive systems because the independently acting modules that make up this system arise at different times in evolution, repurposing loosely common genome regulatory circuits to accomplish a common task. The 'limbic

immune system' functions to integrate information relayed by the innate sensory immune system about the local tissue environment and to provide context to downstream effector innate and adaptive immune responses. The multiple modules add robustness and evolvability to this limbic system to keep abreast of the ever-changing environment and the quick-evolving microbes, especially of those members of an otherwise symbiont community that turn pathobiont without much notice.

Author contributions

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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.2023.1117825/full#supplementary-material

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Innate immunity and early liver inflammation

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The innate system constitutes a first-line defence mechanism against pathogens. 80% of the blood supply entering the human liver arrives from the splanchnic circulation through the portal vein, so it is constantly exposed to immunologically active substances and pathogens from the gastrointestinal tract. Rapid neutralization of pathogens and toxins is an essential function of the liver, but so too is avoidance of harmful and unnecessary immune reactions. This delicate balance of reactivity and tolerance is orchestrated by a diverse repertoire of hepatic immune cells. In particular, the human liver is enriched in many innate immune cell subsets, including Kupffer cells (KCs), innate lymphoid cells (ILCs) like Natural Killer (NK) cells and ILC-like unconventional T cells – namely Natural Killer T cells (NKT), γδ T cells and Mucosal-associated Invariant T cells (MAIT). These cells reside in the liver in a memory-effector state, so they respond quickly to trigger appropriate responses. The contribution of aberrant innate immunity to inflammatory liver diseases is now being better understood. In particular, we are beginning to understand how specific innate immune subsets trigger chronic liver inflammation, which ultimately results in hepatic fibrosis. In this review, we consider the roles of specific innate immune cell subsets in early inflammation in human liver disease.

KEYWORDS

Inflammation, Innate immnuity, Hepatitis (general), NK cells, MAIT cell, Gd T cell, NKT (natural killer T) cell, kupffer cell (KC)

Introduction

Understanding the liver´s architecture and the niches formed by the different hepatic immune cells is equally important to deciphering their immune roles. The liver is subdivided into hepatic lobules, which consist of a portal triad (hepatic artery, portal vein and bile duct), hepatocytes arranged in linear cords between a capillary network (sinusoids) and a central vein (Figure 1). The blood flows from the portal triad to the central vein. The vascular system connecting the portal triad to the central vein is mainly constituted by liver sinusoidal endothelial cells (LSECs). Large fenestrae allow the exchange of macromolecules and components from the sinusoids with hepatocytes (1, 2). Interestingly, hepatocytes have different functions based on their zoning. Close to the portal triad, hepatocytes are the first to interact with gut-derived antigens whereas hepatocytes in proximity to the central vein are associated with detoxification (3). The

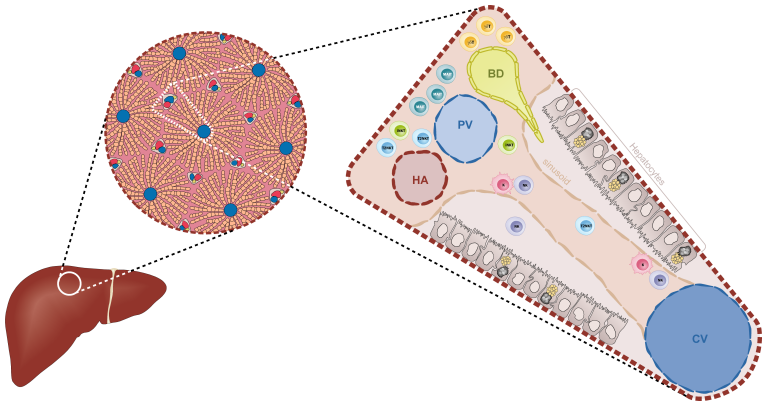

FIGURE 1
Diagrammatic representation of the liver architecture. The classical hexagonal lobule constitutes the anatomic unit of the liver. The lobule´s parenchyma is mainly formed by hepatocytes that are distributed along the sinusoids. The portal triad, formed by the hepatic artery (HA), the portal vein (PV) and the biliary duct (BD), carries the blood supply towards the centroid of the lobule where it is collected by the central vein (CV). Within the sinusoids, Kupffer cells (K) and Natural Killer cells (NK) are located in close proximity to the endothelium (beige). Other ILC-like cells such as iNKT cells and T2NKT cells are constantly surveying the sinusoids. Closer to the triad, especially near the BDs, there is a high frequency of MAIT cells and γδ T cells.

gradual change in blood nutrients, oxygen and antigen load is correlated with significant changes in hepatocytes´ gene expression signature (3, 4). Immune cells could also perform different functions according to their position within the liver. The distribution of innate cells in the liver is based on different chemokines, adhesion molecules and surface receptors (5). KCs are located adherent in the sinusoids and emit extensions into the Disse space. KCs along with LSECs constitute part of the reticuloendothelial system, which clears debris and harmful compounds in the blood. 65% of intrahepatic lymphocytes consist of NK cells, NKT cells, MAIT cells and γδ T cells (6–8) (Figures 2A, B). NK cells are in close proximity to KCs in both mouse and human models, suggesting a physical co-dependence (9, 10). NKT cells are constantly surveilling the liver sinusoids and stop when they detect inflammatory signals (9). CXCR6 was identified as a receptor to regulate mouse intrahepatic NKT cell frequencies and its ligand CXCL16 is overexpressed in macrophages and endothelium near injury areas (10). Human γδ T cells were identified in portal sections and in association with biliary epithelium (11). Human MAIT cells are reported to reside predominantly around bile ducts (12). However, the distribution and frequency of innate cells during inflammation are drastically changed with the recruitment of immune cells to the site of inflammation (9).

Kupffer cells

KCs are liver-resident macrophages that constitute 15% of the total human non-parenchymal liver cell count (13). They represent the primary barrier against pathogens and toxic compounds coming from portal circulation (14). KCs are antigen-presenting cells (APC) and play a crucial role in inducing liver tolerance through cell-to-cell contact, cytokines and other mechanisms such as dioxygenase-dependent sequestration of tryptophan (15). Under physiological conditions, KCs are the major reservoir of macrophages in the liver and can self-renew independently from the bone marrow (16). Upon activation, KCs secrete chemokine ligand 2 (CCL2) which promotes the infiltration of human circulating monocyte-derived macrophages. Increased frequency of $CCR2^+$ monocytes participates in liver fibrosis in mouse models (17, 18) and is indicative of pathology in human acetaminophen-induced acute liver injury (19). However, it is not yet clear whether liver-resident and circulating macrophages are two distinguished populations with different functions. The majority of pathogens coming from portal circulation are trapped in the liver by KCs phagocytosis. KCs cooperate with other non-parenchymal liver cells to clear potential infections (20). KCs can also sense damage-associated molecular patterns (DAMPs) expressed in hepatocytes that induce the secretion of a variety of cytokines and chemokines to efficiently restore homeostasis (20). When liver diseases compromise KCs function, aggravation of the diseases can be foreseen due to secondary infections (21).

Mucosal-associated invariant T cells

MAIT cells are an abundant subset of hepatic T lymphocytes. They constitute up to 30-40% of human hepatic $CD8^+$ T cells (6, 7). Their roles in pathogen defense and tissue repair have been previously reported (22–24). MAIT cells have an invariant T cell receptor (TCR) that recognizes the nonpolymorphic class Ib major histocompatibility (MHC) class I-related protein (MR1) when loaded with antigens. MAIT cells recognize riboflavin derivatives which are necessary for metabolism of many bacteria. These cells are considered an evolutionary system to defend hosts from pathogens since mammals do not produce these metabolites. Under inflammatory conditions, hepatocytes present the riboflavin derivative 5-A-RU to MAIT cells and also secrete IL-7 which is known to shape MAIT cells towards a pro-inflammatory state (7, 25). Upon activation, MAIT cells secrete large amounts of

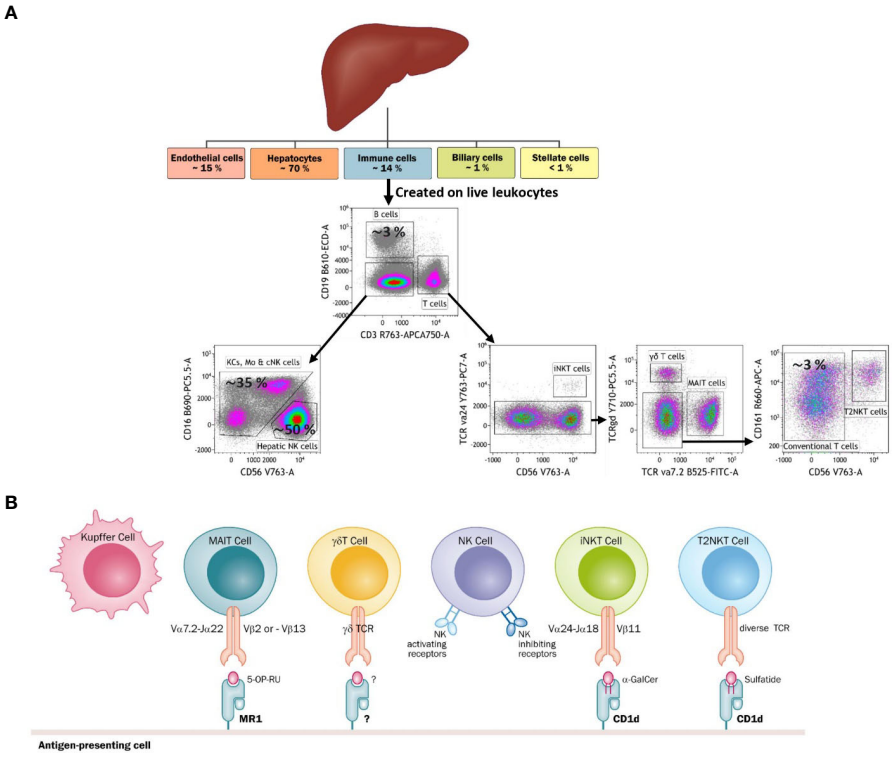

FIGURE 2
(A) Diagram tree of the approximate frequency of liver-resident cells and a FACS-based gating strategy to identify each cell type. The liver is mainly constituted by parenchyma (hepatocytes) and ILCs. Among ILCs, Kupffer cells and NK cells are the most abundant immune cells. The liver is also characteristic for having a niche of unconventional T cells, namely iNKT cells, T2NKT cells, γδ T cells and MAIT cells. **(B)** The main types of antigen recognition by unconventional T cells through their T-cell receptors (TCRs), Kupffer cells and NK cells. Kupffer cells and NK cells are activated through pattern recognition receptors. Additionally, NK cells have receptors that can sense healthy and stressed or dead cells.

pro-inflammatory and pro-fibrogenic cytokines such as IFN-γ, TNF-α and IL-17 (26). Studies in humans demonstrated that triggering MAIT cells in the absence of co-stimulation with cytokines induces wound repair and tissue regeneration (24). These studies suggest that under physiological conditions, MAIT cells probably contribute to tissue repair and regeneration since there is a constant influx of 5-A-RU present in human sera (27) but promote inflammation under acute inflammation. The high sensitivity for cytokines indicates that MAIT cells might be one of the first contributors to early inflammatory responses.

Gamma-delta T cells

γδ T cells are non-conventional subset of T lymphocytes with a limited non-MHC-restricted TCR repertoire. They constitute around 1-10% of human circulating T cells (28). They can recognize a wide variety of antigens and can be activated *via* pathogen-associated molecular patterns (PAMPs), DAMPs or cytokines alone. Upon activation, cells can execute cytotoxic as well as effector functions. Moreover, γδ T cells also play a role in tissue homeostasis (29). In humans, the stratification of γδ T cells is based on the Vδ gene segments used to produce their TCR. Vδ1$^+$ T cells are abundant in the epithelium (30) and protect tissues *via* recognition of non-classical MHCs such as CD1a, CD1c and CD1d (31). Vδ2$^+$ T cells are the most abundant subtype in circulation and can clear infections in periphery organs (28, 32). They recognize phosphoantigens, which are non-peptide low molecular weight antigens. Vδ2$^+$ T cells respond rapidly in a Th1-like fashion to high amounts of self-phosphoantigens (for example in tumor cells) or microbial phosphoantigens (33, 34). The butyrophilin 3A (BTN3A) family can trigger activation of Vδ2$^+$ T cells upon stimulation with phosphoantigens (35). The heterodimer BTNL3/BTNL8 expressed in APC was reported to mediate the TCR-dependent activation of Vδ2$^+$ T cells by binding of the intracellular domain of BTNL3 with phosphoantigens (36). Interestingly, the expression of *BTNL8* was not detectable in human PBMC but it was highly expressed in regulatory T cells after polyclonal stimulation (37). This suggests further investigation into the role of the butyrophilin family in the development of hepatitis and potential role in influencing Vδ2$^+$ T cells. Vδ3$^+$ T cells are a heterogeneous group of T lymphocytes enriched in the liver and also in some diseases such as leukaemia or chronic viral infection (38). They recognize antigens presented by CD1d molecules and respond by producing cytokines and killing of CD1d$^+$ cells (38). Recent evidences suggest that γδ T cells may be involved in liver diseases as previously shown in other autoimmune diseases (28), especially due to the rapid and large secretion of IL-17 (39).

Natural killer T cells

NKT cells are a rare subset of T lymphocytes comprising less than 1% of human peripheral blood T lymphocytes but enriched in the liver (8, 40). NKT cells are known to express NK cell markers like CD56, CD16 and CD161, and produce granzyme (40, 41). Their restricted TCR repertoire recognizes antigenic lipids presented by the MHC class I-like molecule CD1d (42, 43). Based on their TCR, NKT cells have been divided into two subsets. Type I NKT, or invariant (i)NKT cells, are the most studied group because they are enriched in mouse liver and have a semi-invariant TCR. The prototype ligand for iNKT cells is α-galactosylceramide (α-GalCer) (44). Type II NKT cells (T2NKT) consist of a subset with more diverse TCR. The major ligand recognized by T2NKT cells is sulfatide, which is a glycolipid enriched in the myelin of the central nervous system, pancreas, kidney and liver (45). It is difficult to study T2NKT cells because there is a lack of tools to identify and characterize them. Recently, we proposed a novel strategy to isolate and characterize T2NKT cells in humans but the low number of cells in blood is still a limitation (40). The role of iNKT cells and T2NKT cells in liver diseases have been mainly studied using transgenic mice models of CD1d-knockouts or TCRVα14-knockouts, which lack iNKT cells. These studies suggest that, in general, iNKT cells play a pro-inflammatory phenotype whereas T2NKT cells suppress inflammation through direct and indirect inhibition of inflammatory cells, including iNKT cells (46–49). We described a novel subpopulation of T2NKT cells that expresses regulatory T cell markers such as FoxP3 and CD25 (40). FoxP3$^+$ T2NKT cells were found both in the periphery and in the liver and may explain some of the regulatory functions reported previously.

Natural killer cells

NK cells are a major component of the liver's innate immune cell compartment. They account for almost 50% of human intrahepatic lymphocytes (50). Human hepatic NK cells are classified into three different subsets based upon their transcriptional, phenotypical and functional features (50). Liver-resident NK cells are CD56^{bright} CD69$^+$ CXCR6$^+$ CCR5$^+$ and highly cytotoxic (51–54). These cells are long-lived tissue-resident subsets (55). Interestingly, a subset of liver-resident CXCR6$^+$ NK cells was described as having a memory-like responsiveness against -vesicular stomatitis virus (VSV), human immunodeficiency virus (HIV) and influenza (56). Memory-like NK cells produce higher amounts of IFN-γ after rechallenge with the virus. The third NK cell subset is transient circulating NK cells, which are CD56^{dim} CD69$^-$ CXCR6$^-$ CCR5$^-$ and show less cytotoxic activity. They can secrete high amounts of pro-inflammatory cytokines such as TNF-α and GM-CSF (57–59). The regulation of NK cell activity consist on a balance between activating and inhibitory receptors displayed on their surface (60). NK cells survey the liver and induce apoptosis in infected or aberrant cells *via* different mechanisms such as FasL or TRAIL (61, 62). Under inflammatory conditions, NK cells kill hepatic stellate cells (HSCs) to resolve inflammation and limit liver fibrosis *via* granzyme-induced apoptosis and IFN-γ secretion (62, 63). NK cells are fundamental for the proper protection of the liver and aberrant functions have been reported in several liver diseases. Over the past decade, studies on NK cells suggest very heterogeneous populations with distinctive transcriptomes and cellular interactions (64).

Innate immune cell subsets and early liver inflammation

Liver inflammation is the first step to resolving and healing from different hepatocellular stress. When not effective, inflammation can become pathogenic. Hepatitis is a hallmark of liver disease (65) (Figure 3). It is important to identify which cells are precursors of early liver inflammation to avoid unnecessary harm. A recent report highlights the importance of the inflammasome in early inflammation (66). KCs express a variety of pathogen recognition receptors (PRRs) to cover a wide range of dangers. Some of these dangers overactivate the inflammasome, which triggers pyroptosis, a form of cell death accompanied by cell membrane rupture and release of pro-inflammatory IL-1β and IL-18 (67). These cytokines are responsible for the recruitment and activation of innate immune cells (68, 69). The direct cytotoxic and effector functions of innate immune cells can restore homeostasis. However, innate immune cells can also have early involvement in disease processes when the danger is not resolved (e.g. chronic viral infection) or because of repeated insults (e.g. alcohol or drug abuse) (Supplementary Table 1) . Innate immune cells can also recruit other immune cells from the liver and peripheral circulation. Overall, innate immune cells are suggested to be the precursors of the inflammatory niche because of their optimal location, preactivated state, enrichment in the liver and strong effector functions.

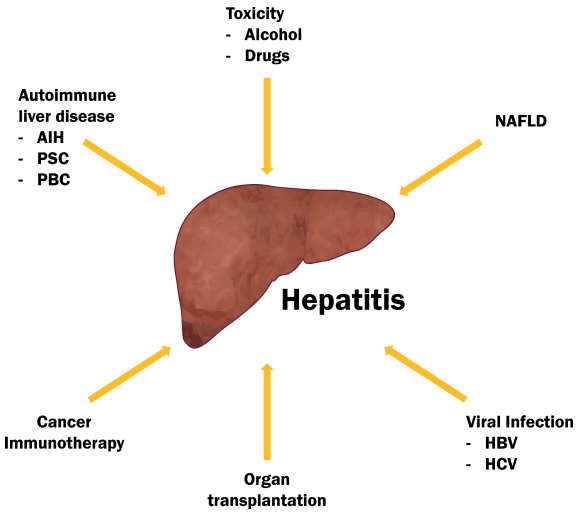

FIGURE 3
Hepatitis is a hallmark of the majority of liver diseases.

Viral hepatitis

Hepatotropic viruses such as hepatitis A, B, C, D and E (HAV, HBV, HCV, HDV and HEV) possess mechanisms to escape from the hosts´ antiviral immunity. When the viruses replicate, often the innate immunity detects viral components, hence triggering an acute inflammatory response resulting in the killing of infected hepatocytes. Since the infection is not properly resolved, viruses remain in a latent state and replicate opportunistically. This progressively leads to chronic liver inflammation (70). In particular, HBV and HCV are the main causes of chronic liver disease and are estimated to affect 257 million (data from WHO 2015) and 115 million people (71), respectively. Together they represent the most common cause of liver cirrhosis, liver cancer and viral hepatitis-related deaths (72).

HBV is directly mutagenic and induces low-grade inflammation progressing into HCC (73). HBV-infected hepatocytes release PAMPs such as glycoproteins, secreted HBsAg or free viral nucleic acids that are recognized by the innate immune system. Human KCs release pro-inflammatory cytokines to orchestrate an antiviral response which also arrests hepatocyte replication, hence viral replication (74). Studies in mice demonstrated the antiviral roles of NK cells and NKT cells (75, 76). HBV patients present higher levels of NK cells in blood compared to HBV-negative controls (77, 78) and are deemed as the major contributors to HBV clearance (79). A positive correlation was found between NK cell activation levels and HBV clearance (79). NK and NKT cell numbers from peripheral blood correlated to the frequency of HBcAg-specific cytotoxic T lymphocytes (CTLs) (80). However, infiltration of circulating NK cells can contribute to liver injury (81). NK cells from HBV patients produced higher levels of TNF-α and induced *in vitro* expression of TRAIL in hepatocytes (82). This study showed that infiltrated circulating NK cells could induce apoptosis of non-infected hepatocytes *via* TRAIL (82). Additional studies in mice and patients show that NK cells could also exacerbate liver injury *via* TNF-α, Fas/FasL and NKG2D/NKG2DL pathways (83, 84). NKT cells and KCs secrete induced nitric oxide synthase (iNOS) as a viral eradication mechanism (85, 86). Moreover, the frequency of NKT cells was increased to normal values with virus clearance (80). These results suggest that circulating NK cells and NKT cells are recruited in the liver causing a reduction in their frequencies in blood. In contrast, peripheral MAIT cells were significantly decreased in HBV-related liver failure patients compared with chronic HBV patients (87). The study suggested that MAIT cells are recruited in the liver and promote a strong inflammatory response damaging the liver. MAIT cells were also reduced in patients with middle/late-stage compared with early-stage liver failure (87). Similar to NK cells and NKT cells, patients that showed disease improvement had an increment in the frequency of MAIT cells (87). In another two studies exploring changes in peripheral γδ T cells in HBV patients, γδ T cells were less abundant in liver failure patients and correlated with disease severity (88). Activation of γδ T cells with PMA/Ionomycin induced the greatest amount of pro-inflammatory TNF-α and IL-17 in liver failure patients (89). However, another study indicated that γδ2 T cells exhibited impaired proliferation and chemotaxis (90). The same study showed *in vitro* that γδ2 T cells inhibit Th17 T cells through cell-to-cell contact and produce high amounts of IFN-γ (90). These results suggest that NK cells and NKT cells are the first-line of defense against HBV infection. Failing to clear the infection, MAIT cells and γδ T cells contribute to chronic inflammation. IFN-α therapy is effective in 20-30% of chronic HBV patients (91). The low response rates may be attributed to the wide spectrum of different clinical conditions. Based on the current understanding of the role of NK cells in HBV clearance, IFN-α is likely to improve the cytotoxic function of liver-resident NK cells by targeting HSC cells and reduce fibrosis (92). It is necessary to investigate whether IFN-α therapy response is subjected to the frequency of circulating NK cell infiltration.

HCV-induced inflammation is partly triggered by non-structural proteins of the virus (93) but the major contributor to HCV-hepatitis are the inflammatory immune cells. *In vitro* studies show that HCV-infected hepatocytes produce several pro-inflammatory cytokines including IL-6, IL-8, MIP-1α and MIP-1β as a response to IL-1β secreted by HSCs (94) or IL-1β and TNF-α by KCs (95). Similar to HBV infection, human circulating MAIT cells were generally reported to be depleted with markers of exhaustion and hyperactivation (96–98). Additional studies suggest that hepatic MAIT cells are major contributors to hepatitis and fibrosis given the nature of the cells. Repetitive IL-12 stimulation or IL-7 secretion by hepatocytes was a sufficient stimulus to induce secretion of the pro-inflammatory cytokines IFN-γ, TNF-α and IL-17 (7, 26). Intrahepatic γδ T cells were shown to be cytotoxic against human hepatocytes in culture (99). We have recently identified a subset of $CD8^+$ γδ T cells that were more abundant in baseline peripheral blood of melanoma patients that had hepatitis after ICI therapy versus non-hepatitis cohort. ICI therapy might induce γδ T cells cytotoxic activity against hepatocytes as observed in HCV infection. NK cells were shown to be compromised in HCV patients allowing the virus to replicate (78, 100). IFN-α therapy induced activation of NK cells and further improved the clearance of the virus (101). NKT cells were also reported to play a role in HCV resolution and progression. The frequency of activated $CD38^+$ or $CD69^+$ iNKT cells strongly correlated with alanine transaminase levels (102). Increased levels of activated iNKT cells were observed during acute inflammation and chronic HCV infection without apparent functional differences (102). The frequency of activated iNKT cells declined spontaneously in resolving patients (102). These data suggest that HCV infection could be mainly managed by NK and NKT cells. Viral clearance also involves other ILC-like cells such as MAIT cells and γδ T cells. Under inflammatory conditions, host hepatocytes switch to an antiviral state to prevent further viral replication. If the infection is not properly resolved, we propose a model where NK cells and MAIT cells have an exhausted phenotype while iNKT cells and γδ T cells promote pathogenesis by targeting infected hepatocytes.

Alcohol-induced hepatitis and drugs

The liver is vital for the detoxification of substances that are harmful to the body. Liver detoxification consists mainly of

converting ingested drugs into water-soluble metabolites *via* xenobiotic biotransforming enzymes (103). This allows drugs to be efficiently secreted through urine. However, in an attempt to solubilize drugs, some compounds are converted into their active form. Acetaminophen, also known as paracetamol, leads to reactive metabolites causing apoptosis and necrosis of hepatocytes (104). In the case of alcohol, free radicals and acetaldehyde are harmful by-products that can lead to significant liver damage over time. Drugs and alcohol can also damage the intestine barrier leading to more bacteria translocation to the bloodstream (105, 106). The influx of gut microbiota and its metabolites activate the immune system through PAMPs and DAMPs (107–110). KCs were reported to be major contributors to the development of alcohol-related liver disease (ALD). Intestine permeability is directly associated with KC activation (111, 112). Exposing mice to LPS and alcohol-derived reactive oxygen species (ROS) has shown to induce TNF-α secretion by KCs (113, 114). In a paracrine manner, IL-1ß secretion by KCs had a significant effect on the pathological progression of ALD (115). A rat model of ALD with depletion of KCs resulted in impaired progression of the pathology suggesting a key role of KCs (116). NK cells were less frequent in alcoholic patients (117) and were less cytotoxic compared to healthy individuals (118). A reduced expression of the activating receptor NKG2D and production of IFN-γ in mice suggests that NK cells cannot efficiently kill activated HSCs (119). Chronic ethanol feeding in mice increased CD1d by enterocytes (120). Similarly, patients affected by alcohol misuse also show increased expression of CD1d in the small intestine (120). An *in vitro* study showed that CD1d increased the loading of αGalCer following increasing concentrations of ethanol and thus, could increase stimulation of iNKT cells (121). Many studies in mice suggest that iNKT cells have a pathogenic role in the development of ALD. It was reported that iNKT cells crosstalk with KCs through IL-1β, promote inflammation and recruit neutrophils (122, 123). CD1d blocking antibodies could partially prevent liver injury (123). Intestinal iNKT cells were observed to migrate to the liver and, collectively with liver iNKT cells, showed a chronic activated phenotype with downregulation of TCR, increased apoptosis and FasL expression (120). *In vitro* experiments from the same study confirmed that iNKT cells could kill hepatocytes *via* Fas-FasL mechanism (120). Activation of T2NKT cells by sulfatide inhibited iNKT cell hepatic damage (124, 125). In a concanavalin A-induced hepatitis mouse model, injection of lysophosphatidylcholine (LPC) activated T2NKT cells and prevented liver injury by iNKT cells (125). Another study described the crosstalk of T2NKT cells with plasmacytoid dendritic cells and recruitment of anergic iNKT cells to the mouse liver *via* IL-12 and MIP-2 (126). As mentioned above, our group recently identified a novel population of human FoxP3$^+$ T2NKT cells that might exert immunoregulatory functions in this scenario (40). Alcoholic-related cirrhosis and severe alcoholic hepatitis patients had a dramatic depletion and hyperactivated circulating MAIT cells (127, 128). Dysfunctional MAIT cells could explain the susceptibility to infection of these patients (127, 128). In another study, MAIT cells had an exhausted phenotype and partially recovered with patient´s alcohol abstinence (129). MAIT cells may contribute to the pathogenesis of ALD *via* IL-17 secretion (129). Surprisingly, only a few reports have described the role of γδ T cells in ALD. In a mouse study following binge ethanol drinking, γδ T cells were described to produce higher amounts of IL-17A than non-binge ethanol-drinking mice (130). The activation of γδ T cells was IL-1ß-dependent, possibly by KCs (130). However, under acute-on-chronic ethanol consumption, γδ T cells did not produce further IL-17A. Instead, CD4+ T cells were the major contributors. This suggests that KCs could play a predominant role in the development of ALD. KCs orchestrate an inflammatory response that involves pro-inflammatory iNKT cells and γδ T cells. Alcohol could directly affect MAIT cells and NK cells causing depletion and impaired functions such as the inactivation of HSCs by NK cells, and tissue repair by MAIT cells.

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD), characterized by an excessive accumulation of fat in hepatocytes, is the most common indication for liver transplant in Western countries and the leading cause of liver transplantation in women (131, 132). It is estimated that 23-25% of the global population have NAFLD to some degree (133). Etiologically, it is suggested that the adipose tissue from patients with NAFLD predisposition release free fatty acids (FFA) and pro-inflammatory mediators into the circulation (134, 135). As a result, an inflammatory response is triggered in the liver. Lipotoxicity, mitochondrial dysfunction and endoplasmic reticulum stress are key inducers of the inflammatory cascade (136). Higher frequencies of KCs were observed in liver biopsies of non-alcoholic steatohepatitis (NASH) patients (137). Depletion of KCs in rats exposed to a high-fat diet (HFD) prevented the development of steatosis (138). *In vitro* experiments showed that TNF-α was responsible for the increased accumulation and the reduced oxidation of fatty acids in hepatocytes (139). Immunohistological stainings revealed a complex crown-like structure consisting of KCs surrounding dying steatotic hepatocytes. Cholesterol crystals are accumulated in the center of these structures (140). Interestingly, previous exposure of KCs to cholesterol crystals showed to precondition the cells towards a pro-inflammatory innate memory-like state (141). Similar observations were taken from macrophages cultured with oxidized low-density lipoproteins (142). Likewise to the effect of alcohol, NK cells of obese individuals had lower NKG2D expression (143) and impaired cytotoxicity (144, 145). Another study showed that there were no differences between NK cells from healthy individuals and NAFLD, while higher expression of NKG2D in NK cells was found in NASH patients (146). Data from mice and humans suggest that iNKT cells have a dual role in NAFLD. More specifically, it is hypothesized that iNKT cells have a protective role during early stages of simple steatosis. In different mouse models of hepatosteatosis, like ob/ob mice, animals fed with HFD or a choline-deficient diet, iNKT cells were apoptotic and showed decreased intrahepatic frequency (147–149). Adoptive transfer of hepatic mononuclear cells but not CD1d$^{-/-}$ mononuclear cells regulated hepatic steatosis *via* IL-10 (150). However, in other instances, opposite results were reported. Mice fed with HFD developed adipose tissue inflammation and glucose intolerance (151). This was significantly exacerbated by

αGalCer-dependent activation of iNKT cells (151). In the liver, iNKT cells could be directly activated *via* hepatic CD1d molecules, exacerbate steatosis and decrease insulin sensitivity by promoting a pro-inflammatory cytokine environment (152). This could suggest that iNKT cells play a protective role during early stages of simple steatosis but exacerbate the disease in chronic steatosis. It would also be interesting to study the potential effect of iNKT cell migration from tissues like the intestines as discussed earlier. T2NKT cells might also play dual roles. In HFD mice, T2NKT cells initiate inflammation in the liver and adipose tissue and promote obesity and insulin resistance (153). However, adoptive transfer of T2NKT cells in HFD obese mice induced prolonged weight loss and glucose tolerance (154). The heterogeneity and impact of fat in intrahepatic T2NKT cell populations remains unclear. The frequency of human NKT cells is decreased in steatosis (155) but increased accordingly to the progression of NAFLD, especially IFN-γ^+ and IL-4^+ cells (156–158). NASH patients had a 4-5 fold relative increase in liver NKT cells (158). CD1d expression was reported to be increased in liver immunohistochemical samples of NAFLD and correlated with disease progression (156). Taken together, NKT cells are reduced in the early stages of simple steatosis. A pro-inflammatory response is protective against obesity. In advanced NAFLD, NKT cells are increased and pathogenic. Circulating MAIT cell frequency was reported to decrease while the number of intrahepatic MAIT cells was increased in NAFLD patients' livers and it tended to be greater with disease progression (159). MAIT cells from NAFLD patients had increased secretion of IL-4 and reduced expression of IFN-γ and TNF-α (159). The current knowledge about the role of γδ T cells in NAFLD is mostly based on mice models. γδ T cells can recognize molecules presented by CD1d and its differentiation is dependent on hepatocyte CD1d (160). γδ T cells are high producers of IL-17A in steatohepatitis (161), a key cytokine known to induce fibrosis and ROS production (162, 163). In HFD mice, IL-17^+ γδ T cells are elevated (164). Additionally, adoptive transfer and gene knockout experiments in HFD mice demonstrated that γδ T cells exacerbate steatohepatitis and liver damage (160, 161). In humans, NAFLD patients showed decreased frequencies of V$\delta2^+$ T cells, but elevated frequencies of V$\delta2^-$ T cells compared to healthy controls (143). Overall, the progression of NAFLD to NASH is a process derived from the increased cellular oxidative stress that leads to the activation of inflammatory pathways (165). Accumulation of ROS induces the expression of TNF-α which can trigger necrotic cell death (166). In line with these results, NK cells were suppressed by ROS (167). KCs develop an apparent pro-inflammatory immune memory state by contact with cholesterol crystals. γδ T cells promote pathogenesis through IL-17 secretion, while NKT cells and MAIT cells exacerbate steatosis by secretion of Th2 cytokines which also contributes to fibrosis (168).

Liver autoimmunity

The three main autoimmune liver diseases are autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). AIH affects portal tracts and liver lobules by lymphoplasmacytic infiltrates while PSC and PBC mainly affect bile ducts. The etiologies of these diseases are yet unknown, but several studies suggest a common immune-mediated liver injury. The dysregulation of immune regulatory networks causes the activation and expansion of autoreactive T cells and B cells (169, 170). The innate system plays an important role in the regulation of the adaptive system. In AIH, an increased frequency of cytotoxic circulating NK cells in the liver was observed in an experimental mouse model of AIH (171). In humans, the frequency of circulating $CD56^{bright}$ NK cells was higher in untreated AIH, while the frequency of circulating $CD56^{dim}$ NK cells was reported to be reduced in active AIH patients or while in remission (171, 172). Our knowledge about NKT cells in liver autoimmunity is mainly based on mouse models. In AIH, concanavalin-induced hepatitis is the preferred model. iNKT cells were reported to upregulate FasL expression to mediate cytotoxicity against hepatocytes (173). Activation of iNKT cells *via* α-GalCer exacerbates the disease and is suggested to be carried out *via* IL-4 and TNF-α secretion (174, 175). Inflammation was also promoted *via* the secretion of IL-17 (176). MAIT cells were reported to be depleted and exhausted in the periphery in patients (177). Chronic stimulation of MAIT cells due to an increased influx of bacteria antigens and chronic inflammation may lead to MAIT cell function impairment. Induction of the exhausted state by repetitive stimulation with IL-12 and IL-18 showed that MAIT cells reduced IFN-γ production but maintained expression of the proinflammatory cytokine IL-17 (177). The frequency of circulating γδ T cells was increased in patients with AIH, PSC and PBC (8). V$\delta1^+$ T cells, known to produce high levels of IFN-γ and granzyme B, were especially incremented in patients with AIH (178). Another study showed that γδ T cells with low expression of TOX were enriched in AIH patients and had prediction potential (179). TOX deficiency was suggested to promote the expression of IL-17A in γδ T cells (179). In general, IL-17 secretion was reported in iNKT cells, MAIT cells and γδ T cells. Although the clinical profile of the distinctive autoimmune liver diseases is different, current studies support common immunological pathways. Taking for instance the role of circulating NK cells, the frequency of these cells was reported to be increased and a higher expression of cytotoxic molecules such as perforin was found in PBC and PSC patients compared to healthy individuals (180, 181).

Liver transplantation

Liver transplantation represents a major hepatic injury. One of the unavoidable injuries is caused by oxygen deprivation. After liver resection, blood flow is restricted for a period of time and the organ becomes hypoxic. This leads to different forms of cell death like apoptosis, ferroptosis, pyroptosis and necrosis (182). After reperfusion, innate immune cells from the recipient migrate to the liver and induce inflammation or tolerance (183). The degree of ischemia-reperfusion injury (I/R) is correlated to the risk of liver rejection (184, 185). I/R injury increased the expression of monocyte chemoattractant protein-1 (MCP-1) and it was associated with poorer graft function (186). This observation was correlated with the increased recruitment of monocytes 2 hours after reperfusion (186). The role of NK cells is dependent on activating and inhibitory

receptors expressed in hepatocytes as well as cytokines secreted by neighbour cells. In I/R injury, components of the inflammasome in KCs like NLRP3 and AIM2 are hyper-activated (187, 188). Inflammasome-derived IL-18 secretion can induce FasL (189) and IFN-γ production in NK cells (190). IFN-γ was reported to induce expression of Fas receptor in hepatocytes and neutralization of IFN-γ secretion by NK cells could protect mice from tissue damage (191). Due to the increased demand for livers and the increasing prevalence of NAFLD, the debate of using steatotic livers for transplant is on the table (192). Steatosis is deemed to cause oxidative stress in the liver, which worsens the graft´s condition with I/R injury. In a retrospective, exploratory study, steatotic livers showing signs of I/R had a significantly worse one-year survival rate, while the survival rate was not conditioned in healthy livers´ by I/R injury (193). In this study, γδ T cells were suggested to exacerbate liver rejection in steatotic livers (193). NKT cells were reported to promote I/R injury. After reperfusion, NKT cells rapidly expand in the liver and produce IFN-γ (194, 195). Depletion of NKT cells with antibodies or both NKT cells and NK cells significantly reduced I/R injury (196). The role of MAIT cells in liver I/R injury remains to be elucidated. In focal cerebral ischemia, MAIT cells were reported to play a pro-inflammatory role (197).

Immunotherapy-associated liver reactions

Cancer immunotherapies, especially immune checkpoint inhibitor (ICI) therapy, have opened new clinical perspectives for cancer patients and is fast becoming one of the main pillars of cancer treatment. ICI therapy uses monoclonal antibodies blocking T cell receptors that are used by cancer cells to evade the immune system. Immune-related adverse events (irAEs) are the result of immune activation derived from ICI therapy. The incidence of ICI-derived hepatitis is approximately 1-3% for programmed cell death 1 (PD1) inhibitors and 3-9% in cytotoxic T-lymphocyte-associated protein 4 (CTLA4) inhibitors (198). The combination of α-PD1/CTLA4 increases the rate of hepatitis (198). CTLA4 plays an important role in downregulating the immune response. The expression of CTLA4 is upregulated in T cells after activation and competes with the costimulatory receptor CD28 to bind to its ligand CD80/CD86 on APC (199). PD-1 is expressed on T cells and B cells and it promotes self-tolerance. Upon binding to its ligand PD-L1, it drives T cell apoptosis or regulatory phenotype. Thus, ICI therapy can arguably impair liver immunotolerance. In acute liver injury, α-PD1 therapy improved the bacterial clearance function of KCs (200). A study treating melanoma patients with α-PD1 showed that NK cell frequency in blood was not affected while NKT frequency was significantly increased (201). Another study observed no changes in either the number or function of MAIT cells in melanoma patients treated with α-PD1 therapy (202). γδ T cells showed no apparent functional changes upon PD-1 blockade *in vitro* (203). The frequency of γδ T cells in melanoma patients treated with a combination of α-PD1/CTLA4 remained unchanged (204). Overall, these data suggest that innate immune cells are not drastically affected by ICI therapies, with the exception of KCs and NKT cells. Immune-suppressive KCs expresses PD-1 to suppress T lymphocytes in acute liver injury (200). α-PD-1 therapy has shown to invigorate bacteria clearance, but it also suggests that KCs may have impaired tolerogenic function to self-antigens reactive T cells. NKT cells also responded to α-PD-1 therapy and exert increased anti-tumor functions by secretion of IFN- γ secretion of inflammatory cytokines (205).

Innate immune cells as diagnostic and therapeutic targets

The innate immune system is also involved in immune homeostasis and healthy tissue turnover. This is accomplished *via* three steps consisting of early inflammation, amplification of the inflammatory signal and resolution. Liver fibrosis is a consequence of inflammation and inefficient resolution. Liver biopsy is the gold standard for diagnosing cirrhotic liver disease, yet it is estimated to miss 10-30% of cases (169). Additionally, biopsy is not ideal because of invasiveness, pain, hypertension and bleeding (206). An optimal approach would be to identify early inflammation before fibrosis development. This could improve patient's treatment and prognosis. Blood markers bring promising perspectives to detect liver damage and abnormal functions (207). The current scoring system for diagnosis and prognosis of fibrosis includes serum proteins (albumin), bilirubin, liver enzymes (aminotransferases, alkaline phosphatase, γ-glutamyl transferase) and direct markers of extracellular matrix turnover (type IV collagen, matrix metalloproteinases). However, there is room for improvement regarding specificity (etiology) and sensitivity (disease stages) (206). The immune system has emerged as an interesting diagnostic and therapeutic target in liver inflammation. Innate immune cells are the frontline defenders in the liver and participate in the initiation, amplification and resolution of inflammation. Identifying immune changes in innate immune cell´s surface expression markers and frequencies can bring future perspective to the diagnosis of low-grade inflammation and also novel therapies. As discussed in this review, depletion of innate immune cells in mice models with hepatitis was able to attenuate several liver diseases. Noteworthy, the close relationship between innate immune cells with DAMPs and cytokines signaling suggests taking into consideration all three factors for the future of liver immunomonitoring and therapies.

Author contributions

JYZ performed literature search and wrote the manuscript. The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of interest

The author declares 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.2023.1175147/full#supplementary-material

SUPPLEMENTARY TABLE 1
Innate immune cells in liver inflammation.

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MR1 deficiency enhances IL-17-mediated allergic contact dermatitis

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Major histocompatibility complex (MHC) class Ib molecules present antigens to subsets of T cells primarily involved in host defense against pathogenic microbes and influence the development of immune-mediated diseases. The MHC class Ib molecule MHC-related protein 1 (MR1) functions as a platform to select MR1-restricted T cells, including mucosal-associated invariant T (MAIT) cells in the thymus, and presents ligands to them in the periphery. MAIT cells constitute an innate-like T-cell subset that recognizes microbial vitamin B_2 metabolites and plays a defensive role against microbes. In this study, we investigated the function of MR1 in allergic contact dermatitis (ACD) by examining wild-type (WT) and MR1-deficient ($MR1^{-/-}$) mice in which ACD was induced with 2,4-dinitrofluorobenzene (DNFB). $MR1^{-/-}$ mice exhibited exaggerated ACD lesions compared with WT mice. More neutrophils were recruited in the lesions in $MR1^{-/-}$ mice than in WT mice. WT mice contained fewer MAIT cells in their skin lesions following elicitation with DNFB, and $MR1^{-/-}$ mice lacking MAIT cells exhibited a significant increase in IL-17-producing αβ and γδ T cells in the skin. Collectively, $MR1^{-/-}$ mice displayed exacerbated ACD from an early phase with an enhanced type 3 immune response, although the precise mechanism of this enhancement remains elusive.

KEYWORDS

innate T cells, delayed-type hypersensitivity, neutrophils, gamma/delta T cells, allergy

Abbreviations: MHC, major histocompatibility complex; MR1, MHC-related protein 1; MAIT, mucosal-associated invariant T; ACD, allergic contact dermatitis; DNFB, 2,4-dinitrofluorobenzene; DNBS, dinitrobenzene sulfonic acid; dLN, draining lymph nodes; Mo/Mϕ, monocyte/macrophage; Der γδ T, dermal gamma-delta T; Epi γδ T, epidermal gamma-delta T.

1 Introduction

The skin is repeatedly exposed to various antigenic substances of natural origin, cosmetics, metal accessories, and medical products of artificial origins, in the broad context of the environment (1), and the immunogenicity of these substances as sensitizers has been investigated (2, 3). Antigen-presenting cells (APC) in skin exposed to sensitizers migrate to the draining lymph nodes (dLN) *via* lymphatic vessels and present them in the context of gene products of self-major histocompatibility complex (MHC) class Ia and II to antigen (Ag)-specific T cells (4). Thus, Ag-specific $CD8^+$ and $CD4^+$ T cells are primed, and these Ag-specific T cells within the memory fraction may be activated upon Ag re-exposure and migrate to the site of Ag entry to induce allergic contact dermatitis (ACD). ACD is transferable with T cells but not with antibodies and is thus classified as T cell-mediated type IV hypersensitivity according to the Gell and Coombs classification (4).

An experimental model of ACD is often employed in mice by painting chemicals such as dinitrohalobenzene onto the skin to study the sensitization and elicitation phases in detail (4). In addition to T cells, various other immune and non-immune cells in the skin are involved in the pathogenesis of ACD, and the crosstalk among them has been studied (5–7). Keratinocytes are the main type of non-immune cells in the skin, constituting a barrier layer since they not only form a physical barrier against the entry of foreign substances and pathogens but also secrete IL-1β when sensing insults against the skin to transmit signals downstream to immune cells (5). The cells of innate immunity include Langerhans cells, dermal dendritic cells, macrophages, neutrophils, and mast cells, which present Ag information and affect the intensity of ACD (6). Natural killer (NK) cells and innate lymphoid cells (ILCs), lymphocytes without rearranged Ag-specific receptors, potentiate (NK and ILC1 in particular) or regulate (ILC2 in particular) the immune and inflammatory responses at both the sensitization and elicitation phases of ACD depending on the context (6, 7).

Innate-like lymphocytes with rearranged TCRs are also important components in ACD. Murine skin is known to harbor a special γδ T-cell population referred to as dendritic epidermal T cells (DETCs) expressing invariant Vγ3Vδ1 (in Garman Nomenclature [GN], Vγ5Vδ1 in Heilig-Tonegawa Nomenclature [H-TN]) TCR in the epidermis (8). However, humans do not have an equivalent epidermal T-cell population, although they harbor $V\delta1^+$ and $V\delta2^+$ T cells in the epidermis and dermis (9). Murine DETCs express NKG2D, which recognizes stress molecules such as RAE-1 induced in keratinocytes when sensitizing chemicals are applied to the epidermis (10). Moreover, keratinocyte-derived IL-1β induces IL-17 expression by DETCs (11) and $V\gamma2^+$ (in GN, Vγ4 in H-TN) or $V\gamma4^+$ (in GN, Vγ6 in H–TN) γδ T cell subsets, including others (collectively referred to as Tγδ17 cells) in the dermis (9), where the latter appear to play a more important role in ACD.

The skin also harbors innate-type T cells with αβ-type TCRs, including natural killer T (NKT) cells (12), and mucosal-associated invariant T (MAIT) cells (13), whose reactivities are restricted by MHC class Ib molecules, cluster differentiation 1d (CD1d), and MHC-like protein 1 (MR1), respectively. These T-cell subsets are also categorized as preset T cells and resemble each other in several ways (14): 1) They recognize non-peptide antigens of microbial origin in the context of the restricting class Ib (glycolipids/CD1d vs. vitamin $B_{2,9}$ metabolite/MR1), 2) major subsets utilize invariant Vα chain (mouse Vα14Jα18/human Vα24Jα18 vs. mouse Vα19/Jα33/human Vα7.2Jα33) with limited yet diverse Vβ chains, respectively, 3) the invariant subsets of T cells exhibit effector/memory phenotypes and may function as either effector or regulatory cells in health and diseases (15). Studies of these invariant T cells may provide insights as to controlling ACD with low-molecular-weight ligands without concerns about MHC barriers because the restriction molecule is homogenous in an allogeneic relationship and highly conserved even in xenogeneic combinations (14).

The role of NKT cells in ACD has already been investigated by employing gene knockout ($^{-/-}$) mice, $CD1d^{-/-}$ (whole NKT cell-deficient), or $J\alpha18^{-/-}$ (invariant NKT [iNKT] cell-deficient) mice compared with wild-type (WT) mice with a C57BL/6 or BALB/c background (16–18). Initial studies demonstrated that ear swelling was reduced in both $CD1d^{-/-}$ and $J\alpha18^{-/-}$ mice, suggesting that iNKT cells appear to function in the initiation and enhancement of ACD through prompt induction of IL-4 after Ag exposure, with involvement of IgM^+ B-1 B cells and effector αβ T cells (16, 17). Subsequent studies revealed that the differential functions of iNKT cells were dependent on the contact sensitizers employed in each study, with iNKT cells playing either pathogenic or regulatory roles (18, 19). Human studies have also demonstrated that iNKT cells are detected in ACD lesions, implying some critical roles (20, 21).

The involvement of another innate αβ type T cell, MAIT cells, in ACD has been limited to date and has been reported for palladium allergy in the foot pad lesions of BALB/c mice, where MAIT TCR was detected with iNKT TCR and presumed to display Ag-specificity (22). The role of MAIT cell accumulation in the lesion remains elusive in the development of ACD as a player in either inflammatory or regulatory responses. Thus, in the present study, we examined the effect of MR1/MAIT deficiency on ACD by comparing DNFB-induced ACD in WT versus $MR1^{-/-}$ mice to probe for altered responses in $MR1^{-/-}$ mice. The involvement of other subsets of innate-like T cells was also revealed in $MR1^{-/-}$ mice, and their relevance in ACD is discussed.

2 Materials and methods

2.1 Mice

C57BL/6 (B6) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and $B6.MR1^{-/-}$ mice were kindly provided by Dr. Susan Gilfillan (Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA) (23) and housed and maintained in an animal facility at the Analysis Center for Integral Genomic Functions at Kitasato University School of Medicine. The mice were provided food and water *ad libitum*. All animals were humanely treated and housed under pathogen-free conditions. All experimental procedures involving mice conformed to the guidelines of the Animal Experimentation

and Ethics Committee of Kitasato University School of Medicine (#2017-143, 2018-119, 2019-025, and 2022-079).

2.2 Induction of allergic contact dermatitis with 2,4-dinitrofluorobenzene

Mice were sensitized at shaved abdomen sites with 25 µL of 0.5% 2,4-dinitrofluorobenzene (DNFB) (Sigma-Aldrich, MO, USA) dissolved in a 1:4 mixture of olive oil (Nacalai Tesque, Inc., Kyoto, Japan): acetone (Fujifilm Wako Pure Chemical Co. Ltd., Osaka, Japan), as previously described (24). Five days after sensitization, the right pinna was painted with 20 µL of 0.3% DNFB, and the left pinna was painted with 20 µL of vehicle alone (elicitation/challenge). Each pinna was measured with a digital micrometer (Mitutoyo Corp., Kawasaki, Japan), and the net pinna thickness (Δthickness = thickness of right pinna – thickness of left pinna) was calculated at 0 (before), 1, and 2 days after challenge.

2.3 Cell preparation from pinnae and lymph nodes of treated mice

Right and left pinnae and inguinal lymph nodes (draining lymph nodes [dLN]) on the right and left sides of the mice were obtained after euthanasia using a confirmed procedure. The pinnae were used for histology, flow cytometry, functional analyses of infiltrated cells, and gene expression analyses. A single-cell suspension was prepared according to the protocol previously described with slight modifications (25). In brief, the removed pinnae were cut into pieces and incubated with 100 µg/mL Liberase® and 400 ng/mL DNase I (both from Roche Diagnostics, K.K., Tokyo, Japan) at 37°C with gentle shaking for 1 h. The digestion was stopped by adding ice-cold phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS (-)], and the solution was layered on Lympholyte®-M medium (Cedarlane Laboratories Ltd., Ontario, Canada) followed by centrifugation at 1,800 × *g* for 20 min. Cells at the interface were collected, washed with medium, and used for flow cytometry and cell culture. The lymph nodes were gently dispersed using a frosted-glass homogenizer to obtain a single-cell suspension, which was used for flow cytometry and cell culture.

2.4 Flow cytometric analysis

A single-cell suspension prepared as above was incubated with TruStain FcX™ anti-mouse CD16/32 antibody (BioLegend, CA, USA) and stained with the following mAbs: anti-mouse Ly-6G (1A8), CD11b (M1/70), TCRβ (H57-597), CD3 (2C11), CD4 (GK1.5), γδ TCR (GL3), Vγ2 (in GN; UC3-10A6), B220 (RA3-6B2), IL-17A (TC11-18H10.1), IFN-γ (XMG1.2), and T-bet (4B10) purchased from BioLegend, anti-mouse CD45.2 (104), RORγt (Q31-378BD) purchased from BD Biosciences, and anti-mouse Foxp3 (FJK-16s) purchased from Invitrogen. 5-OP-RU loaded MR1 tetramer was provided by the National Institute of Health Tetramer Core Facility at Emory University (Atlanta, GA, USA). Cells positive for 7-amino actinomycin D (BioLegend) were electronically gated as dead cells and excluded from the analysis. For transcription factor staining, the cells were initially stained with surface markers, and then fixed and permeabilized with the True-Nuclear™ Transcription Factor Buffer Set (BioLegend). For intracellular cytokine staining, cells were stimulated with PMA (50 ng/mL, Sigma-Aldrich) and ionomycin (500 ng/mL, Sigma-Aldrich) for 4 h in the presence of brefeldin A (x1000; BioLegend) before cell surface staining. The samples were washed and filtered and then analyzed by FACS. After surface staining, the cells were fixed and permeabilized with Fixation Buffer (BioLegend) and Intracellular Staining Permeabilization Wash Buffer (BioLegend), followed by staining with anti-cytokine mAbs. The stained cells were subjected to flow cytometry (FACSVerse™, BD Biosciences) and analyzed using FlowJo software (FlowJo, LLC, CA, USA). The flow cytometry was performed as described previously (25).

2.5 Cell culture and stimulation with dinitrobenzene sulfonic acid

At day 5 of DNFB sensitization, dLN cells were harvested in RPMI-1640 medium (Sigma-Aldrich) containing 10% FCS, 50 µM β-mercaptoethanol (GIBCO, MA, USA), 100 units/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich). One million (1×10^6) cells were cultured in the presence of 100 µg/mL dinitrobenzene sulfonic acid (DNBS) (Sigma-Aldrich) for 3 days (24) and the supernatant was collected for cytokine measurement, as described in section 2.6.

2.6 Quantification of cytokines

The concentration of Th1/Th2/Th17 cytokines in the culture supernatant was quantified by flow cytometry using a BD CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, CA, USA) according to the manufacturer's protocol.

2.7 Quantitative real-time PCR

Total RNA was extracted using the TRIzol® reagent (Thermo Fisher Scientific). cDNA was synthesized from the total RNA using PrimeScript™ RT Master Mix (TaKaRa Bio Inc., Kusatsu, Japan). Real-time PCR was performed using TB Green® Premix Ex Taq™ II (TaKaRa Bio Inc.) and a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. The target gene expression was normalized to *β-actin* and calculated using the $2^{-\Delta\Delta CT}$ method. The primers were as follows: *Actb* (forward 5′-GGCTGTATTCCCCTCCATCG-3′; reverse 5′-CCAGT TGGTAACAATGCCATGT-3′), *Cxcl1* (forward 5′-TTGAAGGT

GTTGCCCTCAGG-3′; reverse 5′-CCAGACAGGTGCCATCAGAG-3′), *Cxcl2* (forward 5′-GGCGGTCAAAAAGTTTGCCT-3′; reverse 5′-CAGGTACGATCCAGGCTTCC-3′), *Csf3* (forward 5′-GTTCCCCTG GTCACTGTCAG-3′; reverse 5′-TGGCTTAGGCACTGTGTCTG-3′), *Il17* (forward 5′-TGAAGGCAGCAGCGATCA-3′; reverse 5′-GGAAGTCCTTGGCCTCAGTGT-3′), *Il1b* (forward 5′-GCAACT GTTCCTGAACTCAACT-3′; reverse 5′-ATCTTTTGGGGTCCG TCAACT-3′) (Hokkaido System Science, Sapporo, Japan).

2.8 Histology and quantitative analyses of microscopic images

The pinna tissue was fixed with buffered formaldehyde solution (10%) (Fujifilm-Wako Pure Chemical), followed by the standard protocol for paraffin-embedded sections and hematoxylin-eosin (HE) staining. Images of the HE-stained tissue were captured using a BIOREVO microscope (BZ-X800, KEYENCE Corp., Osaka, Japan), and the thickness of the pinna was quantified using image analysis software (BZ-X) for the microscope, in addition to manual measurement with a digital micrometer, as described in Section *2.2*.

2.9 Statistics

The results are presented as means ± standard deviation (s.d.). Statistical analysis between two groups was performed using the Mann–Whitney U test, and comparison among three groups was performed using ANOVA followed by Tukey–Kramer tests. Values with $p < 0.05$ were considered statistically significant.

3 Results

3.1 MR1$^{-/-}$ mice develop augmented ACD

To examine the role of MR1/MAIT cells in ACD, WT and MR1$^{-/-}$ mice were sensitized with DNFB in an acetone/olive oil solvent on the abdominal skin and challenged five days later on the right pinna, and the increment in thickness of the pinna in each mouse was calculated. MR1$^{-/-}$ mice exhibited a significantly greater increase in ear swelling than WT mice on days 1 and 2 after DNFB challenge (Figure 1A). MR1$^{-/-}$ mice exhibited thicker pinnae, with severe intercellular edema and augmented infiltration of inflammatory cells compared to WT mice, as shown by histopathology (Figure 1B). The inflammatory cells in the DNFB-painted pinnae appeared to consist mainly of polymorphic neutrophils in both WT and MR1$^{-/-}$ mice. The mean ear thickness of pinna painted with DNFB was also quantified using histological images, and that of MR1$^{-/-}$ mice was greater than that in WT mice (Figure 1C). Although the representative histology of the vehicle control in MR1$^{-/-}$ mice was slightly thicker than that in WT mice (Figure 1B), the mean ear thickness in the control group was similar between the WT and MR1$^{-/-}$ mice.

3.2 More neutrophils are recruited into the ACD-induced pinna in MR1$^{-/-}$ mice

To analyze inflammatory cells infiltrating the pinna challenged with control vehicle or DNFB, cells infiltrated into the pinna were obtained by disintegration of the tissue and analyzed by flow cytometry, as described in the *Materials and Methods*. The acquired mononuclear cells were gated as described (Supplementary Figure 1A). The neutrophils in the pinnae were identified as Ly6G^{+}CD11b^{+} cells (Figure 2A). More neutrophils were recruited to the DNFB-challenged pinnae in both WT and MR1$^{-/-}$ mice (Figure 2A, right panels) than to the control pinnae (Figure 2A, left panels). Furthermore, the number and frequency of neutrophils in the challenged pinnae of MR1$^{-/-}$ mice were significantly higher than those in the pinnae of WT mice (Figure 2B). The expression of genes related to neutrophil migration and survival, such as *Cxcl1, Cxcl2*, and *Csf3*, was significantly increased or tended to be increased in MR1$^{-/-}$ mice compared to WT mice (Figure 2C). *Il17*, which stimulates the expression of these genes, also tended to be increased in MR1$^{-/-}$ mice after two days of DNFB challenge (Figure 2C).

The Ly6G$^{lo-(-)}$CD11b^{+} population that appeared straight below the neutrophil gate in Figure 2A was further separated into Ly6C^{hi}F4/80^{lo} (monocyte/macrophage; Mo/Mϕ) and Ly6C^{-}F4/80^{+} (macrophage; Mϕ) cells (Supplementary Figure 2A). Although tissue-resident Mϕ appeared to be the main cell type in the Ly6G$^{lo-(-)}$CD11b^{+} population, Mo/Mϕ became dominant presumably *via* migration and Mϕ appeared to be markedly reduced by contrast (Supplementary Figure 2A, flow panels) and as a percentage (Supplementary Figure 2B graphs) during the elicitation phase.

3.3 Increased dermal γδ T cells in the pinnae of MR1$^{-/-}$ mice

To examine another major population of cells residing in control pinnae or infiltrating inflamed pinnae, we analyzed αβ- and γδ-type T cells of vehicle- and DNFB-treated pinnae by flow cytometry based on gating, as shown in Supplementary Figure 1B. Both αβ and γδ T cells were detected in vehicle control and DNFB-painted pinnae, and γδ T cells were clearly separated according to the fluorescence intensity as epidermal (Epi: TCR^{hi}) and dermal (Der: TCR^{lo}) γδ T cells (Figure 3A) (25). Notably, in the pinnae of the vehicle control group, the contour of αβ T cells was more evident in WT mice, whereas that of Der γδ T cells was more evident in MR1$^{-/-}$ mice (Figure 3A, upper and lower left panels). Elicitation by DNFB caused a reduction (dense contour to scarce one or dots) of Epi and Der γδ T cells, whereas a clear population of αβ T cells was observed in both WT and MR1$^{-/-}$ mice (Figure 3A, upper and lower right panels). When the number of T cells was analyzed further, the αβ T cells in the pinnae significantly increased after challenge with DNFB in both WT and MR1$^{-/-}$ mice at similar levels, suggesting that the sensitized population of αβ T cells was

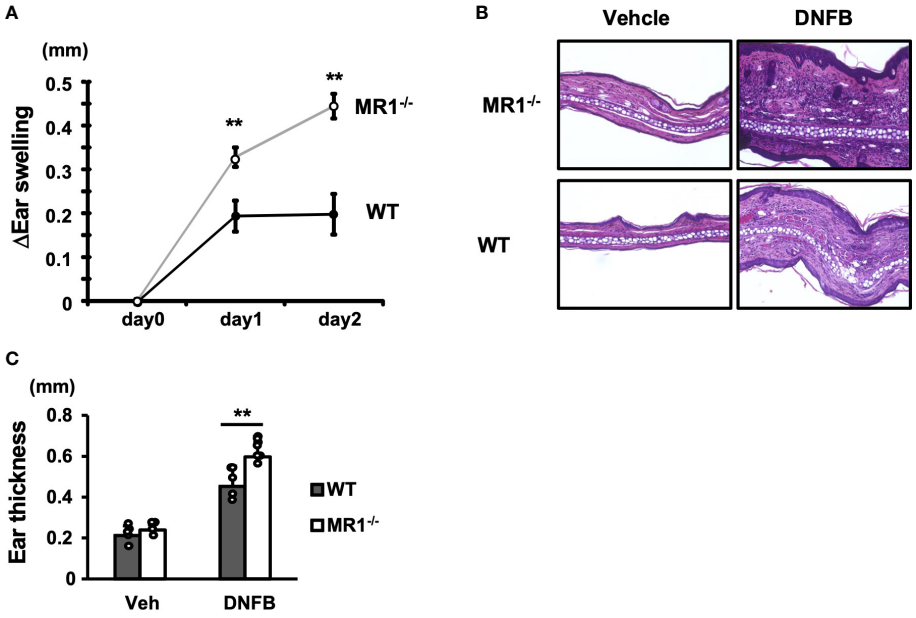

FIGURE 1
$MR1^{-/-}$ mice develop augmented ACD compared with WT mice. **(A)** Wild-type (WT, C57BL/6; B6, closed circle) mice and B6.$MR1^{-/-}$ ($MR1^{-/-}$, open circle) mice were sensitized with 0.5% DNFB and challenged after five days on the left pinna with vehicle only or on the right pinna with 0.2% DNFB. The thickness of the pinnae was then measured with a digital micrometer on day 0 (day of challenge), day 1, and day 2. The increment in thickness of the sensitized pinna was presented as ΔEar swelling (mm), as described in the Materials and Methods. **(B)** Histology of vehicle-painted (control) and DNFB-painted (experimental) pinnae obtained from WT and $MR1^{-/-}$ mice. **(C)** The net thickness of the pinnae including that of vehicle control deduced from the measurements of morphometric analyses of histological specimens is presented as Ear thickness (mm). Representative data of at least three experiments of four to five mice/experiment. Mann–Whitney *U* test. $^{**}p < 0.01$.

vigorously recruited into the pinnae after painting in both strains of mice (Figure 3B, lower right panels), although the percentage of αβ T cells of $MR1^{-/-}$ mice was significantly lower than that of WT mice due to the increased Der γδ T cells as described below (Figure 3B, lower left panels). Accordingly, the percentage of Epi γδ T cells was markedly decreased in the DNFB-challenged pinnae compared to the vehicle controls (Figure 3B, upper left panels). The number of Epi γδ T cells also appeared to be decreased in the DNFB-challenged group, whereas the extent was not as marked as that of the frequency, and $MR1^{-/-}$ mice exhibited higher numbers than WT mice (Figure 3B, upper right panels). Another subset, Der γδ T cells, appeared to be decreased in frequency in WT and $MR1^{-/-}$ mice in the DNFB-treated group under the influence of the dominant recruitment of αβ T cells, whereas the frequency was significantly higher in $MR1^{-/-}$ mice than in WT mice in both the control and DNFB groups (Figure 3B middle left panel). Moreover, the number of Der γδ T cells was not reduced, even in WT mice, and was significantly increased in DNFB-challenged pinnae in $MR1^{-/-}$ mice compared with WT mice (Figure 3B middle right panel). In contrast, the cells of interest in the present study, MAIT cells detected by 5-OP-RU/MR1 tetramer, were reduced in DNFB-challenged pinnae in comparison with those in the vehicle control, (Figure 3C) both in terms of frequency and cell number (Figure 3D). Since $MR1^{-/-}$ mice lack MAIT cells due to the *Mr1*-disruption, there was no difference between the control and DNFB groups.

The increment and reduction of each T cell subset, as compared with other immune cells among the different panels, are not evident, because the cell numbers during the pre- and post-elicitation stages of each cell number differ over several log scales. To better visualize the relationship of each subset of cells in the pinnae of vehicle- and DNFB-painted WT or $MR1^{-/-}$ mice, the cumulative graph of cells for lymphocytic and phagocytic lineages is shown in Supplementary Figure 3. The majority of cells infiltrating the pinnae consisted of neutrophils, Mo/Mϕ, and αβ T cells in both WT and $MR1^{-/-}$ mice, although there was a difference in the composition between WT and $MR1^{-/-}$ mice in the vehicle control and DNFB-challenged pinnae.

3.4 Enhanced Th17 immune responses in $MR1^{-/-}$ mice

To examine the effect of MR1/MAIT cell deficiency on T-cell cytokine production in ACD, the whole draining LN (dLNs; inguinal) cells of abdominal skin from WT and $MR1^{-/-}$ mice were stimulated with 2,4-dinitrobenzene sulfonic acid (DNBS) *in vitro*. The level of IL-17A in the culture supernatant when stimulated with DNBS was significantly higher for the LN cells of $MR1^{-/-}$ mice than for those of WT mice (Figure 4). The production of other cytokines such as IL-10, TNF-α, IFN-γ, and IL-6 was comparable between WT and $MR1^{-/-}$ mice (Figure 4), and IL-4 production was almost undetectable (data not shown).

Next, we examined the frequency and number of T helper (Th) subsets in the dLNs of WT and $MR1^{-/-}$ mice after 5 days of sensitization. There were no differences in the frequencies or the numbers of $CD4^+CD3^+$ (T) cells in dLNs between WT and $MR1^{-/-}$

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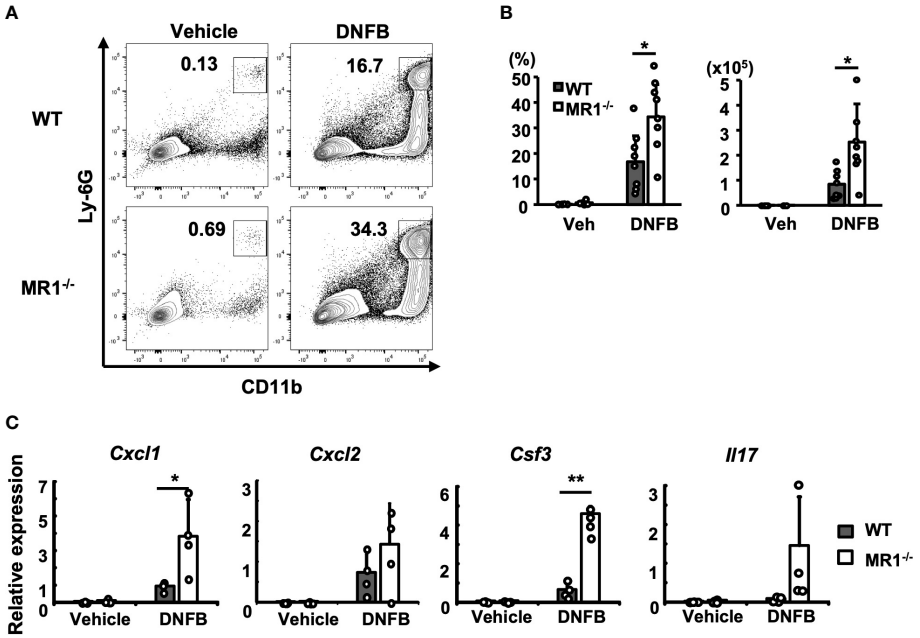

FIGURE 2
More neutrophils are recruited into the ACD-induced pinnae in MR1$^{-/-}$ mice than WT mice. **(A)** Representative flow cytometric profiles of the cells infiltrated into the pinnae prepared two days after challenge with enzymatic degradation, as described in the Materials and Methods and analyzed according to the gating described for Supplementary Figure 1A. The square gate (CD11b^{+}Ly6G^{hi} cells) indicates neutrophils. **(B)** Frequency of the CD45^{+} fraction and cell number in MR1$^{-/-}$ mice compared with the fraction and cell number of WT mice represented by panel **(A)**. **(C)** The expression of *Cxcl1, Cxcl2, Csf3,* and *Il17* related with neutrophil recruitment was examined with mRNA obtained from the left pinnae (vehicle control) and the right pinnae (DNFB) two days after challenge. Representative data of at least three experiments of four to eight mice/experiment. Mann–Whitney *U* test. *$p < 0.05$, **$p < 0.01$.

mice (Figure 5A). When T-bet^{+} cells (Th1) were analyzed among CD4^{+}CD3^{+} cells, the frequency of T-bet^{+} cells in the dLNs of MR$^{-/-}$ mice was lower than that in WT mice, and the number of T-bet^{+} CD4^{+} T cells also exhibited a decreasing trend (Figure 5B). The CD4^{+} T cells were analyzed for RORγt and Foxp3 expression (Figure 5C). The frequencies and numbers of RORγt^{+}Foxp3^{-} (Th17) cells were significantly higher in MR1$^{-/-}$ than in WT mice (Figure 5D, upper left and lower left panels). There were no differences in the frequency and number of RORγt^{-}Foxp3^{+} (Treg) cells between WT and MR1$^{-/-}$ mice (Figure 5D, upper and lower middle panels). Of note, RORγt^{+}Foxp3^{+} cells, which may represent stable Treg effector cells (26), although a small population in comparison with RORγt^{-}Foxp3^{+} cells, appeared more frequently (2×) in MR1$^{-/-}$ mice than in WT mice, as shown in Figure 5C. However, there were no statistical differences in the mean frequencies and cell numbers of the population between WT and MR1$^{-/-}$ mice (Figure 5D, upper and lower right panels). Additionally, there were no differences in each fraction of Th cells in unsensitized mice (Supplementary Figures 4A, B). Consistent with the above findings, staining for intracellular cytokines in CD4^{+} T cells treated *in vitro* with PMA and ionomycin for 4 h (Figure 5E) demonstrated that the CD4^{+} T cells of MR1$^{-/-}$ mice exhibited a higher frequency and number of IL-17A^{+} cells than those of WT mice (Figure 5F, upper and lower left panels), whereas frequency and number of IFN-γ^{+} cells differed between WT and MR1$^{-/-}$ mice (Figure 5F, upper and lower right panels). These cytokine profiles are consistent with the data obtained from the culture experiments in Figure 4. Comparable production of IFN-γ protein was found in the culture supernatant detected by CBA (Figure 4), as intracellular protein detected by flow cytometry (Figure 5E), whereas T-bet^{+} T cells were reduced in frequency (Figure 5B).

3.5 Increased IL-17A-producing dermal γδ T cells in MR1$^{-/-}$ mice

We then examined the population of T cells in the pinnae of MR1$^{-/-}$ mice, because the source of IL-17A production was assumed to be Th17 cells as well as Tγδ17 cells (27). Notably, the pinnae of the vehicle control mice contained dermal T cells at a higher frequency in MR1$^{-/-}$ mice (Figures 3A, B). Der γδ T cells, especially Vγ2^{+} γδ T cells, contain the Tγδ17 cell population in the skin (26). Thus, we examined Der γδ T cells for IL-17 expression in pinnae of unsensitized WT or MR1$^{-/-}$ mice after stimulation with PMA and ionomycin *in vitro*. Not only total T cells but also Der Vγ2^{+} T cells exhibited a high frequency of IL-17A^{+}-T cells in the pinnae of WT mice and an even higher frequency in MR1$^{-/-}$ mice than in WT mice under unsensitized conditions (Figures 6A, B). When Vγ2^{-} Epi γδ and Der γδ T cells were analyzed for IL-17A in the same settings as in Figure 6A (gated as Vγ2^{-} for Supplementary Figure 5A), Vγ2^{-} Der γδ T cells in MR1$^{-/-}$ mice were also significantly increased, but to a lesser extent than Vγ2^{+} Der γδ T

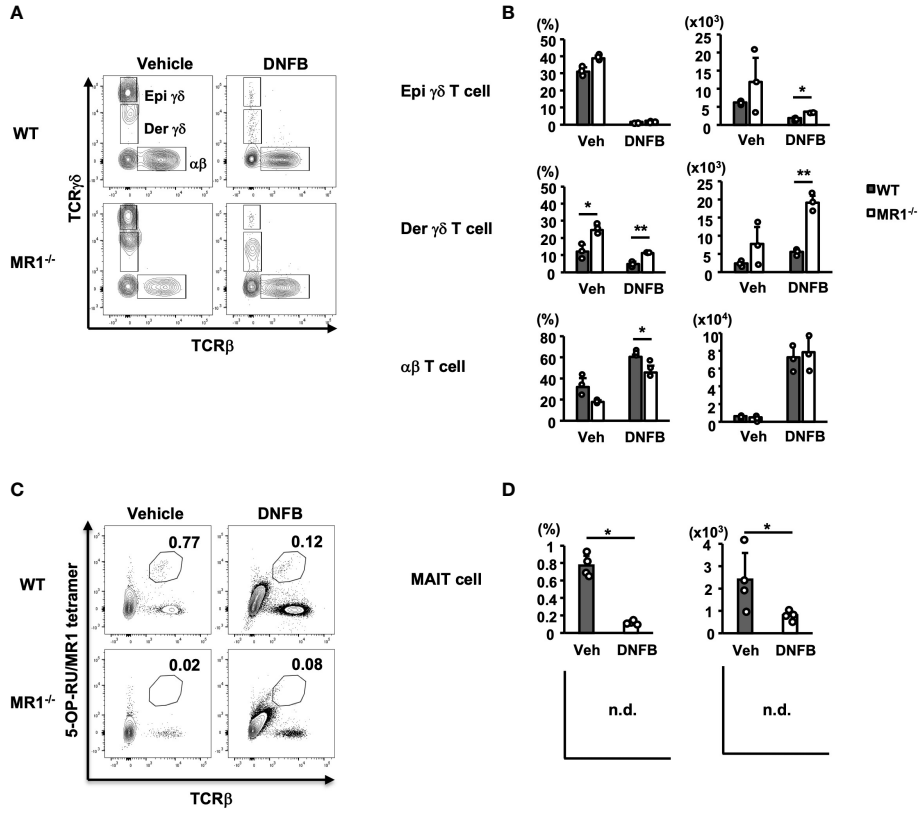

FIGURE 3
T-cell subsets in both vehicle control and DNFB-painted pinnae two days after challenge in WT and MR1$^{-/-}$ mice. **(A)** Representative flow cytometric profiles of T-cell subsets in vehicle- and DNFB-painted pinnae. The cells were prepared as for Figure 2 and analyzed according to the gating of Supplementary Figure 1B for γδ T and αβ T cells. The γδ^{hi} fraction is designated epidermal γδ T cells (Epi γδ) and the γδ^{lo} fraction as dermal γδ T cells (Der γδ). **(B)** Graphs of the frequencies and cell numbers for the αβ T, Epi γδ T, and Der γδ T cells represented in panel **(A)**. **(C)** Representative flow cytometric profiles of MAIT cells, analyzed with the gated fraction of lymphocyte CD45$^+$ cells stained with 5-OP-RU/MR1-tetramer (kindly provided by NTCF, Atlanta, GA, USA) and anti-TCRβ mAb in vehicle control and DNFB-painted pinnae of WT mice two days after challenge. **(D)** Graphs of the frequencies and cell numbers for MAIT cells for the WT mice represented in panel **(C)**. MR1$^{-/-}$ mice lack MR1-restricted cells, including MAIT cells, graphs were not demonstrated (n.d.), with no difference between control and DNFB groups in trace amounts. Representative data of at least three experiments of three to four mice/experiment. Mann–Whitney *U* test. *$p < 0.05$, **$p < 0.01$.

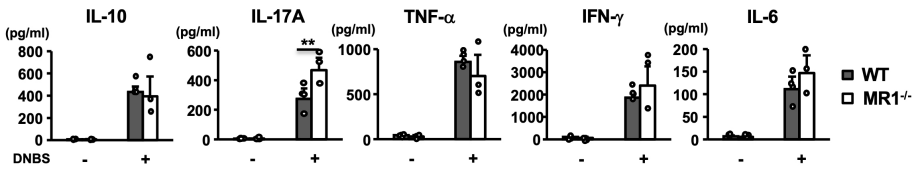

FIGURE 4
Cytokine production by antigen-specific T cells in draining lymph nodes. Lymph node T cells harvested from inguinal lymph nodes at day 5 in vehicle- and DNFB-painted WT and MR1$^{-/-}$ mice were cultured for three days in the presence and absence of DNBS (100 μg/mL). Cytokines (IL-10, IL-17A, TNF-α, IFN-γ, IL-6) in the supernatant were quantified as described in the *Materials and Methods*. Representative data of two experiments of three to four mice/experiment. Mann–Whitney *U* test. **$p < 0.01$.

cells, whereas the Vγ2$^-$ Epi γδ T cells exhibited a decreasing trend ($p = 0.05$) in MR1$^{-/-}$ mice compared to WT mice in terms of the frequency of IL-17A$^+$-cells (Supplementary Figures 5B, C).

We next examined Der γδ T cells for the expression of Vγ2 in DNFB-challenged pinnae two days after elicitation (Figure 6C). The number of Der γδ T cells that expressed the Vγ2 chain increased, even with a decreasing trend for the infiltration of αβ T cells (Figure 6D).

To examine CD4$^+$ T cells in DNFB-challenged pinnae, we analyzed the cells obtained on day 2 of elicitation, and a similar frequency was observed for WT and MR1$^{-/-}$ mice, although an increasing trend in the number of Th cells was observed in MR1$^{-/-}$ mice compared to WT mice (Figure 6E). The CD4$^+$ T cells were also analyzed for the expression of Foxp3 and RORγt (Figure 6F). Both the frequency and cell number of all subsets, RORγt$^+$Foxp3$^-$ (Th17; Figure 6G, left panels), RORγt$^-$Foxp3$^+$ (Treg; Figure 6G, middle panels), and RORγt$^+$Foxp3$^+$ (stable Treg

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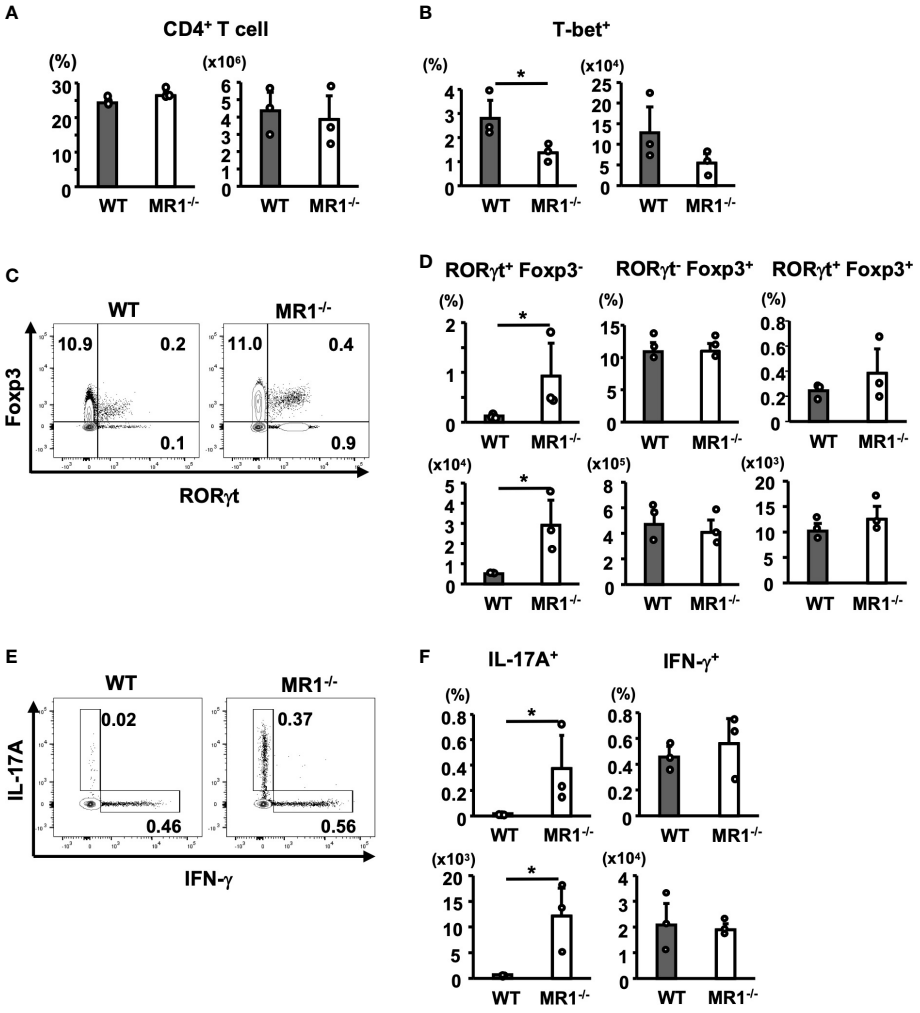

FIGURE 5
T-helper (Th) cell subsets in draining lymph nodes from WT and MR1$^{-/-}$ mice. Cells in inguinal lymph nodes after five days of sensitization were obtained and stained for analyses described in the Materials and Methods. **(A)** Frequency of the CD3$^+$ population and number of CD3$^+$CD4$^+$ cells (Th cells) in WT and MR1$^{-/-}$ mice. **(B)** Frequency and number of T-bet$^+$ (Th1) cells (upper right panels) in the Th gate shown in **(A)**. **(C)** Representative flow cytometric profiles of CD3$^+$CD4$^+$ Foxp3$^+$ and RORγt$^+$ cell populations in WT or MR1$^{-/-}$ mice. **(D)** Frequencies and numbers of RORγt$^+$Foxp3$^-$ (Th17; left panels), RORγt$^-$Foxp3$^+$ (Treg; middle panels), and RORγt$^+$Foxp3$^+$ (stable Treg effector; right panels) cells in WT and MR1$^{-/-}$ mice represented by panel **(C)**. **(E)** Representative flow cytometric profiles of IL-17A or IFN-γ intracellular staining in CD3$^+$CD4$^+$ cells. Intracellular staining of IL-17A and IFN-γ in Th cells following stimulation with PMA and ionomycin for 4 h. T cells were obtained from draining lymph nodes of WT or MR1$^{-/-}$ mice five days after sensitization at shaved abdominal skin sites. **(F)** Frequencies and cell numbers of IL-17A$^+$ (left panels) or IFN-γ$^+$ cell populations (right panels) represented by panel **(E)** Representative data of at least three experiments of three mice/experiment. Mann–Whitney *U* test. *$p < 0.05$.

effector; Figure 6G, right panels), were increased in MR1$^{-/-}$ mice compared with those in WT mice. To explain the upstream events that led to the above differences, the relevant cytokine mRNAs were analyzed 6 h after elicitation. Both *Il17* and *Il1b* expression were significantly increased in the pinnae of MR1$^{-/-}$ mice compared with WT mice as early as 6 h (Figure 6H), suggesting that the expression of IL-1β might enhance the responses of both Th17 and Tγδ17 cells. The ear swelling induced by DNFB challenge in MR1$^{-/-}$ mice was already augmented at 6 h (Supplementary Figure 6A), and the expression of genes relevant to neutrophils, such as *Csf3*, *Cxcl1*, and *Cxcl2*, was also increased (Supplementary Figure 6B), although neutrophil recruitment was comparable at this time point between WT and MR1$^{-/-}$ mice (Supplementary Figure 6C).

4 Discussion

In the present study, we demonstrated that ACD was augmented in MR1$^{-/-}$ mice compared to WT mice because of the increased numbers of Th17 and Tγδ17 cells in MR1$^{-/-}$ mice. MAIT cells were markedly reduced upon elicitation with DNFB in WT mice. MAIT cells (5-OP-RU/MR1 tetramer$^+$ cells) in the dLN on day 3 of DNFB challenge expressed Nur77 in Nur77^{gfp} mice (data not shown), suggesting that MAIT cells were activated during the elicitation phase. Furthermore, the deficiency of MAIT cells appears to be related to an altered distribution and/or number of T cells and a bias towards the type 3 immune response in a direct or indirect manner, although the mechanism remains elusive.

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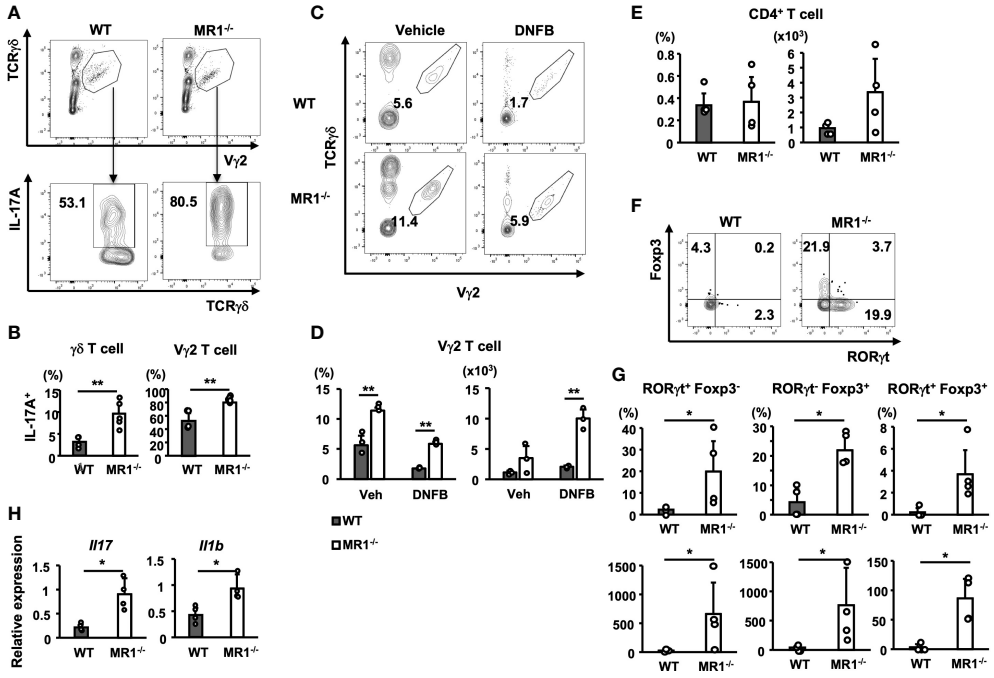

FIGURE 6
T-cell subsets in pinnae of unsensitized or sensitized mice and gene expression in sensitized pinnae. **(A)** Flow cytometric profiles of $V\gamma2^+$ γδ T cells (gated as the polygon; upper panels) in γδ T cells and intracellular IL-17A in gated $V\gamma2^+$ T cells (lower panels) in WT and $MR1^{-/-}$ mice. T cells obtained from unsensitized pinnae were stimulated with PMA and ionomycin *in vitro* for 4 h. The expression of intracellular IL-17A was then analyzed in the $V\gamma2^+$ population in the total γδ T cells by flow cytometry. **(B)** Frequency of IL-$17A^+$ population in the total γδ T cells (left panel) or in the $V\gamma2^+$ cells (right panel) in WT and $MR1^{-/-}$ mice represented by panel **(A)**. **(C)** Representative flow cytometric profiles of dermal $V\gamma2^+$ γδ T cells (gated as the polygon) in vehicle control and DNFB-painted pinnae in WT and $MR1^{-/-}$ mice two days after challenge. **(D)** Frequencies and numbers of $V\gamma2^+$ γδ T cells in vehicle-painted (veh) and DNFB-painted (DNFB) pinnae. **(E)** Frequencies and numbers of $CD4^+CD3^+$ cells (Th) in DNFB-painted pinnae of WT and $MR1^{-/-}$ mice. **(F)** Flow cytometric profiles of RORγt and Foxp3 staining for the Th cells exhibited in E for WT (left panel) and $MR1^{-/-}$ (right panel) mice. **(G)** Frequencies and numbers of $ROR\gamma t^+Foxp3^-$ (Th17; left panels) cells and $ROR\gamma t^-Foxp3^+$ (Treg; middle panels) cells represented in panel **(F)**. **(H)** Relative expression of *Il17* (left panel) and *Il1b* (right panel) mRNA in pinnae 6 h after DNFB challenge in WT and $MR1^{-/-}$ mice. Representative data of at least two experiments of three to five mice/experiment. Mann–Whitney *U* test. $*p < 0.05$, $**p < 0.01$.

MR1 deficiency may cause wider defects in MR1-restricted T cells (MR1T) (28) besides MAIT cells, as the diversity of MR1T (including MAIT and MR1-reactive T) cells extends to six different groups with unique modes of recognition, binding, and reactivity (29), most of which are αβ type but include a γδ type, such as Vδ3Vγ8 T cells that bind and recognize MR1 at its membrane proximal region, similar to an α3 domain-recognizing antibody (30, 31). Notably, the significant role of MR1T cells have been shown to play an important role in antitumor immunity (32) and have already been implicated in infectious and autoimmune diseases (33). It should be noted that T cells obtained from $V\alpha19J\alpha33^{Tg}C\alpha^{-/-}$ mice that overexpress MAIT cells were previously tested for suppression of delayed-type hypersensitivity (34). Transfer of invariant $V\alpha19^+$ T cells but not control non-transgenic T cells suppressed foot pad swelling induced by sheep red blood cells in B6 hosts prior to sensitization, accompanied by a reduction of serum IL-17 and IFN-γ. Nevertheless, further studies will be needed to explore the mechanisms by which MR1T/MAIT cells interact with other immune cells to suppress ACD response.

The significant increase and bias towards Tγδ17 cells we observed in the skin of $MR1^{-/-}$ mice is likely associated with the enhanced ACD response, although the timing and site of the developmental characteristics (35) in the biased distribution of $V\gamma2^+$ Tγδ17 in $MR1^{-/-}$ mice remain to be determined. Interestingly, MAIT cells and γδ T cells have a reciprocal relationship, similar to the expansion of MAIT cells in NKT cell-deficient mice (36). For instance, a patient with a homologous MR1 mutation at position 31 Arg to His substitution (position 9 in mature MR1 protein: $MR1^{R9H}/MR1^{R9H}$) was discovered to display primary immunodeficiency due to functional MR1 deficiency, with no circulating MAIT cells (37). Notably, the patient had increased circulating T cells expressing Vγ9Vδ2 with the $CD27^-CD28^-$ phenotype. Conversely, MAIT cells have been reported to be increased in $\gamma\delta^{-/-}$ mice (38). These results indicate that there is an equipoise among the three types (NKT, MAIT, and γδ) of innate-like T cells by competing with a homeostatic factor or niche (39). Hence, it is tempting to speculate that NKT cells, MAIT cells and γδ T cells all contribute in a reciprocal manner, as a ménage à trois, to various inflammatory diseases such as ACD. The present study demonstrated that in $MR1^{-/-}$ mice, the dominant $V\gamma2^+$ Tγδ17 cells in the skin (Figure 6) and increased Th17 cells (Figure 5) upon sensitization in the dLN migrated to the skin and enhanced ACD. It is intriguing that skin MAIT cells are biased towards IL-17 production (MAIT17) and promote tissue repair (38), and that

their deficiency appears to be compensated by the dominance of Tγδ17 cells in the skin. It is not known whether there are any direct interactions between MAIT cells and γδ T cells that limit each other's effector functions. However, one may speculate that MAIT cells and γδ T cells compete with each other for homing niches within the dermis, where MAIT cells localize near the dermal-epidermal interface (38) and γδ T cells localize to also in superficial regions (40). The most critical factor for MAIT cell tissue homing and homeostasis is likely their early life exposure to and sustained interaction with the microbiota that synthesize riboflavin (38). Tγδ17 cells are similarly influenced by microbes for their expansion and functional activity (41), suggesting that skin commensals affects the balance between MAIT cells and γδ T cells. Furthermore, cytokines such as IL-1β and IL23 (38) in the environment are also thought to be important factors that affect the balance between these T cell subsets.

The macroscopic and microscopic appearances of skin pathology were markedly enhanced with edema and cellular infiltration in MR1$^{-/-}$ mice compared with WT mice (Figure 1). In severe cases in MR1$^{-/-}$ mice, the elicited pinnae were covered with crustae by frequent scratching, and a large area of the inflammatory lesion was sometimes lost, presumably due to necrosis or injury, which was not observed in WT mice. Thus, the skin thickness data for severe cases were inevitably unincorporated in the analyses. The severity of dermatitis may permit use of ACD in MR1$^{-/-}$ mice as an intractable model system to study disease pathogenesis and testing immune therapies. Notably, MAIT cells have been reported to display tissue repair functions, as wound healing by punch biopsy was significantly delayed in the absence of MAIT cells (38). If the keratinocytes injured during ACD by cytotoxic lymphocytes fail to be replaced with newly proliferated cells, the epithelial defect may cause infections and further damage the skin. A recent study also revealed that amphiregulin, a member of the epidermal growth factor family produced by MAIT cells, accelerated wound closure, but in an MR1-independent manner (42). In experimental autoimmune uveoretinitis, MAIT cells ameliorated disease, which was associated with anti-inflammatory/neuroprotective activities of IL-22 as well as IL-22-independent repair functions upon stimulation with 5-OP-RU (43). Accordingly, the severity of ACD response in MR1$^{-/-}$ mice observed in our study may result in part from defective repair due to MR1T/MAIT cell deficiency.

In the absence of exogenous stimulation, MR1$^{-/-}$ mice exhibited a similar pinna thickness compared to WT mice (Figure 1C), suggesting that MR1$^{-/-}$ mice do not develop spontaneous dermatitis. However, increased production of IL-1β in mutant mice than WT mice was detected at pinnae after 6 h of elicitation with DNFB (Figure 6H), since the barrier function of the skin was presumably weakened in MR1$^{-/-}$ mice due to MAIT cell deficiency (38, 44). The ear swelling in MR1$^{-/-}$ mice was more enhanced than WT mice at 6 h of elicitation (Supplementary Figure 6A), whereas the level of neutrophil migration was similar between the two strains (Supplementary Figure 6C), suggesting that edematous changes at the very early phase appeared to be different between MR1$^{-/-}$ and WT mice.

The cellular infiltrates consisted mainly of Mo/Mϕ, neutrophils, and αβ T cells in both MR1$^{-/-}$ and WT mice after DNFB elicitation (Figure 2 and Supplementary Figures 2, 3). Notably, there were significantly more neutrophils in terms of percentage and actual cell numbers in MR1$^{-/-}$ mice than WT mice. The recruitment of infiltrates was concordant with the enhanced expression of cytokines and chemokines by Th17 and Tγδ17 cells in the pinnae stained with DNFB, which supported neutrophil generation, recruitment, and activation (Figures 2C, 4). Resident Mϕ were reduced in percentage due to, in part, dilution by the recruitment of Mo/Mϕ and a reduction in the actual cell number in both WT and MR1$^{-/-}$ mice (Supplementary Figures 2, 3). As for eosinophils in pinnae, the cell number per 10 mg tissue was not significantly increased in DNFB-challenged pinnae in MR1$^{-/-}$ mice compared with WT mice (data not shown). Although these changes result from the MR1T/MAIT cell deficiency, the underlying mechanisms remain to be further investigated.

When the T cells were compared in MR1$^{-/-}$ and WT mice, a large number of αβ-type T cells specific for the sensitizer Ag in pinnae was equally recruited in both MR1$^{-/-}$ and WT mice after challenge with DNFB. Thus, the percentage of Epi and Der γδ T-cell fractions decreased accordingly after challenge (Figure 3B). The apparent reduction was simply due to dilution by the migrated αβ T cells into the pinnae, whereas the number of γδ T cells in each fraction increased after challenge to enhance the ACD response *via* production of cytokines and chemokines from Th17 and Tγδ17 cells. Notably, MR1$^{-/-}$ mice harbored a significantly higher percentage of Der γδ T cells, even in unsensitized states, and exhibited a higher percentage of IL-17A^{+} cells in both the Vγ2^{+} and Vγ2^{-} fraction (Vγ2^{+} > Vγ2^{-}) upon *in vitro* stimulation with PMA and ionomycin (Figures 6C, D; Supplementary Figures 5B, C). The abundance of Tγδ17 cells in the skin of MR1$^{-/-}$ mice may result in a robust type 3 immune response at the site of ACD since more Tγδ17 cells during the initiation phase in the dermis effectively boosted the response compared with WT mice.

The frequency of MAIT cells in mouse skin is strikingly different from that in human skin, with approximately 10% of αβ T cells being MAIT cells in mice and 0.5%-2% of αβ T cells being MAIT cells in humans, with the remainder being the conventional type and NKT cells (38, 44). Therefore, the present results must be considered when assessing whether they are readily applicable to human cases of ACD. However, the involvement of innate T cells in ACD is not compromised in humans, as iNKT cells presumably participate as effectors (21) and the role of NKT cells in ACD may vary depending on different sensitizers (19). MAIT cells were detected in palladium allergy in a previous report (22), and the involvement of iNKT cells has already been demonstrated in allergies to metals such as nickel, cobalt, and chromium (21, 45–47) that are present in accessories, biomedical devices, and food constituents (1). It is intriguing to consider whether MAIT cells and iNKT cells adopt a common or distinct pathway that affects the ACD response. Notably, MAIT cells have been examined as promising targets for immunotherapy in the skin for phototherapy of atopic dermatitis (48) and as effectors of a major inflammatory disease, psoriasis (13). The utilization of MAIT

ligands as therapeutic agents may be associated with low resistance by patients, since they are vitamin B-related compounds with either inhibitory (VB_9 -folate) or stimulatory (VB_2 -riboflavin) activities (14, 49). To examine whether MAIT cells can be modulated to protect against ACD, further investigations that clarify their immunoregulatory role will be required.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine (#2017-143, 2018-119, 2019-025, and 2022-079).

Author contributions

NI, MS and KI designed the studies and wrote the manuscript. NI, MS, EC and KY performed and analyzed the experiments. MG and KI supervised the work. 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1215478/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Gating strategy for the flow cytometric analyses performed in the present study. A. Gating strategy for detecting granulocytes. The acquisition was mostly ungated except for small particles (with very low FSC-A) and the acquired cells were widely gated with FSC-A/SSC-A as shown to include larger cells with intracellular granules. Cells were removed from doublets and dead cells followed by gating for the $CD45^+$ population as shown in sequence. Finally, $CD11b^+Ly6G^+$ cells in the $CD45^+$ cell population were designated as neutrophils. B. Gating strategy for detecting T-cell subsets. The acquired cells were lymphocyte-gated in tighter FSC-A/SSC-A than that of A, as shown. The $CD45^+$ population after removal of doublets and dead cells was further analyzed with TCRβ/TCRγδ or TCRγδ/Vγ2 to discriminate αβ T, γδ T, and $V\gamma2^+$ T subsets in the γδ T cell population.

SUPPLEMENTARY FIGURE 2
Monocytes (Mo) and macrophages (Mϕ) in the ACD-induced pinnae in WT and $MR1^{-/-}$ mice. Cells infiltrated into the pinna were prepared two days after challenge with enzymatic degradation as described in the *Materials and Methods* and analyzed by flow cytometry as described for Supplementary Figure 1A. Flow cytometric profiles of inflammatory cells in the vehicle- and DNFB-painted pinnae in WT and $MR1^{-/-}$ mice, as shown in . The cells of the $CD11b^+Ly\text{-}6G^{lo\text{-}(-)}$ population were further separated into $Ly\text{-}6C^{hi}F4/80^{lo}$ (Mo/Mϕ) and $Ly\text{-}6C^-F4/80^{hi}$ (Mϕ). B. Frequency of Mo/Mϕ (upper panel) or Mϕ (lower panel) in vehicle- and DNFB-painted pinnae of WT and $MR1^{-/-}$ mice at day 2 after elicitation represented by panel A. Representative data of at least three experiments of three mice/experiment.

SUPPLEMENTARY FIGURE 3
Cellular composition of mononuclear cells obtained from vehicle- and DNFB-painted pinnae of WT and $MR1^{-/-}$ mice. Each fraction of cells was recapitulated from the results of flow cytometric analyses according to the gating described for Supplementary Figure 1. Neu: neutrophil ($CD11b^+Ly\text{-}6G^{hi}$); Mϕ: macrophage ($CD11b^+Ly\text{-}6G^{lo\text{-}(-)}Ly\text{-}6C^{lo}/F4/80^{hi}$); Mo/Mϕ: monocyte/macrophage lineage ($CD11b^+Ly\text{-}6C^{lo\text{-}(-)}Ly\text{-}6C^{hi}/F4/80^{lo}$); αβ: αβ T cells ($CD3^+TCR\beta^+TCR\gamma/\delta^-$), $\gamma\delta^{hi}$: epidermal γδ T cells ($CD3^+TCR\beta^-TCR\gamma/\delta^{hi}$); and $\gamma\delta^{lo}$: dermal γδ T cells ($CD3^+TCR\beta^-TCR\gamma/\delta^{lo}$). Representative data of at least three experiments of three mice/experiment.

SUPPLEMENTARY FIGURE 4
T-helper (Th) cell subsets in draining lymph nodes from unsensitized WT and $MR1^{-/-}$ mice. Cells in inguinal lymph nodes were obtained from each unsensitized strain of mice and stained for the analyses according to the *Materials and Methods*. **A**. Representative flow cytometric profiles of $CD3^+CD4^+$ cells of the $Foxp3^+$ and $ROR\gamma t^+$ population in WT and $MR1^{-/-}$ mice. **B**. Frequencies and cell numbers of $ROR\gamma t^+Foxp3^-$ (Th17; left panels), $ROR\gamma t^-Foxp3^+$ (Treg; middle panels), and $ROR\gamma t^+Foxp3^+$ (stable Treg effector; right panels) cells in WT and $MR1^{-/-}$ mice represented in panel A. Representative data of at least two experiments of four mice/experiment.

SUPPLEMENTARY FIGURE 5
IL-17A expression in epidermal γδ T cells and $V\gamma2^-$ dermal γδ T cells in unsensitized mice. **A**. Gating of $V\gamma2^-$ epidermal and dermal γδ T cells in flow

panels of WT and MR1$^{-/-}$ mice. T cells obtained from unsensitized pinnae were stimulated with PMA and ionomycin *in vitro* for 4 h. **B**. The expression of intracellular IL-17A was analyzed in the Vγ2^{-} population in epidermal and dermal γδ T cells by flow cytometry. **C**. Frequency of the IL-17A^{+} population in Vγ2^{-} epidermal (upper panel) and dermal γδ T cells (lower panel) in WT and MR1$^{-/-}$ mice represented in panel B. Representative data of at least three experiments of four mice/experiment. Mann–Whitney *U* test. *$p < 0.05$.

SUPPLEMENTARY FIGURE 6

MR1$^{-/-}$ mice develop an augmented response at 6 h after DNFB challenge. **A**. WT (closed circle) and MR1$^{-/-}$ (open circle) mice were sensitized and challenged on the left pinna with vehicle only or on the right pinna with DNFB. The thickness of the pinnae was then measured with a digital micrometer 6 h after challenge. The increment in thickness of the sensitized pinna represented as ΔEar swelling in Figure 1A. **B**. The expression of *Cxcl1, Cxcl2,* and *Csf3* related with neutrophil recruitment and activation was examined with mRNA obtained from the left pinnae (vehicle control) and the right pinnae (DNFB) of either WT (closed bar) or MR1$^{-/-}$ mice (open bar) at 6 h after challenge. **C**. Frequency of neutrophils in the CD45^{+} fraction in MR1$^{-/-}$ mice compared with those of WT mice as in 6 h after challenge (vehicle: closed bar, DNFB: open bar). Representative data of at least two experiments of four mice/experiment. Mann–Whitney *U* test. *$p < 0.05$

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Cytotoxic innate intraepithelial lymphocytes control early stages of *Cryptosporidium* infection

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Background: Intraepithelial lymphocytes (IELs) are the first immune cells to contact and fight intestinal pathogens such as *Cryptosporidium*, a widespread parasite which infects the gut epithelium. IFN-γ producing $CD4^+$ T IELs provide an efficient and a long-term protection against cryptosporidiosis while intraepithelial type 1 innate lymphoid cells limits pathogen spreading during early stages of infection in immunodeficient individuals. Yet, the role of T-cell like innate IELs, the most frequent subset of innate lymphocytes in the gut, remains unknown.

Methods: To better define functions of innate IELs in cryptosporidiosis, we developed a co-culture model with innate IELs isolated from *Rag2*$^{-/-}$ mice and 3D intestinal organoids infected with *C. parvum* using microinjection.

Results: Thanks to this original model, we demonstrated that innate IELs control parasite proliferation. We further showed that although innate IELs secrete IFN-γ in response to *C. parvum*, the cytokine was not sufficient to inhibit parasite proliferation at early stages of the infection. The rapid protective effect of innate IELs was in fact mediated by a cytotoxic, granzyme-dependent mechanism. Moreover, transcriptomic analysis of the Cryptosporidium-infected organoids revealed that epithelial cells down regulated Serpinb9b, a granzyme inhibitor, which may increase their sensitivity to cytolytic attack by innate IELs.

Conclusion: Based on these data we conclude that innate IELs, most likely T-cell-like innate IELs, provide a rapid protection against *C. parvum* infection through a perforin/granzymes-dependent mechanism. *C. parvum* infection. The infection may also increase the sensitivity of intestinal epithelial cells to the innate IEL-mediated cytotoxic attack by decreasing the expression of Serpin genes.

KEYWORDS

gut, innate intraepithelial lymphocytes, cryptosporidium, organoids, cytotoxicity

1 Introduction

Intestinal intraepithelial lymphocytes (IELs) are tissue resident memory cells which localize within the epithelial layer all along the digestive tract. Owing to their strategic position, their effector and regulatory functions, IELs are considered as the guardians of the gut. Notably, IELs play a potent role in host defense as they can respond rapidly and efficiently to a large variety of pathogens such as viruses, bacteria, fungi, and parasites (1, 2). This property certainly relies on the heterogeneity of the IEL population which is mainly formed by two T cell subsets named conventional ($_{C}$IEL) and nonconventional ($_{NC}$IEL) IELs. Conventional IELs are similar to the effector/memory TCRαβ+ cells from the other compartments and are stimulated by microbial peptides presented by MHC. In contrast, in mice, $_{NC}$IELs express the homodimer CD8αα with either a TCRαβ or a TCRγδ. Most of them recognize self-antigens or proteins from pathogens independently of a classical MHC (1, 2). The IEL compartment also contains lymphoid cells which do not express a TCR. This subpopulation of IELs is mainly composed of type 1 innate lymphoid cells (ILC1) expressing the natural cytotoxicity receptor NKp46 and of peculiar innate lymphocytes with T cell features, named T-cell-like innate IELs (3, 4). The latter population is dominant and expresses intracellular CD3γ and the integrin CD103 ($\alpha_E\beta7$). Around half of them are CD8αα$^+$ (iCD8α) (4, 5). While ILC1s express high level of IFN-γ, T-cell-like IELs produce granzymes and are cytotoxic (4). ICD8α also have the capacity to produce osteopontin encoded by *Spp1* which sustains the homeostasis of ILC1 (6), to phagocyte bacteria and to process and present antigens to MHC class II-restricted T cells (5). Yet, the role of T-cell-like innate IELs in infection remains poorly studied.

Cryptosporidium is an apicomplexan parasite and an opportunistic pathogen that infects the gut epithelium. It is recognized as one of the most important waterborne contaminants in the world and a major cause of diarrhea in human and animals. Since *Cryptosporidium* infects enterocytes by their apical side and replicate within the epithelium, IELs are crucial to detect and fight the parasite (7–9). *Cryptosporidium* specific CD4+ T $_{C}$IELs are able to eliminate the parasite by secreting IFN-γ and thus provide an efficient and a long-term protection (9). When the adaptive immune response is impaired the infection is chronic and much more severe (10–13). However, in immunodeficient individuals, innate mechanisms limit the replication of the parasite. Notably, a number of studies have pinpointed the protective roles of intestinal epithelial cells (iEC), mononuclear phagocytes, neutrophils and conventional Natural Killer (cNK) cells (14, 15). Yet, there are conflicting data about the contribution of cNK cells in cryptosporidiosis since the depletion of cNK cells using anti-asialoGM1 antibodies in immunodeficient SCID or *Rag2*$^{-/-}$ mice does not impact the course of the infection (16–18). Actually, ILC1s but not cNK cells seem to protect against *Cryptosporidium*. Indeed, a recent work showed that ILC1s limit the expansion of the parasite in *Rag2*$^{-/-}$ animals through their secretion of IFN-γ (18).Yet, the role of innate IELs in cryptosporidiosis remains poorly studied. Herein, we developed an *in vitro* model to specifically investigate their functions during the infection. The model is based on the co-culture of innate IELs isolated from *Rag2*$^{-/-}$ mice with murine 3D intestinal organoids infected with *Cryptosporidium parvum*. Using this original experimental assay, we showed that innate IELs rapidly prevent the expansion of the parasite. Interestingly, the protection mediated by IFN-γ produced by ILC1s was not essential during the very early stage of the infection. Instead, we found that the protective effect mostly depends on perforin and serine proteases such as granzymes. Moreover, we also found that infected iEC down regulate the natural granzyme inhibitor serpinb9b and thus could be more sensitive to IELs mediated cytotoxicity.

2 Material and methods

2.1 Mice

Females *Rag2*$^{-/-}$C57BL/6 and C57BL/6 WT mice were obtained from a colony bred at the Pasteur Institute of Lille (France) and regularly controlled for microbial or parasitological pathogens. Animals were housed in groups in covered cages and maintained under aseptic conditions with standard laboratory food and water. The animal experiment ethics committee approved the experimental animal study protocol (APAFIS#30539).

2.2 *In vivo* infection of *Rag2*$^{-/-}$ C57BL/6 mice

Eight-week-old *Rag2*$^{-/-}$mice were infected by oral gavage with $5x10^4$ *C. parvum* oocysts (Iowa strain) per mouse (n=15 infected and n=14 controls). Twenty-four hours post infection (PI), mice were euthanized and the small intestine from each mouse was collected. Ileal sections were collected to quantify the number of innate immune cells in the epithelium by immunohistochemistry and to quantify the parasitic load and the expression of cytokines by RT-qPCR. Innate IELs were isolated as described below and used to quantify gene expression by RT-qPCR and to define their phenotype by flow cytometry.

2.3 Isolation of intestinal crypts and culture of intestinal organoids

Intestinal crypts were isolated from small intestine of female C57BL/6 mice as described by Sato et al. (19). Briefly, small intestine fragments were incubated with PBS 1X containing 8mM of EDTA and shaked for 1h on ice using a rocking platform. Then, EDTA buffer was removed, tissue fragments were vigorously resuspended in cold PBS 1X and supernatant was collected to quantify the number of crypts. One thousand crypts were cultured in 30µl of Matrigel (Corning). The Matrigel was polymerized for 10 minutes at 37°C, and 600µl/well of LWRN conditioned medium was added. The Rho-associated kinase inhibitor Y-27632 (10 µmol/L; Tocris) was included in the medium for the first 2 days to avoid anoikis.

Organoids were passed once a week by dissociating the Matrigel for five minutes at 37°C with TrypLE Express (Gibco, Life Technologies).

2.4 Microinjection of intestinal organoids with *C. parvum* oocysts

C. parvum IOWA oocysts were purchased from WaterborneTM, Inc. (New Orleans, Louisiana). Oocyst solution was stored in the shipping medium (phosphate buffered saline or PBS with penicillin, streptomycin, gentamycin, amphotericin B and 0.01% Tween 20) at 4°C until use. For microinjection, 250 oocysts/µl of the stock solution were centrifuged at 2000 g for 10 minutes. After treatment with 0.025% of Trypsin pH=2.4 (Sigma) for 20 minutes at 37°C, oocysts were resuspended with excystation medium containing RPMI 1640 with 2 mM of L-glutamine (Gibco), 1% of fetal calf serum, 100 mg/ml of penicillin/streptomycin (Gibco), 0.25 mg/ml of Gentamycin (Dutscher), 0.2mg/ml of Bovine Bile (Acros Organics), 1mg/ml of glucose (BioXtra), 0.25µg/ml of folic acid (Alfa Aesar), 1µg/ml of 4-aminobenzoic acid (VWR), 8.75µg/ml of L-Ascorbic acid (Sigma Aldrich) and 0.5µg/ml Calcium Pantothenate (Acros Organics) (20).

A sterile glass capillary of 15µm diameter was used for microinjection (Transfer tip eppendorf). The capillary was loaded with oocysts (250 oocysts/µl) suspended in their excystation medium containing 25 µg/ml of Fast green dye (Sigma) in order to visualize micro-injected organoids. Approximately 200 nl of suspension was injected into each organoid using the Leica DMI 4000B microinjector. For each experiment 20 to 30 organoids were cultured in IbiTreat microdish (Ibidi) and 50% of them were microinjected.

2.5 Isolation of innate IELs and co-culture with intestinal organoids

Isolation of murine innate IELs was performed according to the method described by Schulthess et al., 2012 (21). Briefly, small intestines of 8 weeks old *Rag2*$^{-/-}$ mice were removed and washed with cold PBS 1X. Mesenteric fat and Peyer's patches were removed. The intestine was then opened longitudinally and cut into 0.5 cm fragments which were then incubated in 50 ml of RPMI (Gibco) containing 10% FCS for 2h at 37°C with vigorous agitation. The supernatant is passed through a glass wool column to remove part of iECs. Cells were then separated on a gradient 40/80% of Percoll (GE Healthcare). The innate IELs ring was then collected, washed and taken up in 1ml RPMI-10% FCS. Then 10^5 innate IELs were co-cultured with infected or non-infected organoids 24 hours PI. The co-culture was stopped after 24h. To inhibit IFN-γ or cytolytic activities, anti-IFN-γ Ab (10 µg/ml) (clone XMG1.2 Biolegend) or granzyme B inhibitor I (10µM) (Merck) or aprotinin (2µg/ml) (Sigma) was added in the co-culture simultaneously with IELs. Concanamycin A treatment: innate IELs were isolated from *Rag2*$^{-/-}$ mice and treated for 3h with 50nM of concanamycin A (CMA) (Biotechne) at 37°C. Treated-innate IELs were then washed 2 times and co-cultured with infected organoids for 24h.

2.6 RNA isolation and RT–qPCR

Total RNA was extracted from organoids using a Nucleospin®RNA II kit (Macherey-Nagel) according to manufacturer's protocol. Complementary DNA was synthesized from 1 µg total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real time PCR was performed using Power SYBR Green PCR Master Mix in a StepOne plus system (Applied Biosystems). Gene expression was quantified using the ΔΔ Ct method for rRNA 18s. Cp18S forward, 5'- TGCCTTGAATACTCCAGCATGG-3'; Cp18S reverse, 5'-TACAAATGCCCCCAACTGTCC-3'. The expression of other genes was quantified using ΔCt method. The gene coding for murine beta-actin (*β-actin*) was used as housekeeping gene (Table 1).

2.7 Confocal microscopy

Innate IELs isolated from the *Rag2*$^{-/-}$ mice were labeled with 5 µM CellTrace CFSE (Invitrogen) for 20 min at 37° C. One hundred thousand cells were co-cultured with organoids. After 24 h of culture, organoids were fixed with 4% paraformaldehyde (Microm microtech) for 30 min at RT. Organoids were then permeabilized with PBS 1X containing 1% of triton 100X (Sigma) for 10 min at RT. After washing, organoids were labeled with DAPI (Thermo Fisher) and 1.65 µM of phalloidin Alexa Fluo 647 (Invitrogen) for 1 hour at RT. The co-culture was visualized under a Leica Sp8 confocal microscope.

2.8 Flow cytometry and Cell-sorting

Cells were first incubated with anti-mouse CD16/CD32 Ab (clone 2.4 G2, BD Biosciences) for 10 min at 4° C, then washed and labeled with a cocktail of antibodies for 20 min at 4°C in dark (see Table 2). Cells were washed and treated with BD FACS Lysing Solution (BD

TABLE 1 Forward and reverse primer sequences for RT-qPCR.

Gene	Primer sequences
Actb	F: CCTTCTTGGGTATGGAATCCT R: CTTTACGGATGTCAACGTCAC
Ifng	F: ATGAACGCTACACACTGCATC R: CCATCCTTTTGCCAGTTCCTC
Spp1	F: TCTGATGAGACCGTCACTGC R: AGGTCCTCATCTGTGGCATC
Serpinb9b	F: GATGATTGCCAGCTAGATTG R: TGACCACATAATGTCTGGTTTG
Ifna	F: GTGCTGGCTGTGAGGACA R:GGCTCTCCAGACTTCTGCTCT
Gzmb	F: CAGCAAGTCATCCCTATGGT R: TACTCTTCAGCTTTAGCAGCAT
Cd8a	F: TTTACATCTGGGCACCCTTG R: CTTTCGGCTCCTGTGGTAG
Itgae	F: GACAAAGACTCAGGACCACAC R: GGCCACGGTTACATTTTCTTT
Ncr1	F: GATCAACACTGAAAAGGAGACT R: TGACACCAGATGTTCACCGA

TABLE 2 Antibodies used for flow cytometry.

Manufacturer	Cat#	Antibodies	Clone
Sony	1115540	FITC anti-mouse CD45	30-F11
Biolegend	100713	APC/Cyanine anti-mouse CD8a	53-6.7
Sony	1207125	Pe/Cy7 anti-mouse CD103	2E7
Biolegend	137611	Brilliant Violet 421 anti-mouse CD335 (NKp46)	29A1.4

Biosciences) for 5 minutes at RT. After washing, cells were analyzed on the LSR Fortessa X20 cytometer (Becton Dickinson).

For cell sorting, innate IELs were labeled with a cocktail of antibodies (CD45 FITC (Sony), CD103 PeCy7 (Biolegend) and NKp46 BV421 (Biolegend). Four populations ($CD45^+$, $CD45^+NKp46^-$, $CD45^+CD103^-NKp46^-$, and $CD45^+CD103^+$) were sorted using a BD FACSAria II SORP cell sorter (Becton Dickinson).

2.9 Immunohistochemistry

Ileal samples from all mice of each group were fixed with 4% paraformaldehyde and then embedded in paraffin. Four micrometer-thick sections were incubated with citrate 1X antigen repair solution (Skytec) at 95°C for 20 min after dewaxing and hydration. Then endogenous peroxidase was blocked by Bloxall blocking solution (Vector) for 10 min at RT. Nonspecific antigens were blocked with 5% goat serum for 30 min. The sections were exposed to primary anti-CD3γ (Abcam) and anti -CD8α (Cell Signaling Technology) Abs overnight at 4°C. After washing with Tris-buffered saline solution containing 0.05% Tween, sections were incubated for 30 min with the detection kit "Immpress peroxidase Polymer anti rabbit IgG" (Vector). Negative controls were incubated with irrelevant serum. The staining was revealed using the peroxidase substrate, DAB (Cell Signaling Technology). Hematoxylin counterstain was performed before mounting the slides in an aqueous medium. Slides were analyzed using a microscopy (Leica).

2.10 Quantification of IFN-γ

The secretion of IFN-γ was measured in supernatants of co-cultures using "Mouse IFNγ ELISA MAXTM Deluxe set" kit (Biolegend).

2.11 LDH release assay

Cell death was determined using Cytotox96 non-radioactive cytotoxicity assay (Promega) following the manufacturer's protocol. The colorimetric assay quantified lactate dehydrogenase (LDH) activity released from the cytosol of damaged target cells into the supernatants. Briefly, after 48h of infection, 50µl of co-culture supernatant were incubated with 50 µl of CytoTox 96 reagent for 30 min. The reaction was stopped and the absorbance was recorded at 490nm on Fluostar Omega spectrophotometer (BMG Labtech).

2.12 RNA sequencing

Starting from 4µl of total RNA we add 1µl of ERCC spike-in control. Library generation is then initiated by oligo dT priming, from total RNA (between 50 and 200 ng). The primer already contains Illumina- compatible linker sequences (Read 2). After first strand synthesis the RNA is degraded and second strand synthesis is initiated by random priming and a DNA polymerase. The random primer also contains 5' Illumina-compatible linker sequences (Read 1). At this step Unique Molecular Identifiers (UMIs) are introduced allowing the elimination of PCR duplicates during the analysis. After obtaining the double stranded cDNA library, the library is purified with magnetics beads and amplified. During the library amplification the barcodes and sequences required for cluster generation (index i7 in 3' and index i5 in 5') are introduced due to Illumina- compatible linker sequences. The number of cycles depends on the starting quantity, between 14 cycles for 200ng of total RNA and 16 cycle for 50ng of total RNA. If the quantity is less than 50 ng, the number of cycles will be increase (for example for 17ng, 17 cycles). The final library is purified and deposed on High sensitivity DNA chip to be controlled on Agilent bioanalyzer 2100. The library concentration and the size distribution are checked.

Each library is pooled equimolarly and the final pool is also controlled on Agilent bioanalyzer 2100 and sequenced on NovaSeq 6000 (Illumina) with 100 cycles chemistry. Different chips can be used for sequencing, it depends on the number of libraries pooled, the objective is to obtain a minimum of 20 M reads by sample.

To eliminate poor quality regions and poly(A) of the reads, we used the fastp program. We used quality score threshold of 20 and removed the read shorter than 25 pb. The read alignments were performed using the STAR program with the genome reference mouse (GRCm39) and the reference gene annotations (Ensembl). The UMI (Unique Molecular Index) allowed to reduce errors and quantitative PCR bias using fastp and umi-tools. Based on reads alignments, we counted the numbers of molecules by gene using FeatureCount. Other programs were performed for the quality control of reads and for the workflow as qualimap, fastp, FastQC and MultiQC. Differential Gene Expression of RNA-seq was performed with R/Bioconductor package DESeq2. The cut-off for differentially expressed gene was p-value padj (BH) < 0.1. RNA sequencing data that support the findings of this study have been deposited in sequence Read Archive (SRA) data (https://dataview.ncbi.nlm.nih.gov/object) with the accession code PRJNA98061.

2.12 Statistical analysis

Data were expressed as the mean ± SD or the median with range. A Wilcoxon matched-pairs signed rank test was used for *in vitro* experiments. Statistical analyses were performed using StatXact software (Cytel Studio 7) and GraphPad Prism software

10.3389/fimmu.2023.1229406

(version 5.0, San Diego, CA, USA). The threshold for statistical significance was set to $p<0.05$.

3 Results

3.1 IFN-γ, osteopontin and granzyme B expressions are up regulated at early stages of *Cryptosporidium* infection in *Rag2*$^{-/-}$ mice

To identify immune mechanisms activated during the very early phase of *C. parvum* infection, we first analyzed the expression of genes related to key effector functions of innate immune cells such as interferons and granzymes in the small intestine of adult *Rag2*$^{-/-}$ mice 24h post-infection (PI). We found that expression of IFN-γ (*Ifng*) and Osteopontin (*Spp1*), two molecules associated with the type 1 immune response, as well as the cytolytic enzyme granzyme B (*Gzmb*) were rapidly increased in the ileum of infected animals. In contrast, the amount of IFN-α mRNA which is mainly produced by enterocytes and DC in infected mice (22) was decreased (Figure 1A). Yet, only the expression of IFN-γ was significantly increased in cells isolated from the epithelium of *Rag2*$^{-/-}$ mice infected with *Cryptosporidium* (Figure 1A). However, levels of *Spp1* and *Gzmb* mRNA were around 50 and 200 times higher in the epithelium than in the whole intestine respectively, indicating that cells expressing these genes are enriched (Figure 1B).

In the intestinal epithelium, ILC1s secrete high amounts of IFN-γ while T-cell-like innate IELs, including iCD8a, are known to produce osteopontin and granzyme B (23). We then studied whether NKp46^{+}CD103^{-} ILC1s (3) and innate CD103^{+}CD8α$^{+/-}$ IELs (4, 5) expand in the epithelium upon infection. Frequencies and absolute numbers of these subsets were identical in the gut epithelium of infected and non-infected *Rag2*$^{-/-}$ mice (Figures 1C, D; Supplementary Figures 1, 2).

Together, these data show that type 1 and cytolytic immune responses are activated in few hours after *C. parvum* infection.

3.2 Innate IELs control *C. parvum* infection in co-culture with intestinal organoids

To demonstrate the protective role of innate IELs during the first stages of *C. parvum* infection, we developed an *in vitro* model in which small intestinal organoids infected with the parasite were co-cultured with innate IELs isolated from naïve *Rag2*$^{-/-}$ mice. Oocysts and sporozoites were microinjected inside the lumen of murine organoids in order to access to the apical side of iEC (Supplementary Figure 3, Supplementary Movie 1). The parasitic load increased gradually and was significantly up-regulated 2 days after the microinjection demonstrating that *C. parvum* infects and replicates within murine intestinal organoids (Figure 2A). Moreover, analysis of organoids' transcriptomes using 3'RNA sequencing (RNA-seq) revealed that *C. parvum* infection

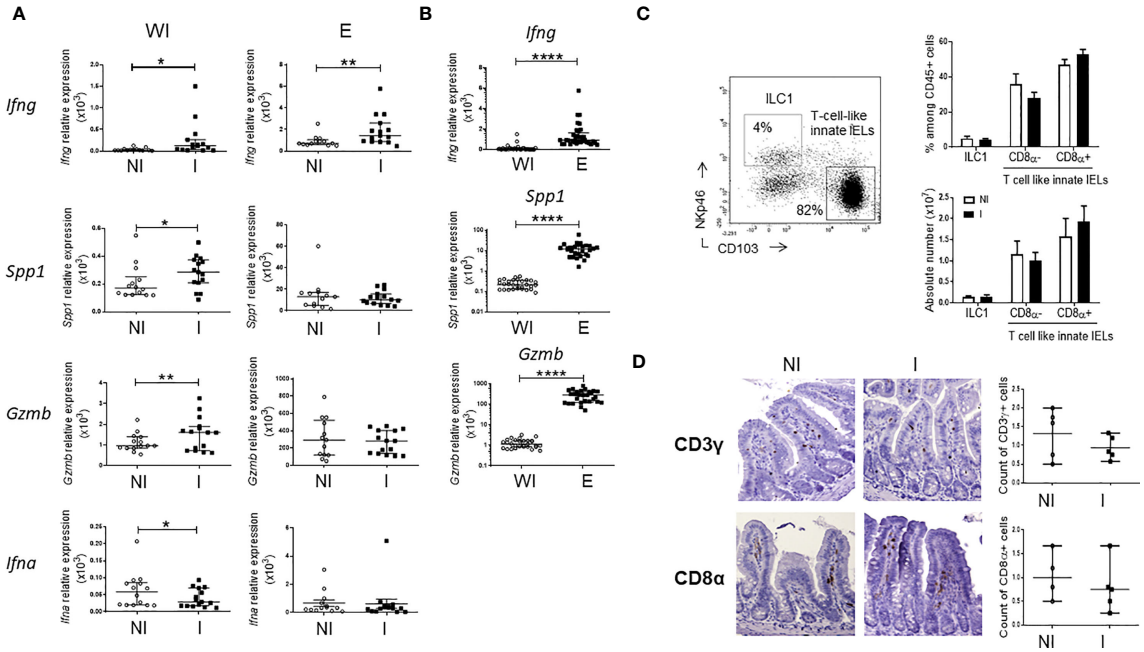

FIGURE 1
Very early immune responses induced by *C. parvum* infection in the ileum of *Rag2*$^{-/-}$ mice. *Rag2*$^{-/-}$ mice were infected by oral gavage with *C. parvum* for 24h. Quantitative RT-qPCR analysis was performed to compare expression of genes in the whole small intestine (WI) and in the epithelium (E) **(A)** between non-infected (NI) (n=14) and infected (I) (n=15) mice and **(B)** between sites. Results were pooled from 3 independent experiments. Medians and ranges are shown. **(C)** Frequencies of innate IELs subsets in the small intestine of NI (n=4) and I (n=5) Rag2$^{-/-}$mice using flow cytometry; representative dot plots and histograms of means values. **(D)** Immunohistochemistry, staining of CD3γ and CD8α on ileal sections from NI (n=5) and I (n=5) *Rag2*$^{-/-}$mice. Scatter plots summarizes results and average values. ****$p < 0.00005$, **$p < 0.005$ and *$p < 0.05$.

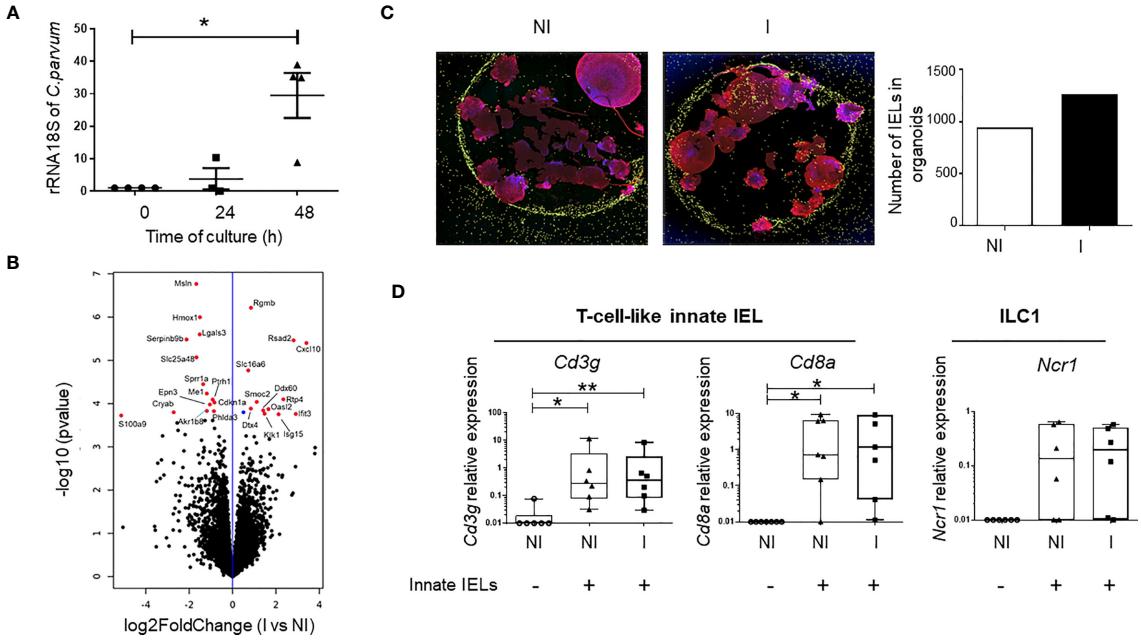

FIGURE 2
Innate IELs controls *C. parvum* infection in murine intestinal organoids. **(A)** The parasitic load was measured at 0, 24- and 48-hours PI in intestinal organoids using RT-qPCR to quantify *C. parvum* 18S rRNA. Results were normalized to the β-actin transcript level and median with range are shown in 4 independent experiments. **(B)** Volcano plot of differentially expressed genes (DEGs) between non-infected (n=4) and infected (n=4) organoids. (blue dot padj ≤ 0.1; red dots FC ≥ 1.5 and padj ≤ 0.1). **(C)** Immunofluorescence of co-cultures with non-infected (NI) and organoids infected (I) for 24h with *C. parvum*. Nuclei are stained in blue (DAPI), the actin in red (phalloidin) and innate IELs in yellow (CFSE). The histogram shows the number of innate IELs present in organoids. **(D)** Expression of innate IELs signature genes (*Cd3g*, *Cd8a* and *Ncr1*) in co-cultures (n=6) with NI and I organoids using RT-qPCR. **p < 0.005 and *p < 0.05).

modified significantly the expression of 26 genes (Figure 2B; Supplementary Figure 4). Most of them were involved in immune responses such as C-X-C motif ligand 10 (*Cxcl10*) gene that promotes the recruitment of immune cells (Figure 2B; Supplementary Table 1). Yet, the number of innate IELs was not significantly increased in infected organoids compared to non-infected ones. In fact, innate IELs migrates spontaneously and similarly into infected and non-infected organoids (Figure 2C; Supplementary Figure 5). Moreover, the expression of gene signatures of T-cell-like innate IELs (i.e. *Cd3g, Cd8a*) and of ILC1 (i.e. *Ncr1*) revealed that the two subsets were present in the same proportion within infected and non-infected organoids (Figure 2D).

Importantly, the co-culture with innate IELs isolated from *Rag2*$^{-/-}$ mice decreased strikingly the amount of *C. parvum* in organoids (Figures 3A, B). To determine which subset protects against *C. parvum* infection, innate IELs from *Rag2*$^{-/-}$ mice were next sorted by FACS and co-cultured with infected organoids. As expected, purified CD45+ innate IELs significantly decreased the parasitic loads. This protective effect was not modified by the depletion of NKp46^{+} ILC1s but it was completely abolished after the removal of both ILC1s and CD103^{+} innate IELs. In addition, co-culture with purified CD103^{+} innate IELs, also tended to decrease the expansion of *C. parvum* within organoids (Figure 3B).

In keeping with the decrease of the parasitic load, the expression of genes which were modified by *C. parvum* infection in organoids was normalized by the presence of the innate IELs (Figure 4A). The unsupervised hierarchical clustering analysis also showed that transcriptomes of infected co-cultures were closer from that of non-infected organoids than from infected ones (Figure 4B).

Overall, these results showed that CD103^{+} innate IELs, the majority of which are T-cell-like innate IELs (4), protected against *C. parvum* infection in a co-culture model with intestinal organoids.

3.3 Co-culture revealed that the early protection against *C. parvum* infection does not depend on IFN-γ

IFN-γ plays a key role in controlling of *Cryptosporidium* infection in both immunocompetent (9, 24, 25) and immunodeficient mice (16, 18). We also found a significant increase of *Ifng* expression in the gut of *Rag2*$^{-/-}$ 24h after the infection with *C. parvum* (Figure 1A), suggesting that the cytokine may also be involved in the early immune response against the parasite. Intraepithelial ILC1 produces high amount of IFN-γ and thereby limits parasite spreading (18). However, those cells were barely detectable in organoids (Figure 2D) and their depletion did not affect the protective effect of innate IELs in co-culture (Figure 3B). Nevertheless, CD103^{+} T-cell-like innate IELs were present in organoids (Figure 2D) and these cells can also produce IFN-γ, although in a smaller amount than ILC1s (6) (Supplementary Figure 6). Thus, we first seek the presence of IFN-γ in co-cultures. IFN-γ was detected in co-cultures with innate IELs but not in organoids alone (Figure 5A). The amount of IFN-γ released by innate IELs in the medium was however similar in co-cultures with organoids infected or not with *C.*

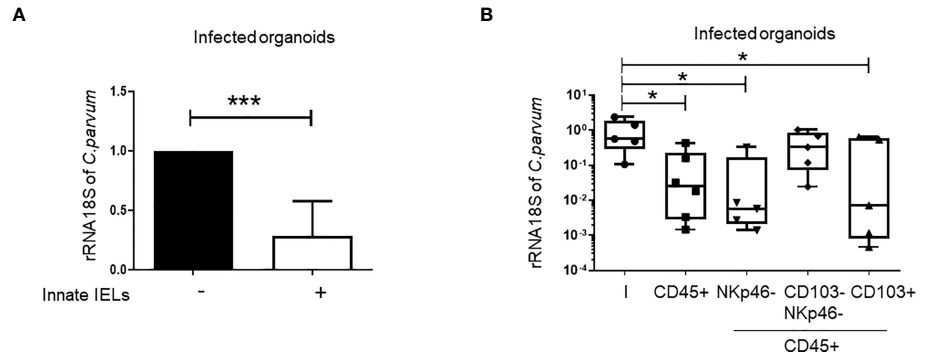

FIGURE 3
Innate IELs limit the development of *C parvum* in intestinal murine organoids. *C parvum* 18S rRNA expression was measured by RT-qPCR in infected organoids co-cultured or not with innate IELs (n=8). Results were normalized to the condition without lymphocytes and histograms present medians with interquartile range **(A)**. Innate IELs were FACS-sorted and co-cultured with infected organoids (n=2); Results are represented using box and whiskers show individual values **(B)**. *p < 0.05; ***p < 0.0005.

parvum (Figure 5B). Moreover, blocking IFN-γ with a neutralizing Ab did not inhibit the protecting effect of innate IELs (Figure 5C).

Thus, very early protection mediated by innate IELs does not seem to rely on IFN-γ secretion.

3.4 Cytotoxic innate IELs provide rapid protection against *C. parvum*

T-cell-like innate IELs are cytotoxic cells (4, 5) and thus, they could reduce *Cryptosporidium* load by lysing infected iEC, alike cytotoxic NK (26, 27) and CD8 T cells (28, 29). In keeping with this hypothesis, we observed that cell death, measured as lactate dehydrogenase (LDH) release, was higher when innate IELs were co-cultured with infected organoids than when they were cultured with non-infected ones (Figure 6A). The level of LDH was also increased in organoids alone upon infection showing that *C. parvum* induces iEC death by itself. However, the quantity of LDH was significantly more elevated in infected organoids in presence of innate IELs indicating that the immune cells are cytotoxic and promote the exclusion of infected EC (Figure 6A).

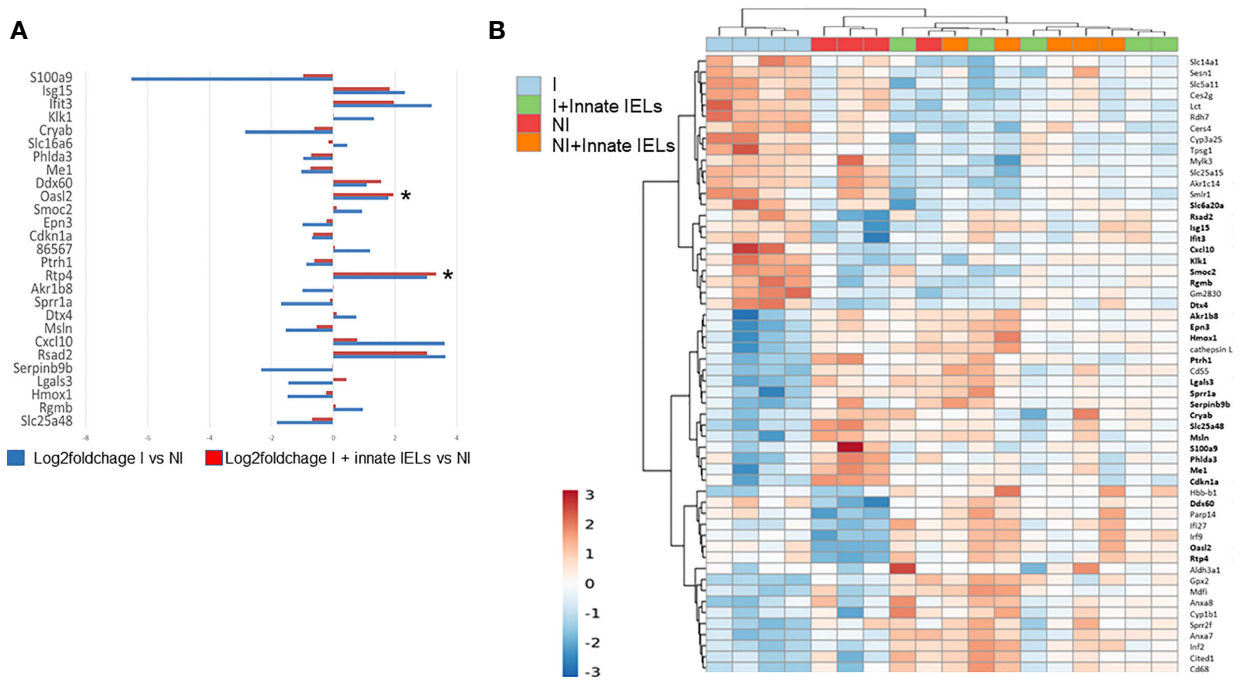

FIGURE 4
Innate IELs normalize expression of genes deregulated by *C parvum* in intestinal murine organoids. **(A)** Medians of log-fold changes of gene expression between infected organoids alone (in blue) or co-cultured with innate IELs (in red) and non-infected organoids. Twenty-seven genes differentially expressed between infected vs non-infected organoids are shown. **(B)** Heat map representing an unsupervised, hierarchical cluster analysis of all experimental conditions (p-value adjusted ≤ 0.1 and FC ≥ 1.5). *p < 0.05.

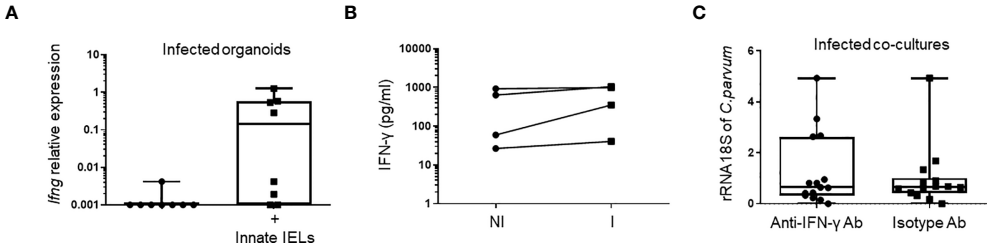

FIGURE 5
The anti-parasitic effect of innate IELs is independent of IFN-γ in co-cultures. Quantification of IFN-γ (*Ifng*) **(A)** using RT-qPCR (n=8) and **(B)** ELISA (n=4) in infected organoids with or without innate IELs. **(C)** Amounts of *C parvum* 18S rRNA in infected organoids co-cultured with innate IELs treated with a blocking anti-IFN-γ mAb or an isotype control Ab. Results show individual points from 3 independent experiments.

To further analyze the cytotoxic mechanism, we next pre-treated innate IELs with the vacuolar type H+-ATPase inhibitor concanamycin A (CMA) before the co-culture with infected organoids. CMA inhibits cytotoxicity as it blocks perforin activity (30, 31). Since CMA-treated innate IELs were unable to control *C. parvum* infection (Figure 6B), we concluded that the immune response against the parasite likely relies on a perforin-dependent cytotoxic mechanism.

Alike perforin, granzyme B is a potent mediator of cytotoxicity in T-cell-like innate IELs (4, 5). Moreover, its expression was rapidly increased in the gut of *Rag2*$^{-/-}$ mice infected with *C. parvum* (Figure 1A) and granzyme B mRNA was detected in co-cultures within infected organoids (Figure 6C). To investigate the impact of granzyme B-mediated cytotoxicity on the infection, we then compared the parasitic load in co-cultures treated or not with the granzyme B specific inhibitor I. We found a small but not significant increase of *C. parvum* 18S rRNA in infected samples treated with the granzyme B inhibitor suggesting that other protective mechanisms are involved (Figure 6D).

Innate IELs also express additional cytolytic granzymes such as granzymes A (5). We then used aprotinin, a non-selective serine-protease inhibitor, to inhibit the activity of all granzymes expressed by innate IELs. Strikingly, aprotinin abolished the protective effect of innate IELs in co-culture indicating that they control the infection through a granzyme-dependent cytotoxic mechanism (Figure 6E).

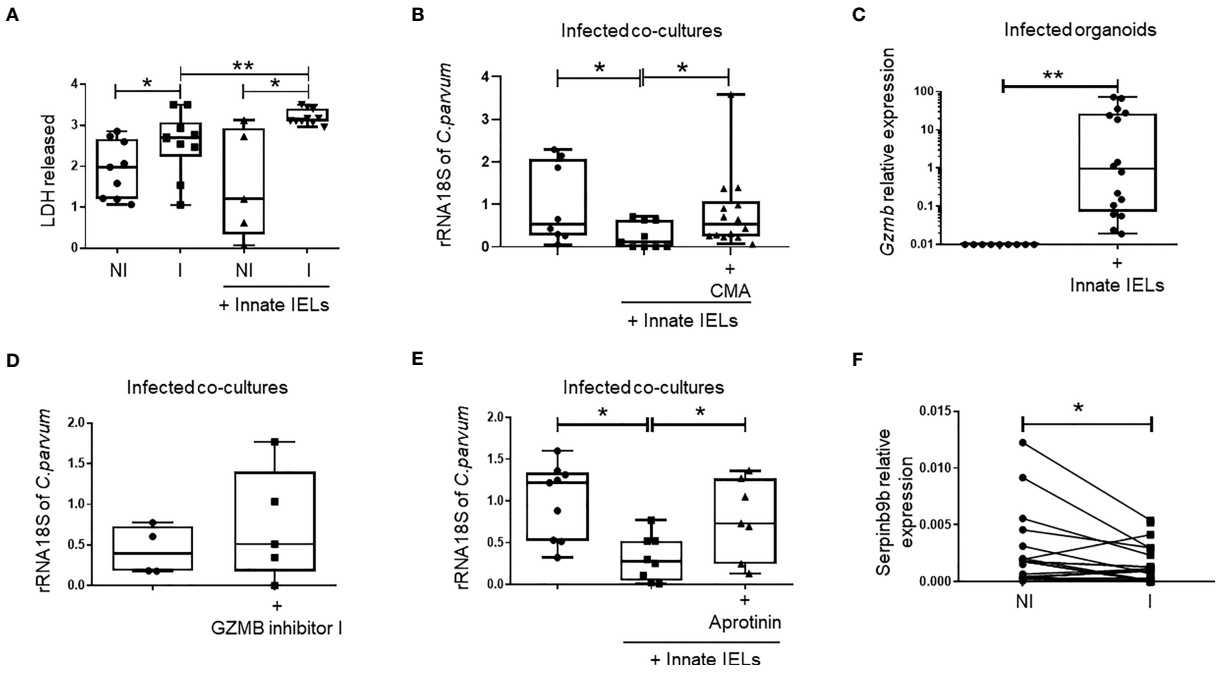

FIGURE 6
Innate IELs limit *C parvum* expansion in intestinal organoids through perforin and a serine-protease dependent mechanism. **(A)** Quantification of LDH release (DO 490 nm) after 48h of infection in supernatant of organoids non-infected (NI), infected **(I)** alone or co-cultured with innate IELs. (n=9). **(B)** Quantification of *C parvum* 18S rRNA using RT-qPCR in infected organoids co-cultured with innate IELs treated or not with the perforin inhibitor Concanamycin A (CMA) (n=2 independent experiments). **(C)** Expression of *Gzmb* in infected organoids cultured with or without innate IELs using RT-qPCR. Quantification of *C parvum* 18S rRNA using RT-qPCR in infected organoids co-cultured with innate IELs treated or not with **(D)** the GZMB inhibitor I or **(E)** with Aprotinin (n=3 independent experiments). Box and whiskers show individual value. **(F)** Expression of Serpinb9b measure by RT-qPCR in organoids infected or not for 24h with *C parvum* (n=10). **p < 0.005 and *p < 0.05.

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Interestingly, RNA-seq showed a significant decreased of serpinb9b expression, a natural serine protease inhibitor, in intestinal organoids infected with *C. parvum* (Figures 4A, B). This result was further confirmed using RT-qPCR (Figure 6F). In addition, the infection seems to down regulate the expression of other serpins such as serpinb9 and b6b which inhibit the granzymes B and A, respectively (32) (Supplementary Figure 7).

Altogether, our data indicated that innate IELs protect against *Cryptosporidium* infection through a serine protease-dependent mechanism and suggest that infected iEC may be more sensitive to granzyme-mediated cytolysis.

4 Discussion

The intestinal epithelium contains many subsets of lymphocytes including adaptive conventional and unconventional T cells and innate IELs which maintains the homeostasis and ensure the protection of the compartment against a wide range of pathogens. Innate lymphocytes play a potent role in early stages of infection (16, 18) and they can also compensate for an immature or an impaired adaptive immunity. These properties are well shown in apicomplexan parasitosis in which NK and ILCs limit parasites spreading and expansion through the secretion of IFN-γ and cytotoxic mechanisms in WT and immunodeficient mice (33). Still, while intestinal Apicomplexa parasites (e.g. *Toxoplasma gondii*, *C. parvum*) infect and replicate in the gut epithelium, the role of innate IELs in these pathologies, which can be chronic and severe in immunocompromised individuals, remains poorly studied.

Investigating functions of intestinal innate IELs is challenging using *in vivo* experimental models since there is no efficient way to specifically deplete or modulate their activity. Moreover, the presence of cells with similar traits such as cNK, ILC3 and ILC1 in the lamina propria can hide their specific role. Thus, to dissect functions of innate IELs in cryptosporidiosis, we developed a co-culture system with murine small intestine organoids infected by *C. parvum*. We showed that *C. parvum* replicated within murine organoids like in human organoids (34) and stimulated immune mechanisms. Notably, the amount of *Cxcl10* mRNA, a chemokine usually induced by IFN-γ, was significantly increased in infected organoids indicating that iECs are a primary source of the chemokine and that the parasite directly stimulates its expression. This mechanism which has been reported by Lacroix-Lamandé et al. in murine intestinal epithelial cell lines (i.e. ICcl2, Mode-K) is supposed to promote a rapid recruitment of immune effector cells in the infected mucosa (35). However, in our co-culture system the number of innate IELs was slightly but not significantly increased in infected organoids compared to the non-infected ones. The composition of the population of innate IELs which infiltrated the organoids was also not modified by the infection. Likewise, no accumulation of immune cells nor modification of the IEL population was observed in the gut of *Rag2*$^{-/-}$ mice 1 day after the infection by *C. parvum*. Altogether these data suggested that the impact of immune cells recruitment was insignificant at this very early stage of the infection.

Actually, innate IELs from naive *Rag2*$^{-/-}$ mice efficiently blocked the expansion of *C. parvum* in infected organoids attesting that the immune cells which reside within the intestinal epithelium were already armed to fight the parasite. Moreover, we showed that the protection is mainly mediated by CD103^{+} T-cell-like innate IELs which is a dominant subset in the gut epithelium of *Rag2*$^{-/-}$ mice (4).

Seeking for the underlying molecular mechanism, we first investigated the contribution of IFN-γ which plays a potent role in cryptosporidiosis. Indeed, several studies have shown that deletion or neutralization of the cytokine increased the parasite burden and aggravate the infection in immunodeficient mice (16–18, 36). In addition, we found that the cytokine was rapidly (i.e. within 24h PI) up-regulated in the whole intestine and in innate IELs isolated from *Rag2*$^{-/-}$ mice. However, the amount of IFN-γ was low and was not increased by *C. parvum* infection in co-cultures. Besides, its neutralization with a blocking anti-IFN-γ mAb did not inhibit the antiparasitic effect of innate IELs. Thus, the protection mechanism mediated by innate IELs in co-culture was independent of IFN-γ secretion. This result might be explained by the small number of ILC1s in intestinal organoids as we detected low or no expression of *Ncr1* in co-cultures. The ILC1 subset represents only around 5% of the IELs isolated from *Rag2*$^{-/-}$ and thus, the number of ILC1s that colonize organoids may not be sufficient to see their effect. Moreover, osteopontin (*Spp1*) which was shown to promote the homeostasis of intraepithelial ILC1 (6) was not detected in co-cultures (data not shown) and thus their survival could also be impaired. Yet, iCD8α IELs which produce osteopontin were present in co-cultures and *Spp1* expression was significantly increased in the gut of infected *Rag2*$^{-/-}$ mice. Further work is then needed to better define the role of osteopontin and ILC1s in cryptosporidiosis.

Nonetheless, ILC1s were not involved in the protective effect observed in co-cultures as their depletion did not affect the growth of *C. parvum* in organoids.

Cytotoxic mechanisms are also involved in the defense against *Cryptosporidium* infections (26–29), we then investigated the role of perforin and granzymes in the protection mediated by innate IELs. Perforin is one of the major effector molecules used by cytotoxic cells to mediate cell lysis since it forms pores in the plasma membrane of target cells that allow the entrance of toxic molecules such as granzymes. Its inhibition impairs cytolytic activity. Herein, we showed that the perforin inhibitor CMA abolishes the protective effect of innate IELs in co-cultures. As shown by others, CMA blocks the acidification of lytic granules and thereby inactivates the cathepsin L required for the maturation of perforin (30, 31). This result indicates that innate IELs can control *C. parvum* growth through a cytotoxic-dependent mechanism. In contrast, we observed a small but non-significant increase of the parasitic burden in co-cultures with innate IELs treated with a granzyme B inhibitor. Thus, Granzyme B has a minor role in this cytotoxic mechanism mediated by innate IELs. Yet, we report a rapid up-regulation of granzyme B expression in the small intestine of infected *Rag2*$^{-/-}$ mice that might reflect the activation of cNK cells of the lamina propria (26, 27).

Finally, using aprotinin, a non-selective serine-protease inhibitor, we almost completely restore the expansion of the parasite indicating that other granzymes contribute to the protection mediated by innate IELs in the co-culture. T-cell-like innate IELs are cytotoxic cells and they not only express high

amounts of granzyme B but also of granzyme A (4, 5). Besides, the transcriptomic analysis of the iCD8α subset suggest that those cells express additional granzymes such as K and M (5). These proteases could then participate to the cytolytic mechanism.

Interestingly, we also found that infected iEC down regulated the expression of serpinb9b, a natural inhibitor of granzyme M (37), and also that of other serpin b family members. Based on these data, it is tempting to speculate that infected iECs decrease their resistance to granzyme-mediated attacks in order to favor the elimination of the parasite.

5 Conclusion

In conclusion, we have developed co-culture model to specifically investigate the role of innate IELs during the very early stages of cryptosporidiosis. This original approach revealed that innate IELs, most likely T-cell-like innate IELs, provide a rapid protection against *C. parvum* infection through a perforin/granzymes-dependent mechanism. Moreover, we showed that the infection modulates functions of iEC that favor the recruitment of effector immune cells and may increase their sensitivity to the cytotoxic attack. Still, further work is needed to detailed the molecular mechanisms involved in these processes.

Data availability statement

RNAseq data have been deposited in sequence Read Archive (SRA) data with the accession code PRJNA980614, https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA980614.

Ethics statement

The animal study was approved by Comité d'éthique en expérimentation animale n°075 APAFIS#30539. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

BM designed and supervised the study. FH, MD, KG, ME, and GC participated in study design and performed research. PZ participated in experiments. BM and FH 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1229406/full#supplementary-material

SUPPLEMENTARY MOVIE 1
Microinjection of murine organoids with *C. parvum* oocysts in excystation medium stained with fast green dye.

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Non-classical HLA-E restricted CMV 15-mer peptides are recognized by adaptive NK cells and induce memory responses

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Introduction: Human cytomegalovirus (HCMV) reactivation causes complications in immunocompromised patients after hematopoietic stem cell transplantation (HSCT), significantly increasing morbidity and mortality. Adaptive Natural Killer (aNK) cells undergo a persistent reconfiguration in response to HCMV reactivation; however, the exact role of aNK cell memory in HCMV surveillance remains elusive.

Methods: We employed mass spectrometry and computational prediction approaches to identify HLA-E-restricted HCMV peptides that can elucidate aNK cell responses. We also used the K562 cell line transfected with HLA-E0*0103 for specific peptide binding and blocking assays. Subsequently, NK cells were cocultured with dendritic cells (DCs) loaded with each of the identified peptides to examine aNK and conventional (c)NK cell responses.

Results: Here, we discovered three unconventional HLA-E-restricted 15-mer peptides (SEVENVSVNVHNPTG, TSGSDSDEELVTTER, and DSDEELVTTERKTPR) derived from the HCMV pp65-protein that elicit aNK cell memory responses restricted to HCMV. aNK cells displayed memory responses towards HMCV-infected cells and HCMV-seropositive individuals when primed by DCs loaded with each of these peptides and predicted 9-mer versions. Blocking the interaction between HLA-E and the activation NKG2C receptor but not the inhibitory NKG2A receptor abolished these specific recall responses.

Interestingly, compared to the HLA-E complex with the leader peptide VMAPRTLIL, HLA-E complexes formed with each of the three identified peptides significantly changed the surface electrostatic potential to highly negative. Furthermore, these peptides do not comprise the classical HLA-E-restriction motifs.

Discussion: These findings suggest a differential binding to NKG2C compared to HLA-E complexes with classical leader peptides that may result in the specific activation of aNK cells. We then designed six nonameric peptides based on the three discovered peptides that could elicit aNK cell memory responses to HCMV necessary for therapeutic inventions. The results provide novel insights into HLA-E-mediated signaling networks that mediate aNK cell recall responses and maximize their reactivity.

KEYWORDS

Adaptive NK cells, memory, dendritic cells, peptides, HLA-E

1 Introduction

Natural Killer (NK) cells are innate immune cells that mediate immune responses against intracellular pathogens and cancer. NK cells do not require prior activation or binding of a specific antigen. Instead, their activation is regulated mainly by a range of inhibitory and activating receptors (1, 2). Nevertheless, a growing number of experimental and clinical studies support the unique role of a subpopulation of NK cells termed adaptive (a)NK cells in possessing an elevated specific response to viral peptides through ligation of the non-polymorphic HLA-E to the activation receptor NKG2C (3, 4). However, little is known concerning the antigen presentation mechanisms underlying the recognition of viral peptides by aNK cells.

It has been demonstrated during the last decade that aNK cells, which have virus-specific immunological memory, accumulate in human cytomegalovirus (HCMV)-infected individuals and recognize peptides derived from HCMV-encoded proteins through HLA-E. These include the UL40 molecule that contains a nonameric epitope with an identical sequence to endogenous HLA-E-binding peptides (3, 5, 6). HCMV is a widespread virus, a member of the Beta-herpesviridae family, that infects 60-90% of the adult population. After a primary infection, the virus establishes life-long latency in its host (7). HCMV has developed multiple immune evasion strategies to avoid and hamper immune responses (8). HCMV has also profound effects on NK cell phenotype, proliferation, and function (9). The unique aNK cell subset was first described in association with the response to HCMV infection. These aNK cells are similar to mature conventional (c) NK cell populations and express CD57, the activating receptor NKG2C, while also displaying downregulation of the inhibitory counterpart NKG2A that binds to HLA-E. aNK cells have common epigenetic signatures resulting in the downregulation of the transcription factor PLZF and the proximal signaling molecules SYK, EAT-2, and FcεR1γ (10, 11). These epigenetic changes have been shown to persist long-term *in vivo* (12). aNK cells have been hypothesized to go through the same memory phases as T cells (clonal expansion, contraction phase, and memory formation). Furthermore, similarly to $CD8^+$ T cells, aNK cells go through mitophagy, which is itself a hallmark of immunological memory formation (13).

HCMV encodes a large number of different proteins, including the tegument protein pp65 and the immediate early protein (IE), that modulate immune responses of NK cells, as well as T and dendritic cells (DC) (14, 15). Similar to T cells, NK cells may also be primed by DCs and enhances NK cell responses (16, 17). Also, aNK cell accumulation in HCMV-seropositive individuals indicates that pp65 may not inhibit the proliferative capacity and the functional activity of aNK cells (18).

HCMV reactivation is associated with adverse clinical outcomes in immunosuppressed individuals, such as post hematopoietic stem cell transplantation (HSCT) patients, and occurs in up to 70% of such HCMV-seropositive recipients (19). HCMV reactivation takes place in severely ill immunocompetent patients and is associated with prolonged hospitalization or death (20). Effective HCMV prophylaxis is warranted for both immunocompromised and immunocompetent patients. We have previously shown that aNK cell presence is associated with anti-tumor effects, reduced relapses, and better clinical responses following HSCT, as well as resistance to suppressive cells in the tumor microenvironment (21–24). Thus, it is highly important to develop a long-lasting and efficient memory response in aNK cells towards HCMV. This could represent one of the best strategies to prevent graft rejection and relapse over time, allowing us to control HCMV reactivation.

In this study, we hypothesized that, similar to T cells, DCs can present pathogenic peptides to aNK cells. We refolded HLA-E with an ensemble of 15-mer overlapping peptides that cover the entire length of the HCMV protein pp65. Using mass-spectrometry, we e discovered three HLA-E binding pp65-derived peptides that provoke aNK cell memory responses specifically towards HCMV. Our results demonstrate that aNK cells require recognition of HLA-

E-restricted peptides presented by DCs that depends on NKG2C and HLA-E interaction, resulting in a significant aNK cell expansion with enhanced capacity to recognize and kill HCMV-infected target cells. Our findings pave the way for new and novel therapeutic inventions that could potentially limit the clinical severe complications caused by HCMV reactivation.

2 Materials and methods

2.1 Blood donors

Peripheral blood mononuclear cells (PBMCs) from healthy HCMV-seropositive or seronegative donors were obtained from Memorial Blood Bank (Minneapolis, MN) and the Stockholm blood bank. All participants gave informed consent to participate in the study before taking part. All samples were de-identified before receipt and approved for use by the university institutional review board in accordance with the Declaration of Helsinki.

2.2 Cell isolation

PBMCs were isolated from buffy coats by density gradient centrifugation using Ficoll-Paque Premium (GE Healthcare). Monocytes were isolated by positive selection using anti-CD14 microbeads (Miltenyi Biotech). Untouched CD3-CD56+ NK cells were isolated using negative selection kits (Miltenyi Biotech). NK cell donors with ≥ 4% aNK cells were used for further analysis.

2.3 Identification of peptide sequences through mass spectrometry

A pool of 138 different 15-mer peptides covering the sequence of full-length pp65, kindly provided by the NIH AIDS reagent program, was refolded with HLA-E and human β2-microglobulin (hβ2m) as previously described (25, 26). The obtained HLA-E/hβ_2m/peptide complexes were isolated using size-exclusion chromatography. Peptides were thereafter eluted from the purified MHC/peptide complexes under acidic conditions (0.1% trifluoroacetic acid, 10% CH3CN), further purified using a 5 Kd Ultrafree-15 centrifugal filter device (Millipore) and concentrated using speed vac (Labconco). All experiments were repeated independently three times. The sequences of all eluted peptides were analyzed using mass spectrometry. Peptide sequences detected in at least two independent experimental replicates were further sent for synthetization. Furthermore, a selection of nonameric peptides predicted through the molecular modeling of the 15-mer in complex with HLA epitopes identified by MS were also produced. The 9-mer sequences were predicted based on each non-conventional residue's ability to fit the HLA-E peptide binding cleft.

TABLE 1 Peptides used in the study and melting temperature measured with nanoDSF.

Sequence	Name	Melting Temperature (Tm)
RGPGRAFVTI	P18-I10, H-2D^d-restricted control peptide	
QMRPVSRVL	hsp60, HLA-E-restricted control peptide	
VMAPRTLIL	UL40, HLA-E-restricted control peptide	
The pool of 129 HIV overlapping 15mer-peptides	All 15mers are derived from the HIV-1 protein Gag	
The pool of 138 overlapping 15mer-peptides	All 15mers are derived from the HCMV protein pp65	
SEVENVSVNVHNPTG[1]	pp65$_{85\text{-}99}$ MS-identified 15mer	62.1°C
TSGSDSDEELVTTER[2]	pp65$_{401\text{-}415}$ MS-identified 15mer	62.6°C
DSDEELVTTERKTPR[3]	pp65$_{405\text{-}419}$ MS-identified 15mer	52.6°C
EVENVSVNV	pp65$_{402\text{-}410}$; 9-mer predicted from [1]	62.1°C
SGSDSDEEL	pp65$_{86\text{-}94}$; 9-mer predicted from [2]	61.9°C
VTTERKTPR	pp65$_{411\text{-}419}$; 9-mer predicted from [3]	53.7°C
DSDEELVTT[4]	pp65$_{405\text{-}413}$; 9-mer predicted from [2] and [3]; Higher binding affinity to HLA-E	54.6°C
DMDEELVLL	pp65$_{405\text{-}413}$(p2M, p9L); Altered peptide ligand version from [4] with predicted higher binding affinity to HLA-E	54.1°C
DQDEELVTL	pp65$_{405\text{-}413}$(p2Q, p9L); Altered peptide ligand version from [4] with predicted higher binding affinity to HLA-E	54.9°C

Underlined: HLA-E restricted motif.
Superscript: The 15-mer peptides reference.

2.4 Cell culture

$CD14^+$ monocytes (M), purified from HCMV-seropositive and -seronegative individuals, were differentiated into immature DC (imDC) in GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) for three days ($2x10^6$/ml in 2 ml/well, 6-well plates). Later, imDC were washed, counted and re-cultured ($1x10^6$/ml in 6-well plate) overnight in DC-CellGro® medium (Cellgenix), supplemented with 2% human AB-serum and differently conditioned; imDC: GM-CSF (100 ng/ml) and IL-4 (20 ng/ml); mature DC (mDC): GM-CSF (final 100 ng/ml), IL-4 (final 20 ng/ml), IFNg (1000 IU/ml), TLR7/8 agonist R848 (2.5 ug/ml), poly IC (20 ug/ml), LPS (10 ng/ml) (Peprotech and Sigma Aldrich), and in the presence or absence of customized HLA-E-binding peptides identified within this study, and other control and a selection of previously known HLA-E-restricted peptides (Table 1, Nordic Biosite, and NIH AIDS reagent program IEDB). A control experiment was performed culturing IL-15-activated NK cells with MRC-5 fibroblast cell line (ATCC) in the presence or absence of pp65 and accessed for aNK cell activities.

Following overnight maturation, DC were washed, counted, and co-cultured with NK cells in 96-well plates at a concentration of 0.05-0.15 x 10^6 cells and 1:10 ratio per well in RPMI-1640 with 10 ng/ml IL-15 for 14 days. In further experiments, NK cells were cultured with mDC+pp65-peptides for 14 days in addition (5 µg/ml) of either a control isotype-matched antibody IgG (clone Poly4053), anti-NKG2C (clone 134522), anti-NKG2A (clone 131411), or anti-HLA-E (clone 3D12), anti-MHC I (clone W6/32), and MHC II (Tü39) blocking antibodies (27–30) (BioLegend, R&D systems) at the primary phase (day 0 and 7) or at the secondary restimulation phase (at day 14, during 6h stimulation).

2.5 Flow cytometry

Cells were stained with fluorochrome-conjugated antibodies against the following antigens: CD14, CD80, CD83, HLA-E, CD56, CD3, CD57, NKG2C, NKG2A, CD45RA, CD45RO, FcεRIγ/Syk, IFN-γ, Ki67 (proliferation), CD107a (degranulation), and TNFα, all from Biolegend. All staining was performed in combination with Live/Dead Fixable Dead Cell Stain (ThermoFisher) to exclude dead cells. Detection of intracellular FcεRIγ/Syk, IFN-γ, Ki67 (proliferation), CD107a (degranulation), and TNFα was performed following fixation and permeabilization (eBioscience) according to the manufacturer's instructions. Cells were acquired on either an LSRII or Fortessa cytometer (BD Biosciences) and data were analyzed using FlowJo (TreeStar) and Cytobank Premium (Beckman Coulter) (31).

2.6 NK cell degranulation and cytokine production assays

For determination of NK cell cytolytic activity (degranulation), as well as IFN-γ and TNFα production, cells were incubated at 37°C for 6 hours at a 1:1 ratio with either the MRC-5 cells that were pre-infected with the HCMV strain VR1814 at MOI 1 for three days, or with uninfected MRC-5 cells followed by flow cytometry analysis. CD107a, GolgiPlug, and GolgiStop (BD Biosciences) were added to the culture media during incubation. Intracellular staining and flow cytometry analyses were then performed.

2.7 NK cell killing assays

To determine NK cell killing capacity, HCMV-infected or uninfected MRC-5 cells were fluorescently labeled with CellTrace Violet (5 uM, Invitrogen), and target killing was evaluated using Live/Dead dye (Invitrogen) following a six-hour incubation at an effector to target (E: T) ratio of 1:1. MRC-5 cell killing was assessed by gating on CD45 negative populations, further gated on CellTrace positive population and assessed for the proportion of Live/$Dead^+$ cells.

2.8 HLA-E/peptide production and purification

The HLA-E*0101 heavy chain and $h\beta_2m$ were expressed individually as inclusion bodies using the BL21 (DE3) *E. coli* strain, following previously published protocols (25, 26). Inclusion bodies were solubilized in 8 M Urea, 100 mM Tris HCl pH 8, and 2 mM EDTA. The refolding of HLA-E/peptide complexes was carried out by the following dilution: 3 mg of peptide and 8 mg of hβ2m were added firstly to the refolding buffer (100 mM Tris pH 8, 450 mM L-Arginine, 5 mM L-Glutathione reduced, 0.5 mM L-Glutathione oxidized, 2 mM EDTA, 0.5 mM AEBSF) and the solution was left at 4°C under stirring for half an hour. 4 mg of unfolded HLA-E was then added in three steps. After 24 hours, the refolding solution was concentrated to approximately 5 mL. The sample was then purified by size exclusion chromatography using a HiLoad 16/60 Superdex 200 pg column equilibrated with 20 mM Tris HCl pH 8 and 150 mM NaCl. The eluted protein was analyzed by SDS-PAGE, frozen in liquid nitrogen, and stored at -20°C.

2.9 Nano differential scanning calorimetry (NanoDSF)

Thermal unfolding experiments were performed by nanoscale differential scanning fluorimetry (nanoDSF) (32). The protein intrinsic fluorescence during the thermal ramp was followed at 330 nm and 350 nm with a Prometheus NT.48 instrument from NanoTemper Technologies with an excitation wavelength of 280 nm 28. Capillaries were loaded with 10 ul of protein at a concentration of 1 mg/mL in 20 mM Tris-HCl, pH 8.0, and 150 mM Sodium Chloride. The temperature ramp measurements were recorded from 20 to 95°C (temperature slope 60°C/hour). Three independent measurements were carried out for each complex. The fluorescence intensity ratio was recorded, and its first derivative was calculated with the manufacturer's software (PR.ThermControl, version 2.1.2).

2.10 Molecular modeling of HLA-E/peptide complexes

The molecular modeling of the three-dimensional structures of HLA-E/peptide complexes was performed using the crystal structure of HLA-E/CD94/NKG2A (33) (PDB code 3CDG) as a preliminary template and assuming that CD94/NKG2C similarly interacts with HLA-E to CD94/NKG2A. The modeling was performed manually in the program Coot (34), followed by model regularization to improve the peptide chain's geometry and remove all possible sterical hindrances. Flanking peptide residues were modeled in an arbitrary conformation to demonstrate that HLA-E can present longer peptides and do not prevent CD94/NKG2C binding. Peptide elongation at the C- and N-terminal of the HLA-E peptide binding cleft was possible using different rotamers for the side chains or residues K146 and W167, respectively. None of the introduced flanking peptide residues interact with the CD94/NKG2C heterodimer.

2.11 HLA-E binding assay

Peptide binding assay was performed in the HLA-E*0101 transfected K562 cell line (K562E*0101), kindly provided by Dr. Jakob Michaelsson (Karolinska Institutet, Center for Infection Medicine, Department of Medicine, Huddinge), and mycoplasma tested before use. These K562E*0101 cells have a constitutive HLA-E expression. Briefly, cells were re-suspended in a medium at 10^6 cells/ml, and indicated peptides were added at a concentration titration of 0-100 µM. After an overnight incubation at 37°C, cells were washed with PBS to remove free peptides. Next, HLA surface expression was monitored after staining with anti-HLA-E (BioLegend) and viability dye. Analysis was done using flow cytometry as described above. Results are reported as flow cytometry histograms or mean fluorescence intensity (MFI) compared to Fluorescence Minus One (FMO) control.

2.12 Data management and statistical analysis

All experiments were repeated independently at least three times. One representative and accumulative data are presented. All numeric data were subjected to a normal distribution test before further statistical analysis. For the comparison within groups, parametric or non-parametric multiple comparison two-way ANOVA or one-way ANOVA tests were performed. Student's T-test was used when comparing two groups only. All statistical tests were two-sided and ± SEM. All p-values or asterisks from multiple comparisons were corrected using the FDR method <0.05. No asterisk or p-values represent not significant data. The Prism v9.2 software (GraphPad) was used for statistical analyses. All dimensional reduction opt-SNE analyses based on flow cytometry data were done utilizing the Cytobank 29 cloud-based platform.

3 Results

3.1 aNK cells expand significantly following co-culture with mature DCs that present a pool of HCMV pp65-derived 15-mer peptides

A previous study revealed that NKG2C$^+$ NK cells specifically recognize distinct HCMV strains that encode a heterogeneous repertoire of the classical UL40 peptides. These peptides control the expansion and differentiation of NKG2C$^+$ aNK cells (3). However, that study did not reveal whether other HCMV-derived peptides can control aNK cell recall responses. Neither did previous studies investigate the involvement of professional antigen-presenting cells (APCs), such as DCs, in priming human aNK cells. Here, we first assessed the recognition specificity of aNK cells towards a large array of overlapping HCMV 15-mer peptides derived from the pp65 protein. Purified NK cells from HCMV-seropositive individuals were co-incubated at a 10:1 ratio with autologous monocytes or imDC representing poor APCs and mDC as professional APC, either loaded with pools of 15-mer peptides derived from the HCMV-associated pp65 protein or unloaded. As a control, we used a control pool of peptides derived from the HIV-associated Gag protein. This high-throughput phenotypic screening allowed us to identify HCMV-specific expansion of aNK cells. Interestingly, only the addition of mDC loaded with the pp65-peptide pool (referred to as mDC$^+$pp65) to the NK cell culture led to a consistent increase in the NKG2C$^+$CD56$^+$ aNK cell pool (CD3$^-$CD56$^+$CD57$^+$FCεRγ$^-$) ($p \leq 0.03$). In contrast, cNK cell frequency (CD3$^-$CD56$^+$CD57$^+$FCεRγ$^+$) did not increase, relative to all other controls (Figures 1A, B). Complementing this observation, we found a significant increase in the proliferation index of aNK cells (% Ki67) compared to cNK cells following the addition of mDC$^+$pp65, which was not seen in the other tested conditions (73.9 ± 12, p= 0.04) (Figure 1C). However, we were unable to establish any functional difference, including degranulation and cytokine production between cNK and aNK cells following generic stimulation with an agonistic anti-CD16 antibody combined with IL-12 and IL-18 recombinant cytokines, suggesting a need for a specific secondary stimulation to induce functional advantage in aNK cells. Further analysis revealed that compared to NK cells cocultured with peptide unloaded control DC, NK cells co-cultured with mDC$^+$pp65 displayed a remarkable increase in the CD45RO population, which has been shown to represent mature and functional NK cells in hematological malignancies and identify memory NK cells (35, 36) (Figure 1D). Our results suggest that peptide priming stimulates and generates memory-like NK cells.

3.2 Peptide recognition by aNK cells is dependent on both HLA-E and NKG2C

Earlier studies have demonstrated the importance of interactions between NKG2C and HLA-E molecules in NK cell responses to HCMV (3, 28). It is also well-established that NKG2A

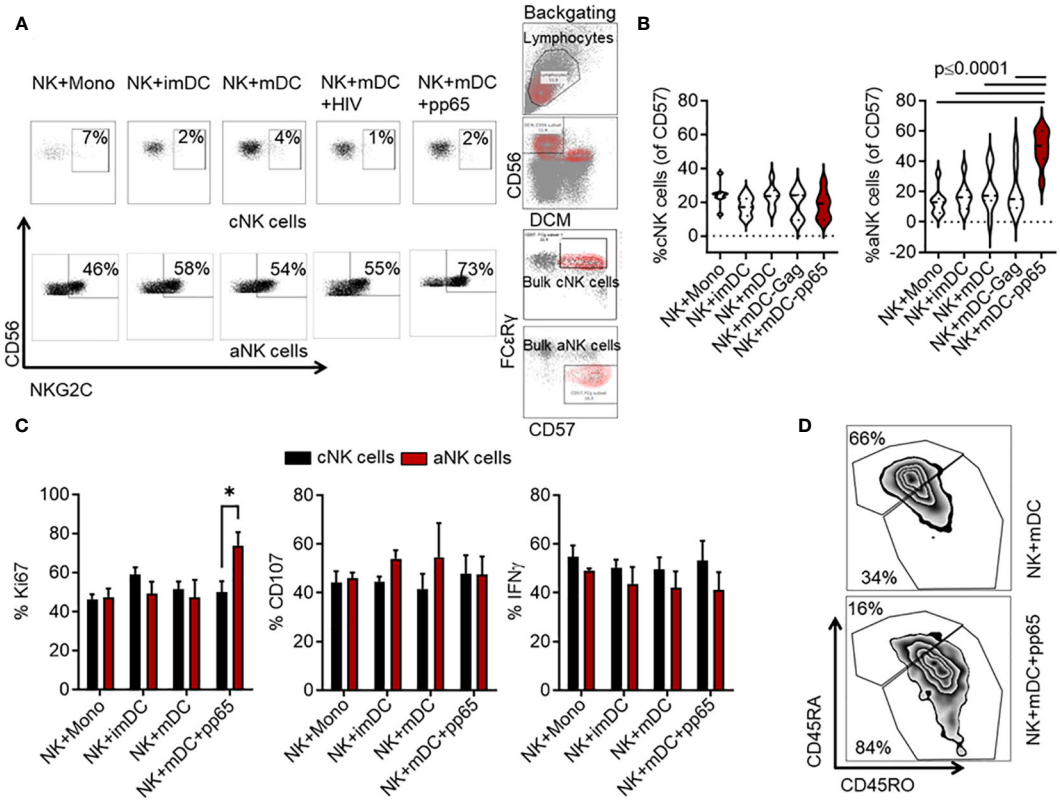

FIGURE 1
Co-culture of mDC loaded with an HCMV-associated pp65-derived peptide pool significantly expands the frequency of aNK cells. NK cells from HCMV-seropositive donors were cultured for 14 days with mDC loaded with the pools of overlapping 15-mer peptides derived from either HCMV-associated pp65 or HIV-1-associated Gag proteins, or left unloaded in the presence of 10 ng/ml IL-15 relative to cocultures with monocytes and imDC. **(A)** Representative flow cytometry plots and gating strategy of CD56/NKG2C cNK and aNK cell expansion are shown. **(B)** Cumulative (n = 10) data showing the percentages of cNK and aNK cells within the total CD57+ NK cell population. Results from five independent experiments are presented as mean ± SEM. A One-way ANOVA test was used for statistical analyses. **(C)** Cumulative (n = 9) data showing the percentages of aNK and cNK cell proliferation (Ki67), degranulation (CD107a), and IFNγ production following co-culture with M, imDCs, mDCs, or mDCs+pp65. The presented results are from three independent experiments. All the cumulative data are shown as mean ± SEM. A two-way ANOVA test was used for statistical analyses. **(D)** Representative data from three independent experiments showing NK cell phenotype based on CD45RA and CD45RO expression levels when in co-culture with mDC or mDC+pp65. * indicating p-values ≤ 0.05.

is an inhibitory receptor, expressed on both NK and T cells, that competes with the activating receptor NKG2C for HLA-E binding (37, 38). Our previous studies revealed that aNK cells have low or no NKG2A expression, making them less susceptible to NKG2A-mediated inhibition (10, 22). Here, we investigated whether aNK cell responses to mDC^{+}pp65 dependent on the interaction of HLA-E with any of these two NK cell receptors. For this purpose, NK cells were cultured with mDC^{+}pp65 in the presence of blocking antibodies specific to either HLA-E (39), NKG2C (40), NKG2A (41), or a control IgG isotype. Our results demonstrated that antibody blocking of HLA-E or NKG2C abolished the expansion of aNK cell population, observed in response to stimulation by mDC^{+}pp65 and the presence of control IgG antibodies. In contrast, we did not observe any difference in the expansion of aNK cells when blocking the NKG2A receptor interaction, confirming that aNK cell responses are independent of NKG2A, instead dependent on the interaction between HLA-E- HCMV- peptide complexes and NKG2C (Figures 2A, B). The reduction in expansion of aNK cells in the presence of anti-HLA-E or anti-NKG2C antibodies was not due to changes in cell viability, rather reflected a lack of specific proliferation (Figures 2C–F). In control experiments, we confirmed the blocking capacity of these antibodies. Using the K562 cell line transfected with HLA-E and loaded with pp65 peptide pool and cocultured with NK cells; we found that aNK cell degranulation was reduced when blocking NKG2C and HLA-E. On the other hand, assessing cNK cell degranulation, we found that blocking NKG2A enhanced their degranulation capacity (Supplementary Figures 1A, B). Thus, these results indicate substantial participation of NKG2C and HLA-E in the aNK cell antigen priming phase by DCs.

3.3 Identification of three pp65-derived HLA-E-restricted 15-mer epitopes that do not comprise the classical motif

Given the rate-limiting role of HLA-E for aNK cell recognition of the pp65 peptide pool, we next sought to identify HLA-E-restricted pp65-peptides. A total of 138 15-mers from the pp65-derived peptide pool were refolded with HLA-E*0101 heavy chain

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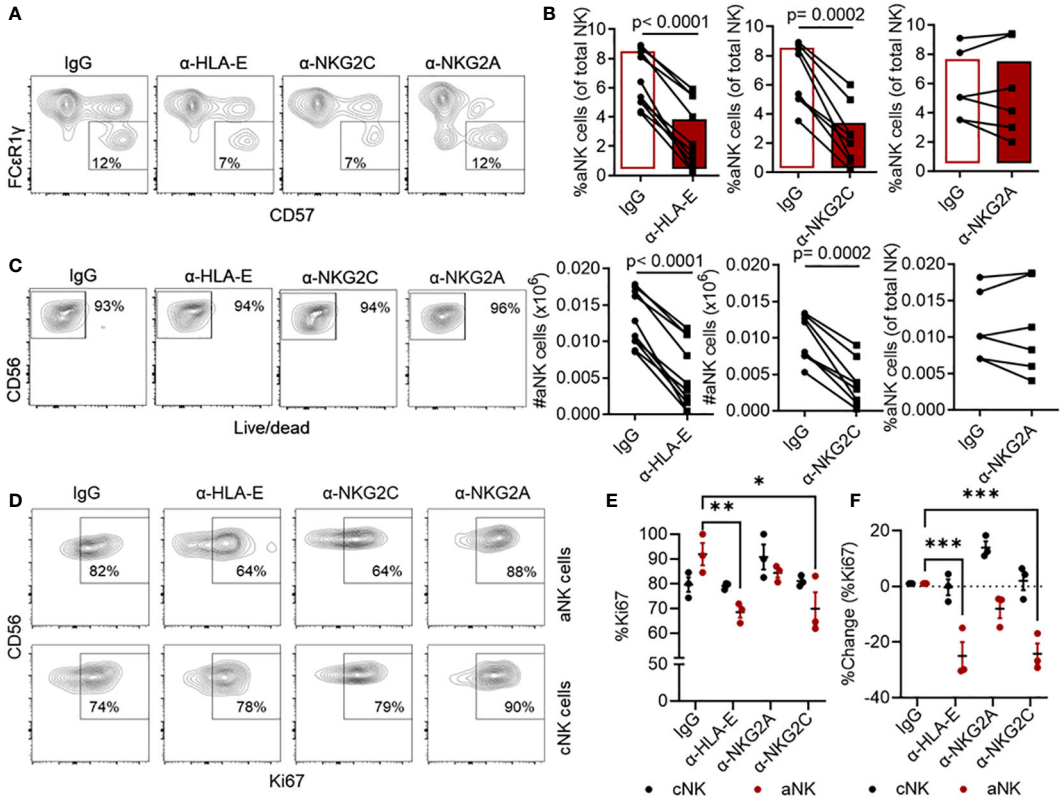

FIGURE 2
aNK cell proliferation is dependent on HLA-E/NKG2C interactions. **(A)** Representative gating strategy of NK cells (n = 6-10) cultured with mDC+pp65 for 14 days in the presence of either a control isotype-matched antibody IgG, or anti-HLA-E, anti-NKG2C, or anti-NKG2A blocking antibodies (5 µg/ml), thereafter assessed for their **(B)** (top panel) frequency and (bottom panel) number of cells, **(C)** viability, and **(D, E)** proliferation by flow cytometry. All blood donors were HCMV seropositive. **(F)** Percentage change (blocking – IgG) from **(E)** is shown. The results from 2-5 independent experiments are presented, and data are shown as representative plots or for each donor, where the donor in **(A, C)** is the same, and the donor in **(D)** is included in accumulative data in **(E)**. Student's T-test and Two-way ANOVA were used for statistical analyses. * indicating p-values ≤ 0.05, ** indicating p-values ≤ 0.001, *** indicating p-values ≤ 0.0001.

TABLE 2 MS identified HLA-E-restricted pp65 peptides.

Sequence	-logP	mass	length	ppm
TPRVTGGGAMAGAST	67.41	1348.641	15	-1.4
TSGSDSDEELVTTER	65.97	1624.706	15	2.1
KAESTVAPEEDTDED	62.97	1634.679	15	-3
SEVENVSVNVHNPTG	62.95	1580.743	15	0.7
TGGGAMAGASTSAGR	56.09	1250.567	15	-0.4
TPRVTGGGAMAGAST	55.71	1332.646	15	-0.5
TVAPEEDTDEDSDNE	55.19	1664.617	15	4.6
ARNLVPMVATVQGQN	50.07	1612.836	15	2.1
EEDTDEDSDNEIHNP	48.6	1757.65	15	1.1
TLGSDVEEDLTMTRN	41.34	1695.762	15	2.6
TGGGAMAGASTSAGR	36.73	1266.562	15	0.3
DSDEELVTTERKTPR	27.67	1774.87	15	3.8

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and hβ$_2$m, yielding a homogenous ensemble of HLA-E/peptide complexes that were isolated using size exclusion chromatography (Supplementary Figure 2A). All bound peptides were then eluted through mild acetic acid treatment, and the sequence identity of all bound epitopes was assessed using mass spectrometry. A total of twelve 15-mers were identified in at least two out of a total of three independent assays (Table 2). Later, three peptides were selected based on sequence overlap with the pp65-derived peptides identified from the HCMV strain AD169 (Inventor: Lewis L. Lanier, Patent Application Number: 16/616,435, Publication number: 20200171135). Interestingly, the sequences of pp65$_{85\text{-}99}$ (SEVENVSVNVHNPTG), pp65$_{401\text{-}415}$ (TSGSDSDEELVTTER), and pp65$_{405\text{-}419}$ (DSDEELVTTERKTPR) do not comprise the classical HLA-E-restriction motifs. Instead, these three HLA-E-restricted epitopes were heavily negatively charged and contained polar residues. Molecular models of HLA-E in complex with the 15-mer peptides, pp65$_{85\text{-}99}$, pp65$_{401\text{-}415}$, and pp65$_{405\text{-}419}$, indicate that the surfaces of these complexes are significantly more electronegative compared to the surface of HLA-E in complex with classical epitopes such as UL40 or hsp60 (Supplementary Figures 2B, C). Molecular models of HLA-E in complex with all 9-mer peptides that we identified and designed based on the sequences and the molecular models from the three 15-mers also display the same electronegative effects on the surface of these pMHC complexes (data not shown). The capacity of each of the three identified peptides to form a complex with HLA-E and hβ$_2$m was demonstrated through the successful refolding of individual HLA-E/peptide in complexes. All three obtained HLA-E/peptide complexes displayed high overall stability as measured by nano differential scanning calorimetry (nano-DSF), with melting temperature (T_m) values stretching from 52.5 to 62°C (Figure 3A, Table 1).

Since all three peptides induced very similar functional activities and recall responses in aNK cells compared to the HLA-E binding UL40 and Hsp60, we hypothesized that the recognition of these specific HLA-E/pp65-peptide complexes could be due to a different binding mode of NKG2C, and/or to specific properties intrinsic to the core of these particular peptides, which would promote aNK cell recall responses.

We next evaluated whether the 15-mer peptides pp65$_{85\text{-}99}$, pp65$_{401\text{-}415}$, and pp65$_{405\text{-}419}$ can bind to HLA-E*0101 on target cells. The K562 cell line transfected with HLA-E*0101 was loaded

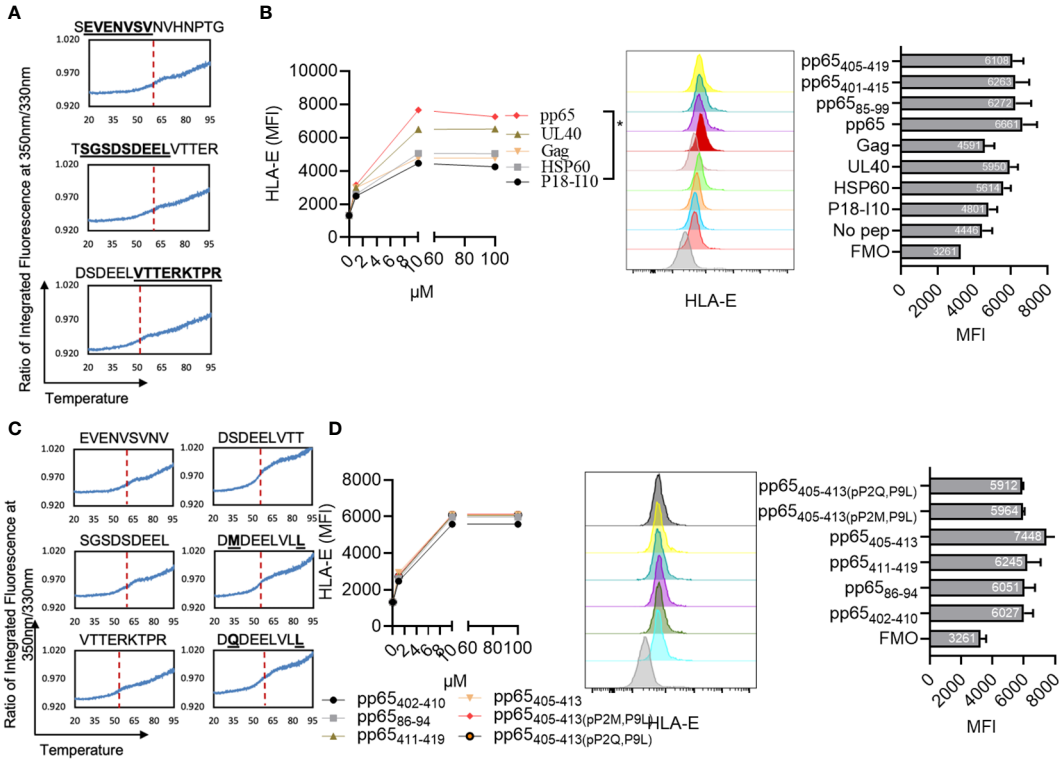

FIGURE 3
Identification of three 15-mer and 9-mer pp65-derived HLA-E-restricted epitopes. **(A)** NanoDSF studies to assess the thermal stability of HLA-E in complex with the following peptides: SEVENVSVNVHNPTG, DSDEELVTTERKTPR, and TSGSDSDEELVTTER. The F350/F330 was plotted against temperatures varying from 20°C to 95°C. Red dashed lines indicate the calculated melting temperatures (Table 1). **(B)** K562 cells transfected with HLA-E*0101 were loaded with the indicated peptides overnight, washed, and analyzed for their HLA-E expression. Peptide titration from three independent experiments and one representative overlay histogram and MFI are presented out of 3 independent experiments (10 μM). **(C)** NanoDSF studies to assess the thermal stability of HLA-E in complex with the following peptides: SGSDSDEEL, DSDEELVTTERKTPR, VTTERKTPR, DSDEELVTT, DMDEELVLL, and DQDEELVLL. The F350/F330 was plotted against temperature varying from 20°C to 95°C. Red dashed lines indicate the calculated melting temperatures (Table 1). **(D)** K562 cells transfected with HLA-E*0101 were loaded with the indicated peptides overnight, washed, and analyzed for their HLA-E expression. Peptide titration and one representative overlay histogram and MFI are presented out of 3 independent experiments (10 μM). Positive (UL40) and negative (no peptide) peptide controls are included in **(B)**. A Two-way ANOVA was used for statistical analyses of cumulative data in **(B, D)**. *Multiple comparisons were corrected by using the FDR method <0.05.

overnight with the three identified peptides as well as other controls including the non-HLA-E binding H-2D^{d}-restricted peptide P18-I10 (RGPGRAFVTI) (42), the HLA-E binding hsp60 peptide QMRPVSRVL, and the UL40-derived peptide VMAPRTLIL. We found that all three 15-mer peptides were able to increase the expression of HLA-E to similar levels compared to the classical UL40 and the pp65 peptide pool and at higher levels compared to all other controls (Figure 3B), which is well in line with the nano-DSF results.

We next addressed whether we would be able to identify 9-mer versions within the 15-mers. The three 9-mer peptides pp65$_{402-410}$ (SGSDSDEEL), pp65$_{86-94}$ (EVENVSVNV), and pp65$_{411-419}$ (VTTERKTPR) were designed as potential candidates (Table 1), following HLA-E binding prediction by the NetMHC server. These predictions were complemented by a visual inspection of how these peptides could fit within the HLA-E binding cleft. Furthermore, as we observed a sequence overlap between pp65$_{402-410}$ and pp65$_{411-419}$, we also designed a fourth epitope pp65$_{405-413}$ (DSDEELVTT). Finally, two altered peptide ligand (APL) variants, pp65$_{405-413}$(p2Q, p9L) were designed, in which we introduced components of the HLA-E motif and therefore both predicted to bind HLA-E with a higher affinity. Refolding with pp65$_{402-410}$, pp65$_{86-94}$, pp65$_{411-419}$, or with pp65$_{405-413}$, pp65$_{405-413}$(p2Q, p9L) and pp65$_{405-413}$(p2M, p9L) resulted in the production of HLA-E complexes with stability that was very similar to their 15-mer counterparts as measured by nano-DSF, thus demonstrating that each nonamer could bind to HLA-E (Figure 3C). Furthermore, our cellular peptide binding assay revealed a similar increase in HLA-E expression levels on K562E*0101 cells loaded with pp65$_{402-410}$, pp65$_{86-94}$ or pp65$_{411-419}$ as well as pp65$_{405-413}$ and the APLs pp65$_{405-413}$(p2Q, p9L), pp65$_{405-413}$(p2M, p9L) (Figure 3D).

3.4 Recognition of specific HLA-E/peptide complexes by aNK cells provokes recall responses

Having demonstrated enrichment of aNK cells when in culture with mDC^{+}pp65, we thereafter tested whether specific recognition of the 15-mer pp65$_{85-99}$, pp65$_{401-415}$, and pp65$_{405-419}$ epitopes could provoke recall responses by aNK cells. Therefore, NK cells were co-cultured with mDC loaded with each peptide or control peptides, including P18-I10, UL40, and Gag, or hsp60 (Table 1). MRC-5 cells, uninfected or infected with HCMV, were used as targets for peptide-primed aNK cells. We observed a higher TNFα production by aNK cells cultured with mDC loaded with pp65$_{85-99}$, pp65$_{401-415}$, or pp65$_{405-419}$ peptides compared to unloaded mDC when restimulated with HCMV-infected MRC-5. Importantly, TNFα production was higher in aNK cells from HCMV-seropositive compared to HCMV-seronegative individuals

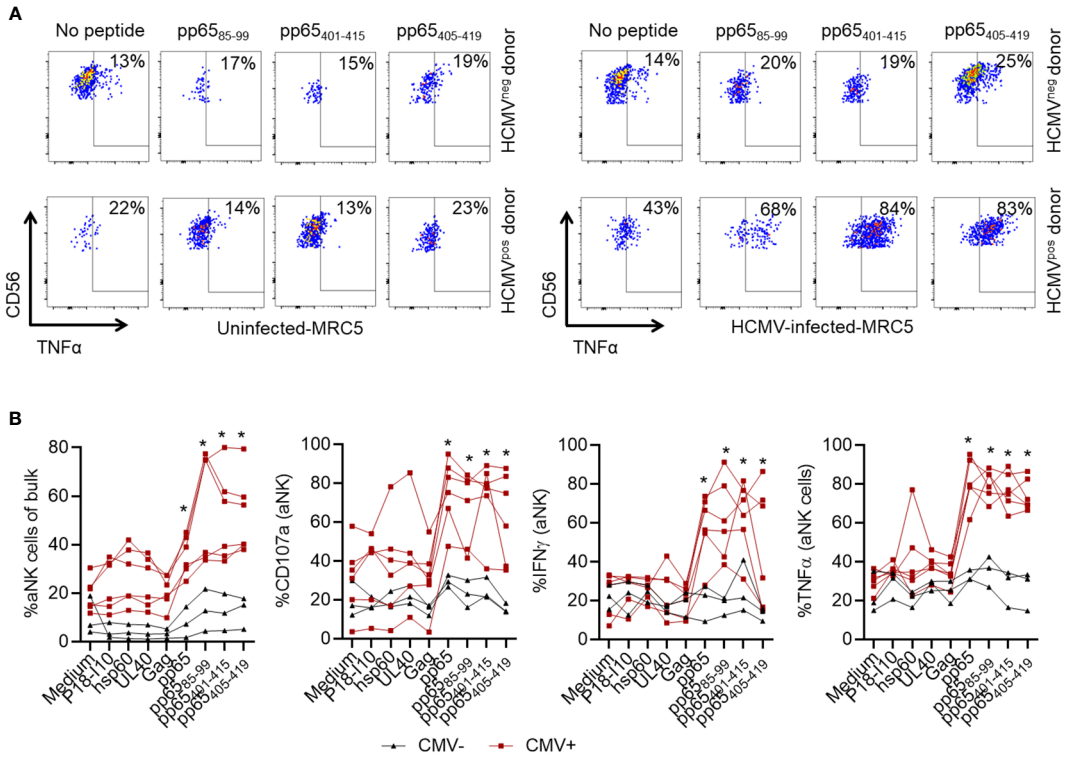

FIGURE 4
aNK cells recognize pp65-derived HLA-E-restricted 15-mer epitopes presented on mDC and perform recall responses. NK cells were cocultured with mDC in the absence of peptides or pulsed with the indicated peptides (10 μM) in the presence of 10 ng/ml IL-15 for 14 days. Later, NK cells were restimulated with HCMV-infected or uninfected MRC-5 cells before analysis of aNK cell function. Blood donors were either HCMV-seropositive (red lines) or -seronegative (black lines). Data are shown from three independent experiments and individual donors (n = 9). Representative plots **(A)** and cumulative data **(B)** are shown. A Two-way ANOVA test was used for statistical analyses and * indicating p-values ≤ 0.05.

(Figure 4A). Notably, following the recognition of these 15-mer peptides aNK cells displayed a marked increase in frequency, degranulation (CD107a), and cytokine production (Figure 4B). The observed increase in aNK cell activity was significantly higher compared to the effects of other classical HLA-E-restricted leader peptides such as UL40, and at least equal to the effects generated by the pp65-derived pool of the 15-mer peptides. Interestingly, all three pp65-derived 15-mer peptides also activated aNK cells from HCMV-seronegative individuals, yet to a much lower extent (Figure 4B). In contrast, the cNK cell population did not respond to the identified peptides (Supplementary Figure 3A). To assess the direct effect in eliciting an antigen-specific secondary response against pp65, we cultured NK cells without DC but with MRC-5 loaded with the pp65 peptide pool and assessed aNK cell responses. aNK cells displayed an increased expansion of NKG2C$^+$ cells and degranulation, demonstrating a direct recognition of the pp65-derived peptides, however, to much lower levels compared to when cultured with loaded mDC (Supplementary Figures 3B, C). These results, suggesting that mDC are excellent presenters of these negatively charged peptides. aNK cells were subsequently cultured with mDC loaded with the nonameric peptides pp65$_{402-410}$, pp65$_{86-94}$, and pp65$_{411-419}$, and our results demonstrated that at least one of these peptides elicited specific recall responses in aNK cells as measured by expansion and cytokine production (Supplementary Figure 3D). Hence, our results also demonstrate the equivalent capacity of 15-mer and 9-mer peptides that are heavily negatively charged to elicit such NK cell memory responses. Altogether, these results demonstrate that aNK cells can specifically recognize 15-mer HLA-E-restricted peptides, resulting in memory recall responses.

3.5 aNK cell antigen priming is dependent on HLA-E but not MHC class I or II

We sought to confirm that the defined 15-mer peptides are also solely presented on HLA-E and to exclude the possibility that these peptides are also presented by other MHC class I and II molecules. NK-DC was, therefore co-cultured in the presence of each of the three peptides and one of the blocking antibodies against HLA-E, MHC I, or MHC II at the priming or the restimulation phase. We found that at the priming phase, HLA-E was still the prominent antigen-presenting molecule associated with aNK cell memory (Figures 5A, B). On the other hand, blocking HLA-E, MHC class I, or MHC II at the secondary stimulation phase with HCMV-infected MRC-5 diminished aNK cell recall responses (Figure 5C). Thus, our findings suggest that aNK cell memory formation is dependent on HLA-E. However, long-term priming may result in enhanced ability of the immune cells to attack multiple epitopes on the infected cells associated with other MHC molecules than HLA-E. This phenomenon can be implied by the broad effect of MHC I,

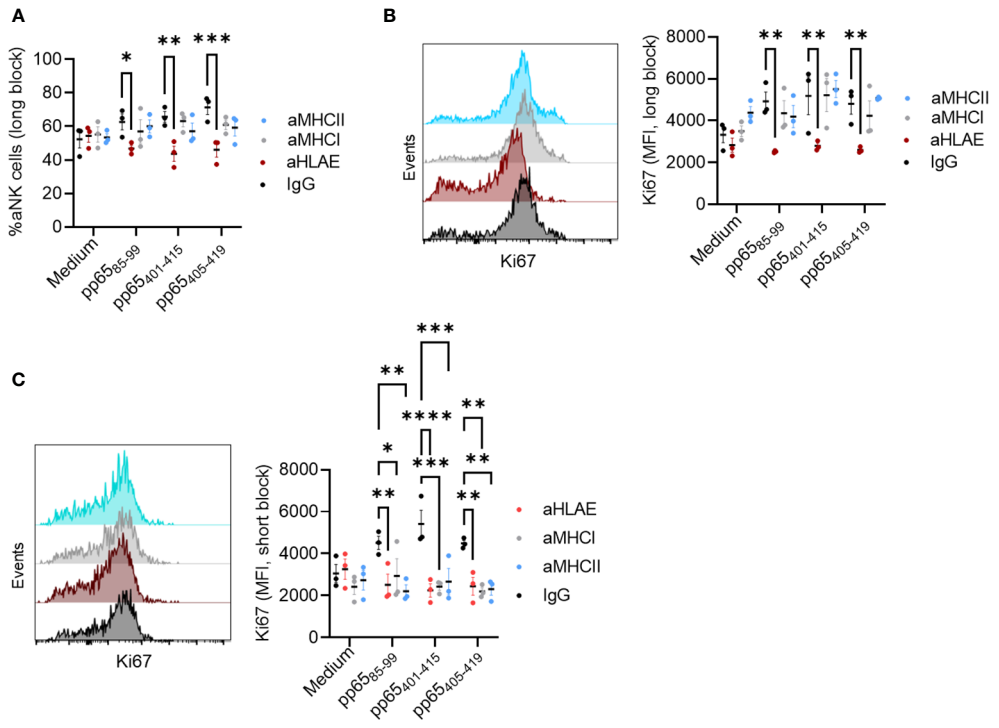

FIGURE 5
aNK cells antigen priming is dependent on HLA-E and no other MHC molecules. NK cells (n = 3) were cultured with mDC+pp65 for 14 days in the presence of either a control isotype-matched antibody IgG, or anti-HLA-E, anti-MHC I, or anti-MHC II blocking antibodies (5 µg/ml), at **(A, B)** the priming phase (day 0 and 7) or at **(C)** the restimulation phase (day 14) and thereafter assessed for their frequency and proliferation by flow cytometry. All blood donors were HCMV seropositive. The results from two independent experiments are presented, and data are shown as representative histograms for each donor. A Two-way ANOVA was used for statistical analyses. * indicating p-values ≤ 0.05, ** indicating p-values ≤ 0.001, *** indicating p-values ≤ 0.0001.

II, and HLA-E blocking at the secondary stimulation phase, which blocks the proliferation of aNK cells, giving rise to other possible mechanistic ways to target and kill the infected cells.

3.6 Multidimensional investigations of aNK cells confirm increased activity in response to the identified 15-mer and 9-mer peptides

We next sought to investigate the dynamic change of NK cell subsets following antigen priming and secondary stimulation. NK cells cultured with mDC or mDC loaded with the selected pp65 peptides were restimulated with CMV-infected MRC-5 and investigated for aNK cell identification (FcεRIγ, CD57, and NKG2C) and functional (Ki67 and TNFα) marker expression by flow cytometry. Flow cytometry data were subjected to dimensional reduction opt-SNE analysis to identify live NK cell ($CD56^{+}CD3^{-}$ live/dead^{-}) clusters potentially associated with specific peptide recognition. The opt-SNE analysis identified six clusters based on the markers' expression density, including CD57, NKG2C, FcεRIγ, CD107a, Ki67, and TNFα (Figure 6A). These six clusters had different expression levels of the selected markers dependent on the loaded peptide (Supplementary Figures 4, 5A). Among these six clusters, aNK cells and cNK cells were identified as clusters P1 and P3, respectively, based on the following characteristics: low versus high FcεRIγ, high CD57, high versus low NKG2C expression levels (Figure 6B, Supplementary Figure 5A). We found that aNK, compared to cNK cells, displayed a substantial increase in CD57 density and high expression levels of NKG2C in response to all the 15-mer and 9-mer pp65 peptides identified in this study. Functionally, these aNK cells exhibited high proliferation (presented as Ki67) and high TNFα production (Figure 6C), as previously shown in the 2-dimensional analysis. In addition, HLA-E-binding pp65-derived peptides, e.g., $pp6_{5401-415}$ and $pp65_{405-413}$ (p2Q, p9L), enhanced the expression of aNK cell-associated markers CD57 and NKG2C, even in HCMV-seronegative individuals following two weeks co-culture with mDC (Figure 6D). Importantly, priming NK cells with $pp65_{405-419}$ or $pp65_{405-413}$(p2Q, p9L) resulted in specific killing of HCMV-infected compared to HCMV-uninfected MRC-5 cells (Figure 6E, Supplementary Figure 5B). In summary, our results demonstrate that aNK cells, like T cells, can form memory against HCMV-infected cells when primed with HCMV-pp65 peptide loaded DC.

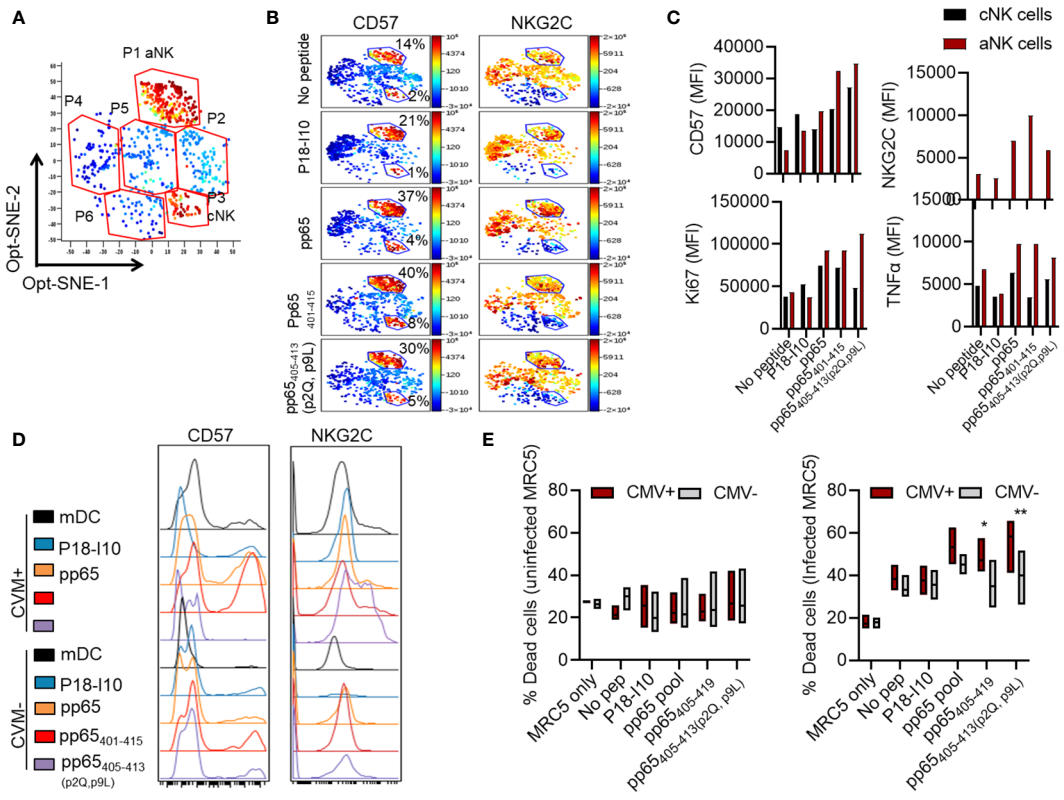

FIGURE 6
Multidimensional investigations of aNK cells confirm their specific function in response to peptides. **(A)** Dimensional reduction opt-SNE analyses of NK cells are shown following coculture with mDC loaded with pp65405-419 (DSDEELVTTERKTPR) peptide and assessed phenotypically distinct clusters. One representative opt-SNE, out of three independent experiments is shown. **(B–D)** Dimensional reduction opt-SNE analyses of NK cells are shown following coculture with mDC unloaded or loaded with different peptides and assessed for the phenotype (CD57 and NKG2C) and function (Ki67 and TNFα) of aNK and cNK cell clusters. One representative out of three independent experiments is shown. **(E)** NK cells were cultured with mDC peptide unloaded or loaded for 14 days and assessed for the killing capacity of infected or uninfected MRC-5 cells 6 hours prior to staining. Pooled data are shown from two independent experiments (n=11). Data are shown in boxplots and statistical analysis was performed using a Two-way ANOVA test * indicating p-values ≤ 0.05, ** indicating p-values ≤ 0.01.

4 Discussion

In contrast to T and B cells, NK cells have been historically regarded as phenotypically static cells with a short lifespan, unable to provide immunological MHC/peptide-specific memory. This view has been reevaluated considering more recent reports describing the phenotypic, epigenetic, and functional heterogeneity that exists among populations of NK cells, particularly in response to viral infections (10, 43–45). In the present study, we demonstrated that aNK cells can recognize 15-mer HLA-E-restricted HCMV peptides with unconventional sequences, and establish a memory response resulting in a clonal-like expansion following a secondary stimulation. This peptide-specific recognition elicited significant recall responses that led to the killing of HCMV-infected targets, thus confirming their unambiguous immunological memory of specific epitopes.

Recent discoveries established that aNK cells are able to respond to specific viral antigens through interaction with antigen-loaded non-polymorphic HLA-E (3). Cell-surface stabilization of HLA-E requires loading with peptides, which can be derived from MHC class I leader peptides or other proteins at steady state (46–48). In addition to host peptides, the UL40 motif in HCMV has been found to encode HLA-E-stabilizing peptides that are partially similar to MHC class I leader sequences (49, 50). Here we show that none of the three peptides comprised the classical HLA-E-restriction motifs, which include a methionine residue that binds to the HLA-E B-pocket or a leucine/valine residue at p9 that would fit in the hydrophobic F-pocket of HLA-E (51, 52). Furthermore, none of these three peptides contain glutamine or a lysine residue that could be used as anchor positions for binding to the B-pocket, as described in HLA-E epitope mapping studies (52, 53), or proline residues at p3, p4, p6 or p7, all shown to enhance the binding capacity of peptides to HLA-E (54). Instead, these three HLA-E-restricted epitopes were all heavily negatively charged and comprised polar residues. None of these three epitopes was predicted to bind to HLA-E by the MHC peptide prediction server NetMHC (55). However, it should be noted that parts of these three peptide sequences have been previously described/predicted in the literature mainly as targets for B cell recognition (56, 57) or as epitopes restricted to classical MHC class I and class II molecules (58–60). To our knowledge, all the previously determined crystal structures of HLA-E present nonameric peptides, which bind stably to HLA-E. However, recent MS analyses have demonstrated that nonameric peptides constitute only 18% of the whole HLA-E immunopeptidome and demonstrated the presence of longer peptides with lengths ranging from 10 to 21 amino acids (53, 61). Interestingly, although the identified peptide sequences do not have homology with the traditional motif, they bind to HLA-E*0101, as demonstrated by both molecular and cellular binding assays. In addition, all three 15-metric peptides identified within the present study induced recall responses in aNK cells that could be due to specific molecular features, including their significant electronegativity. Indeed, similar to our negatively charged 15-mer HCMV peptides, some of the peptides derived from *M. tuberculosis* proteins were acidic and were recognized by CD8 T cells (61).

HCMV-infected cells and several solid tumors overexpress HLA-E as an escape mechanism of cNK and T cell killing through ligation of the inhibitory receptor NKG2A (38, 50, 62). Theoretically, inhibition of NKG2A will allow for the interaction of the NKG2A counterpart, the activating receptor NKG2C with HLA-E and may allow for specific targeting. A phase II clinical trial in which anti-NKG2A was combined with an epidermal growth factor receptor inhibitor in previously treated head and neck carcinomas showed a 31% objective response rate (63). Hypothetically, these clinical responses might be due to the NK cell activation status or tumor-antigen recognition by aNK cells, and whether the patient is an HCMV carrier. Here, we found that inhibition of NKG2A in aNK cells interacting with mDC loaded with HCMV-peptides did not change their activation state, excluding the possibility that the identified peptides bind to NKG2A. In contrast, antibody blocking of HLA-E and NKG2C interactions significantly altered the activation state of aNK cells in coculture with peptide-loaded mDC, indicating an antigen recognition state through HLA-E/peptide/NKG2C complexes rather than a co-stimulation boost.

Here, we hypothesized that similar to T cells, the activation of aNK cells may involve both antigen recognition (signal 1) and co-stimulatory signals combined with signaling from cytokines provided by professional antigen-presenting cells (APC) (signal 2 and 3). We and others have previously demonstrated that both DC and B cells can activate NK cells (64–67). However, whether APC are also essential for aNK cells to display a secondary immune response was unknown. Our results reveal that aNK cells depend on at least signal 1. Further studies are needed to determine the specific DC co-stimulatory receptors (signal 2) and cytokine stimulation for aNK cell recall responses and memory persistence.

Our results have implications for strategies to expand aNK cells with immunological memory *ex vivo* for immunotherapy. Several transplantation studies have shown that NK cells are involved in tumor rejection and protection from relapse, supporting the therapeutic potential of NK cells in tumor eradication (68, 69). Recently, it has been shown that aNK cells with single-KIR +NKG2C+ expanded from selected HCMV infected donors with feeder cells loaded with HLA-G leader-derived peptides have potent reactivity towards HLA-mismatched acute myeloid leukemia cells (4). Despite these encouraging findings, NK cell therapies are limited by the lack of antigen specificity. Also, similar to T cells, resistance to NK cell-mediated killing may also develop due to the recruitment and differentiation of immune suppressive cells, including regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC), as well as overexpression of immune inhibitory checkpoint proteins in the tumor microenvironment (TME). We discovered earlier that aNK cells found in HCMV-seropositive individuals can resist TME-induced suppression. We showed that the mechanisms sparing aNK cells from immune suppression by MDSC and Treg involved the downregulation of the checkpoint molecules T cell immunoglobulin and ITIM domain (TIGIT), programmed death receptor (PD-1) and IL-1R8 (22, 23). In agreement with these findings, we found that reconstitution and expansion of aNK cells in individuals with HCMV reactivation was associated with reduced leukemia relapse and better clinical outcomes following hematopoietic stem cell transplantation

(22, 24). Thus, our 15-mer identified peptides could potentially be used as a therapeutic vaccine strategy to provoke antigen-specific aNK cell responses with persistent memory, combined with the ability to resist immunosuppression. Ongoing studies in our lab evaluate the potential of this strategy in solid tumors.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Karolinska Institutet institutional review board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

NA and BS performed experiments analyzed data, visualization, methodology, writing and editing manuscript. TS, YS, TR, FC performed experiments, collected data, formal analysis, reviewing manuscript. CS-N, JM, AA. Provided study materials, reagents, instrumentation, formal analysis, or other analysis tools, validation, supervision, editing, and revising manuscript. DS Conceptualization, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing and editing manuscript, project administration. 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1230718/full#supplementary-material

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Innate lymphoid cells: a new key player in atopic dermatitis

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Atopic dermatitis (AD) is a common allergic inflammatory skin condition mainly caused by gene variants, immune disorders, and environmental risk factors. The T helper (Th) 2 immune response mediated by interleukin (IL)-4/13 is generally believed to be central in the pathogenesis of AD. It has been shown that innate lymphoid cells (ILCs) play a major effector cell role in the immune response in tissue homeostasis and inflammation and fascinating details about the interaction between innate and adaptive immunity. Changes in ILCs may contribute to the onset and progression of AD, and ILC2s especially have gained much attention. However, the role of ILCs in AD still needs to be further elucidated. This review summarizes the role of ILCs in skin homeostasis and highlights the signaling pathways in which ILCs may be involved in AD, thus providing valuable insights into the behavior of ILCs in skin homeostasis and inflammation, as well as new approaches to treating AD.

KEYWORDS

innate lymphoid cells, atopic dermatitis, inflammatory response, pathophysiology, cytokines

1 Introduction

Atopic dermatitis (AD) is a chronic skin disease characterized by a massive infiltration of inflammatory cells, with intense pruritus, plasmacytic exudates, dry skin, and erythematous papules as the predominant clinical symptoms (1). The onset of AD is not limited by age or race. AD plays a significant role in the global burden of dermatologic diseases and has a detrimental impact on the quality of life of patients and their families. From 1990 to 2017, AD ranked 15th among all non-fatal diseases and the first among dermatological diseases in disability-adjusted life years (DALYs) (2). Traditionally, the pathogenesis of AD is highly complex, involving genetic predisposition, epidermal dysfunction, and T-cell-driven inflammation. The T helper (Th) 2 cells dominate the pathogenesis of AD by secreting pro-inflammatory cytokines such as interleukin (IL)-4 and IL-13. Dupilumab is a monoclonal antibody that selectively blocks IL-4 and IL-13 signaling and received the first global approval for AD treatment in March 2017, representing a major advance in treating patients with moderate-to-severe AD (3, 4). However,

dupilumab is ineffective in some AD patients and might induce new regional dermatoses, ocular complications, alopecia, and other adverse effects (5). Although there is no accurate cure for AD, many novel and targeted therapies promise to slow the disease's progression considerably, especially in patients with refractory AD. In recent years, the detection of innate lymphoid cells (ILCs) in the context of skin homeostasis and inflammation has gained increasing attention (6).

ILCs are a newly found lymphoid lineage component of the innate immune system that differentiates from common lymphoid progenitor cells (CLPs) (7, 8) and produce a range of cytokines associated with subsets of T helper cells (9). Furthermore, ILCs are characterized by the absence of antigen-specific receptors produced by genetic recombination (8), and their growth is typically dependent on the common gamma chain of the IL-2 receptor, Notch, and the transcription factor inhibitor of DNA binding 2 (ID2) (7). ILCs are crucial in generating immune responses, maintaining tissue integrity, and mediating inflammatory responses (8). Recent studies have demonstrated that the pathophysiology of AD is strongly connected to abnormal ILC activation (10, 11).

This review presents the involvement and function of ILCs in the skin, emphasizing the role of several subgroups of ILCs in the pathogenesis of AD, and further discusses the possible associated signaling pathways. The aim is to shed new light on the molecular mechanisms of AD and imply the potential value of targeting ILCs for therapy.

2 The subsets of the ILC family

The ILC family comprises a group of immune cells with pleiotropic functions, which lack somatic rearrangements of immune receptor genes characteristic of T and B cells (12). In the early phases of the study, it was customary to group ILCs into three major categories, with different functions for each subset, namely, natural killer (NK) cells, RORγt$^+$ ILCs, and type 2 ILCs (13). Subsequently, the International Union of Immunological Societies (IUIS) approved the classification of ILCs into five subpopulations, namely, NK cells, ILC1s (group 1 ILCs), ILC2s (group 2 ILCs), ILC3s (group 3 ILCs), and lymphoid tissue-inducing (LTi) cells, based on the various developmental trajectories and transcription factors expressed by ILCs (12, 14, 15) (Figure 1).

NK cells are cytotoxic lymphocytes with a shorter half-life than B and T lymphocytes and occur more frequently in the circulatory system (16). NK cells can directly induce the death of tumor and virus-infected cells without specific immunization, thereby controlling intracellular pathogens (17, 18). NK cells depend on the IL-15 developmental pathway, with differential expression of GATA binding protein 3 (GATA3) and IL-7 receptor α-chain (CD127) (19, 20). Based on the relative expression of surface markers CD16 and CD56, NK cells in human peripheral blood could be subdivided into CD56^{bright} CD16 $^-$ and CD56^{dim} CD16$^+$ NK cells (21). NK cell subpopulations differ in their cytolytic activity and cytokine production capacity. Vosshenrich et al. speculated that the two CD56 NK cell subsets in humans might share characteristics with various NK cells generated by the bone marrow and thymic NK cell pathways in mice (20).

The ILC1s monitor the immune system and defend the host, and they are often non-cytotoxic or weakly cytotoxic (12). ILC1s and NK cells differ in the production and dependence of transcription factors (11, 22). Zhang et al. proposed that NK cells are defined by high levels of co-expression of T-bet and eomesdermin (Eomes), whereas ILC1s are defined by the single expression of T-bet or Eomes (23). Similar to NK cells, ILC1s are developmentally reliant on T-box transcription factor (T-bet) and release type I cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor (TNF) (12). T-bet has been shown to bind to the promoters of protein-coding genes in Th1 cells, activating many critical genes in the Th1 cell response, suggesting that ILC1s may contribute to the Th1 cell response (24). Unlike NK, ILC1s are the first and primary producers of IFN-γ *in vivo* during the early stages of viral infection and do not require IL-18 signaling to optimize IFN-γ production (25). ILC1s produce optimal IFN-γ in a signal transducer and activator of transcription 4 (STAT4)-dependent manner via tissue-resident X-C motif chemokine receptor 1-positive conventional dendritic cells (XCR1$^+$ cDC1), thereby limiting viral replication at the initial site of infection (25). Furthermore, RNA-sequencing analysis suggests that *Itgb3* (encoding CD61) and *Cd200r1* (encoding CD200r1) may be new, reliable specific markers to distinguish peripheral tissue-resident ILC1s from circulating NK cells, providing new insights for future studies (25).

ILC2s are usually considered substantial members of the ILC family involved in innate immune responses and regulation of tissue homeostasis (26). RORα and GATA3 (27), which are ILC2-specific transcription factors, are required for ILC2 formation. ILC2s express IL-7Rα, CD45 (28), BCL11B, and GFI1 (29), and their distinctive characteristic is the secretion of Th2-associated cytokines such as IL-4, IL-5, IL-9, IL-13, and amphiregulin (AREG) (10).

ILC3s depend on RORγt for their functional development, expressing natural cytotoxicity receptors (NCRs) and the surface marker IL-23R (12). According to the expression of NCR NKp44, ILC3s could be categorized into two main subgroups: NCR $^-$ ILC3s and NCR$^+$ ILC3s. NCR $^-$ ILC3 equivalent Th17 cells express RORγt and produce IL-17A/IL-22, and NCR$^+$ ILC3 equivalent Th22 cells express transcripts of RORγt and aryl hydrocarbon receptor (AHR) and produce only IL-22 (30). ILC3s modulate adaptive Th17 cell responses and produce Th17-related cytokines such as IL-17 and IL-22 (31).

LTi cells are crucial for secondary lymphoid organ formation during embryogenesis and act in T- and B- cells' development, activation, and function (32). It is essential for LTi cells to express the chemokine receptors CXCR5 and CXCR6 in order to differentiate to the next stage (33). Additionally, LTi cells are similar to ILC3s, expressing RORγt markers and releasing cytokines that overlap with ILC3, such as IL-17 and IL-22 (34). As a crucial transcription factor for developing ILC3 progenitors, the promyelocytic leukemia zinc finger (PLZF) has a defining role in innate lymphocyte lineage differentiation (35). PLZF expression is not required to form LTi cells, although ILC3s are (33, 36).

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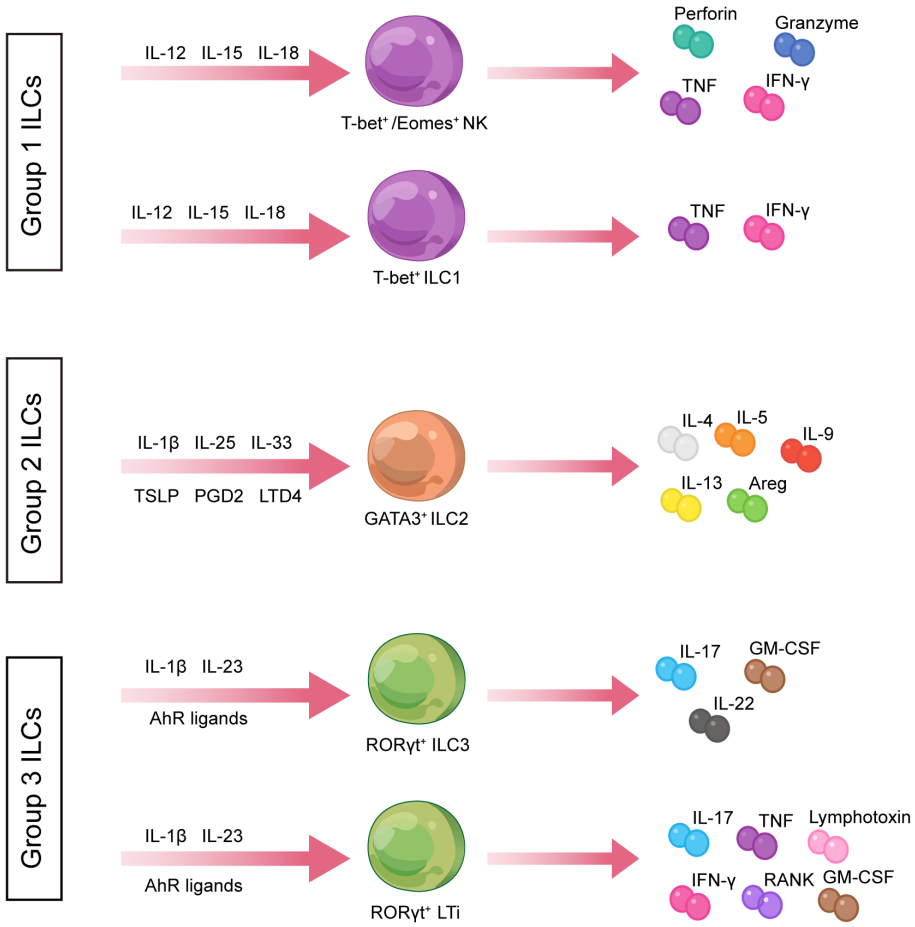

FIGURE 1
Classification of innate lymphoid cells. ILCs are divided into three groups. ILC1s produce type 1 cytokines such as TNF and IFN-γ and express T-bet in response to IL-12, IL-15, and IL-18 stimulation. ILC2s are defined by the expression of RORγt and secretion of Th2-associated cytokines such as IL-4, IL-5, and IL-13. ILC3s express GATA3 and produce IL-17A and IL-22 in the stimulation setting by IL-1β, IL-23, and AHR ligands. AHR, aryl hydrocarbon receptor; Areg, amphiregulin; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN-γ, interferon-gamma; IL, interleukin; ILCs, innate lymphoid cells; LTD4, leukotriene D4; NK, natural killer; PGD2, prostaglandin D2; RANK, receptor activator of nuclear factor kappa B; Th, T helper; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

3 ILCs in skin tissue

ILCs are widely distributed in various organs and tissue types in the human body (37, 38). ILCs are usually preferentially enriched in barrier tissues, such as the skin, intestine, and lung, which facilitate the maintenance of barrier function and response to tissue-derived signals (37). Our understanding of the functional features of skin ILCs is still developing compared with those of the lung and gut (11); thus, certain traits of skin ILCs will be discussed.

The skin is a mechanical and biological barrier for the body, protecting epithelial integrity and maintaining homeostasis. Anatomically, the skin consists of avascular epidermis, dermis, and subcutaneous tissue (39), each layer with specific morphological and physiological functions. Furthermore, the presence of ILCs in the skin is related to the host species and the skin layer's location (Figure 2).

ILC subsets are differentially presented in various layers of mouse and human skin. NKp46 is a receptor found on the surface of NK cells from the early to late stages of differentiation. Luci et al. employed tissue immunofluorescence assay to detect NKp46 expression and discovered that the distribution of NK cells in mouse and human skin was identical at a steady state (40). This work demonstrated that NKp46$^+$ CD3$^-$ NK cells were predominantly present in the dermis and virtually absent from the epithelium, indicating that the proliferating dermal NK cells may be the source of NK cells recruited to inflamed skin during the allergic phase. In contrast, Kobayashi et al. did not find genes associated with NK cells and ILC1s by sorting and performing single-cell RNA sequencing of Lin$^-$ Thy1.2$^+$ ILCs from each skin layer of wild-type (WT) C57BL/6 mice (14). Kobayashi et al. and Luci et al. used mice with the same genetic background. Still, they were controversial about the frequency of NK cells in the skin, probably related to the different technical aspects of the assay. Kobayashi et al. also revealed that in mice, the subcutaneous and epidermal layers were highly enriched in genes characteristic of ILC2s and ILC3s, respectively, and the dermis was characterized by both ILC2s and ILC3s (14).

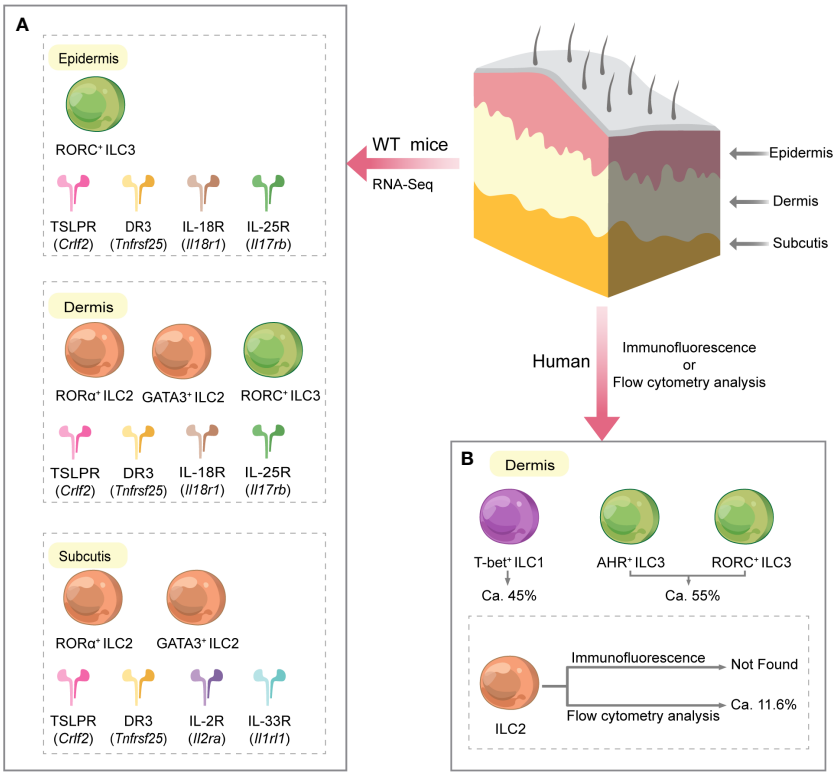

FIGURE 2
Different distribution of ILCs and their related receptors in various layers of normal mouse and human skin. **(A)** In mice, the subcutaneous and epidermal layers were highly enriched in genes characteristic of ILC2s and ILC3s, respectively, while ILC2s and ILC3s characterized the dermis. Furthermore, mouse skin RNA sequencing studies showed that ILCs in all skin layers expressed *Crlf2* (encoding the TSLPR subunit) and *Tnfrsf25* (encoding DR3). Dermal ILCs and epidermal ILCs highly express *Il18r1* (encoding IL-18R subunit) and *Il17rb* (encoding IL-25R). *Il1rl1* (encoding IL-33R subunit) and *Il2ra* (encoding IL-2R subunit) were significantly expressed on subcutaneous ILCs. **(B)** In humans, ILCs are only present in the dermis of normal skin. ILCs in the dermis are mainly composed of ILC1s and ILC3s. Flow cytometry data showed that among all dermal ILCs, ILC1s accounted for approximately 45%, ILC3s for approximately 55%, and ILC2s for approximately 11.6%. However, $GATA3^+$ ILC2 was not detected in skin tissue sections by immunofluorescence. DR3, death receptor 3; IL, interleukin; IL-2R, IL-2 receptor; IL-18R, IL-18 receptor; IL-25R, IL-25 receptor; IL-33R, IL-33 receptor; ILC, innate lymphoid cell; T-bet, T-box transcription factor; TSLPR, thymic stromal lymphopoietin receptor; WT, wild type.

Alkon et al. reported that ILCs from AD skin frequently co-expresses type 2 (*GATA3* and *IL13*) and type 3/17 (*RORC*, *IL22*, and *IL26*) molecular signatures at the single-cell level and can rapidly change their molecular, immunophenotypic, and functional characteristics upon cytokine stimulation, participating in host defense or promotion of disease onset (41). Reynolds et al. showed by single-cell RNA sequencing that ILCs in the epidermis and dermis of AD patients and normal healthy subjects could be classified into four subgroups, namely, ILC1/3, ILC2, ILC1/NK, and NK, with ILC2s (*IL7R*, *PTGDR2*, and *GATA3*) having the most distinct signature (42). Additionally, Brüggen et al. demonstrated that very sparse ILCs are present in the upper dermis of normal human skin with an algorithm-based *in-situ* analysis technique, while the hypodermal areas and epidermis are almost devoid of ILCs (43). Using immunofluorescence, they found that the ILC population in the upper dermis was dominated by ILC1s, followed by AHR^+ ILC3s, and no $GATA3^+$ ILC2s were observed (43). Instead, flow cytometry analysis revealed the presence of ILC2s in normal human skin cell suspensions, accounting for approximately 10% of all ILCs (43). The controversial results of the two methods in this study may be related to factors such as sample collection site, cell migration, changes in cell phenotype during isolation and purification, and the sensitivity of the assay.

Additionally, single-cell RNA-sequencing studies of wild-type C57BL/6 mouse skin showed that ILCs in all skin layers expressed *Crlf2* [encoding the thymic stromal lymphopoietin (TSLP) receptor subunit] and *Tnfrsf25* [encoding death receptor 3 (DR3)] (14). Dermal and epidermal ILCs highly express *Il18r1* (encoding IL-18 receptor subunit) and *Il17rb* (encoding IL-25 receptor) (14). *Il1rl1* (encoding IL-33 receptor subunit) and *Il2ra* (encoding IL-2 receptor subunit) were significantly expressed on subcutaneous ILCs (14) (Figure 2). These findings suggest a layer-specific receptor expression pattern in the skin, implying that cytokine species may be different in skin layers (14), which contributes to a better understanding of the mechanisms of localization of cytokine signaling pathways in the skin.

4 ILCs in atopic dermatitis

4.1 NK cells in atopic dermatitis

As one of the innate lymphocytes, NK cells are important sentinels of the organism to operate the immune system. NK cells exert immunomodulatory functions early in the inflammatory response, mainly by forming crosstalk effects with other immune cells and secreting a large variety of cytokines, such as TNF-α, IFN-γ, GM-CSF, IL-5, IL-6, and IL-10 (44–46). Significantly, NK cells induce Th1 cells to initiate a protective reaction by releasing IFN-γ, which facilitates the maintenance or enhancement of the body's antiviral immunity (44, 47). NK cells have been detected in the damaged skin of patients with atopic dermatitis and MC903-induced AD-like mouse models (a systemic AD-like inflammatory phenotype closely resembling human AD was induced by the topical application of MC903 to the skin) (48). It has been reported that peripheral NK cells were significantly reduced in AD patients, possibly related to chemokine-dependent NK cell recruitment from the periphery to the lesioned skin (45). C motif chemokine receptor 2 (CCR2), C-C motif chemokine receptor 5 (CCR5), and C-X-C motif chemokine receptor 3 (CXCR3) are the primary chemotactic receptors that regulate circulating NK cell migration (49).

Bi et al. demonstrated that NK cell activation inhibited ILC2 amplification and cytokine production *in vitro* and *in vivo* and that this modulation was predominantly mediated by IFN-γ (50). Mature $CD56^{dim}$ NK cells were recovered in most AD patients after dupilumab treatment, indicating that the NK cell deficiency in AD patients was reversible by the blockade of type 2 cytokines (48). Moreover, in a mouse model of NK cell-deficient AD (AD-like disease induced in *Il15* $^{-/-}$ mice by application of MC903), the reduction in NK cell numbers was restored by dupilumab as a Th2 cytokine blocker, suggesting that NK cells may contribute to suppressing the type 2 inflammation in AD (48). The inflammatory effects of ILC2s may be inhibited by IFN-γ released by NK cells, although more validation in animal models and patients at various illness stages is required. Mack et al. demonstrated that NK cell deficiency in mice could lead to the deterioration of pathogenic ILC2 responses *in vivo*, assuming that the NK cell–ILC2 inhibition axis may be a potential regulatory mechanism in the skin barrier (48) (Figure 3). This finding suggests that defects in NK cell numbers or function lead to type 2 inflammation and skin damage, further suggesting that NK cells may be closely associated with AD development.

Furthermore, NK cells also contribute to the body's protective immunity. As important antigen-presenting cells in the immune response, NK cells can selectively edit dendritic cells (DCs) by

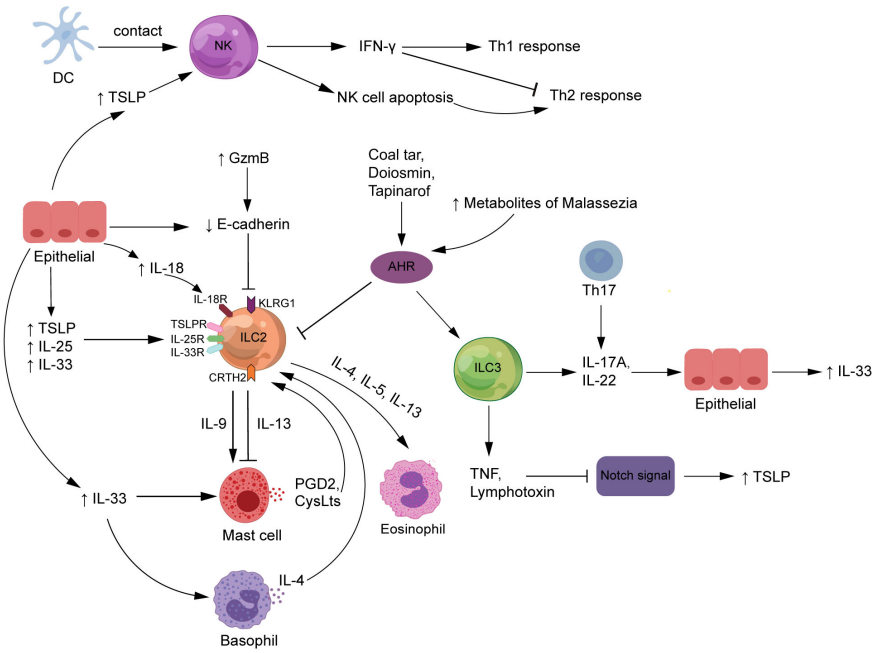

FIGURE 3
The roles of ILCs in atopic dermatitis. NK cells are stimulated by DC exposure or high TSLP levels to enhance Th1 responses, attenuate Th2 responses by producing IFN-γ, and improve Th2-type immunity by promoting auto-apoptosis. The expression of TSLP, IL-33, and IL-25, released by epithelial cells and serve as major ILC2 activators, is increased in AD patients. The interaction of ILC2s with other innate immune cells, such as mast cells and basophils, is critical to the complex mechanics of AD. Also, ILC3s release IL-17A or IL-22, which contribute to the pathogenesis of AD. Increased GzmB expression and FLG deficiency in AD patients both enhance E-cadherin cleavage, which inhibits the interaction between E-cadherin and the KLRG1 receptor expressed on ILC2s, which would strengthen the body's ILC2-related response. ILCs may trigger TSLP secretion by producing TNF and lymphotoxin to downregulate Notch signaling. In addition, diosmin, coal tar, and tapinarof inhibit the action of ILC2s but promote the function of ILC3s by activating the AHR signaling pathway. AHR, aryl hydrocarbon receptor; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; CysLts, cysteinyl leukotrienes; DCs, dendritic cells; FLG, filaggrin; GzmB, granzyme B; IFN-γ, interferon-gamma; IL, interleukin; ILCs, innate lymphoid cells; KLRG1, killer cell lectin-like receptor G1; NK, natural killer; PDG2, prostaglandin D2; Th, T helper; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

killing immature DCs while retaining mature ones, which is required for adaptive immune responses to be initiated successfully (51). NK cells undergo close contact with DCs in the affected tissues of AD patients, suggesting that NK cells are induced to become preferential targets for apoptosis after exposure to activated monocytes, which also enhances the deviation of immune response from Th1 toward Th2 type and contributes to microbial infection (45, 52, 53). However, the activation signals that trigger natural killer cell death *in vivo* are currently unknown. TSLP is an epithelial cell-derived cytokine that is one of the key factors driving the development of the vicious cycle of inflammation in AD (54). TSLP could activate DCs to promote Th2 immune responses and has been reported to act directly on NK cells expressing TSLPR and IL-7Rα to produce IL-13, suggesting that TSLP may be a key factor in the role of NK cells in AD development (55) (Figure 3).

Maintaining a relatively stable number and function of NK cells *in vivo* is critical to the progression of AD, and a clearer understanding of the specific pathways of NK cells in the pathogenesis of AD may provide new strategies for AD.

4.2 ILC2s: the critical ILCs in atopic dermatitis

Kim et al. first found the presence of skin-derived ILC2s in healthy human skin (56). They observed a significant increase in the frequency of ILC2s in lesional AD skin compared with healthy control skin by flow cytometry, suggesting that ILC2s perform a crucial function in developing skin inflammation. ILC2s, generally considered to be the most important ILC subtype in AD pathogenesis, promote the development of Th2 cells by producing characteristic cytokines, such as IL-13 and IL-5, and it has been demonstrated that ILC2 deficiency leads to severe defects in Th2 cell immune responses (57). Interestingly, Alkon et al. showed that cutaneous ILC2 in patients with AD can have cytokine transcripts characteristic of type 17 and/or type 3 immunity that can co-produce cytokines such as IL-5, IL-13, IL-22, and IL-17A (41).

4.2.1 Modulators of activated ILC2s in atopic dermatitis

It is well known that TSLP, IL-33, and IL-25 are major activators of ILC2s, and all of these cytokines have been reported to be elevated in the skin of AD patients (29).

ILC2s express receptors for TSLP, IL-33, and IL-25, all of which have a cascade of regulatory and recruitment effects on ILC2s in AD (58). TSLP, IL-33, and IL-25 can activate ILC2s to secrete various pro-inflammatory factors to induce the development of AD, and this effect can be amplified by the stimulation of allergens such as house dust mite (HDM) extraction (29, 58) (Figure 3). Studies have shown that TSLP can interact directly with T cells from AD patients to enhance Th2 responses by promoting the proliferation of IL-4-producing cells and secretion of IL-4 (59). IL-33 facilitates the survival and function of mast cells and basophils, which may be related to disrupting the skin barrier in AD patients and accumulating these innate immune cells in the skin lesions (60).

Of note, the relative magnitude of the contribution of IL-33 and TSLP in the inflammatory response in AD remains controversial. Kim et al. showed that AD development in mice is heavily dependent on TSLP but independent of IL-33 and IL-25 (56). In the AD-like model of inflammation, deficiency of the TSLP receptor gene in mice significantly reduced the frequency and absolute number of ILC2s, while the IL-25 or IL-33 receptor gene deficiency did not affect the ILC2 response (56). In contrast, Salimi et al. suggested that adding IL-25 and IL-33 but not TSLP increases ILC2s (58). In parallel experiments where the TSLP, IL-33, and IL-25 receptor genes were each individually knocked out in MC903-induced AD mice, the number of ILC2 was sequentially reduced in skin lesions of these mice compared with wild-type mice (58). One explanation for this contradictory finding could be related to the differences in the genetic background of the mice in the two laboratories.

Indeed, most skin ILC2s have low receptor expression for the epithelial cytokines IL-33, IL-25, and TSLP and are primarily activated by IL-18, which is highly expressed in skin ILC2s (61). Ricardo-Gonzalez et al. showed that skin ILC2s can respond to IL-18 to produce type 2 inflammatory cytokines. In AD-like skin inflammation, IL-18-deficient mice had reduced amounts of IL-5- and IL-13-producing ILC2 in skin tissues compared with WT mice (61). Serum IL-18 was elevated in AD patients compared with healthy controls and correlated with disease severity (62), implying that targeting IL-18 may improve type 2 immune activation in AD.

4.2.2 Interaction of ILC2s with other innate immune cells in atopic dermatitis

The interaction of ILC2s with other innate immune cells, such as mast cells and basophils, is key to the etiology of complex AD (Figure 3). Mast cells produce and release various pro-inflammatory mediators such as histamine, chemokines, and cytokines, pivotal in the IgE-mediated skin wheal reaction and its associated AD pruritus (63). Studies have shown that the proportion of mast cells containing TNF-α, IL-4, IL-6, and CD30 ligand immunoreactive is higher in AD lesions than in non-lesioned skin (63). Intravital multiphoton microscopy revealed that normal murine dermal ILC2s (dILC2s) preferentially reacted with skin-resident mast cells and had pro- and anti-inflammatory properties (64). The almost exclusive production of IL-13 by dILC2 in the skin may be associated with AD. The results of *in-vitro* experiments showed that co-incubation of mast cells with recombinant IL-13 had a dose-dependent inhibitory effect on the release of IgE-dependent cytokines from mast cells, suggesting that dILC2 has the potential to modulate mast cell function through IL-13 production (64). However, once stimulated by inflammation, dILC2 exerted a pro-inflammatory effect and was able to promote eosinophil infiltration and mast cell activation in the skin (64). Additionally, a substantial amount of human and mouse research data supports the idea that IL-9 acts as a Th2 cytokine to stimulate type 2 immune responses (65). IL-9 mRNA expression was significantly increased in AD patients' peripheral blood and skin lesions compared with normal subjects (66), and polymorphisms in IL-9 and IL-9 receptor genes were associated with the AD phenotype (67). IL-9 enhances mast

cell proliferation and function and is produced mainly by T cells but also by ILC2s, mast cells, and eosinophils (68). These findings suggest that ILC2s and mast cells may crosstalk through IL-9 in AD pathogenesis.

Flow cytometry data and fluorescence microscopy images indicated that basophils and ILC2s were enriched and aggregated near inflamed lesions of AD patients and AD mouse models (69, 70). Interestingly, Mashiko et al. found that the frequency of basophils in skin lesions of AD patients was positively correlated with cutaneous ILC2s and negatively correlated with circulating ILC2s, suggesting that basophils may promote the migration of circulating ILC2s to the skin of AD patients (71). Moreover, the temporal analysis showed that on day 4 of MC903 treatment, the frequency and the absolute number of basophils in mouse skin lesions were significantly higher compared with controls but not ILC2s, suggesting that the basophil response preceded the ILC2 response in the context of AD-like inflammation (69). Studies have shown that IL-4 from basophils is required for the proliferation of ILC2s and the development of related responses in skin inflammation (69, 72). To determine the role of basophils, an anti-FcεRI monoclonal antibody (MAR-1) was used to deplete basophils (73). Imai et al. systematically conditioned the clearance of basophils using MAR-1 or Bas-TRECK mice [basophils in mice are specifically depleted by a toxin receptor-mediated conditional cell knockout (TRECK) system] and found that ILC2 responses were suppressed along with relief of AD-like inflammation, suggesting that ILC2s mediate the innate immune response in conjunction with basophils in AD (72). The exact mechanism by which cross-regulation between ILC2s and basophils occurs in AD remains unclear, and other upstream innate cellular mechanisms are largely unexplored.

4.3 Role of ILC3s in atopic dermatitis

Type 2 cytokines are usually considered to have a substantial role in AD development, whereas evidence indicates that ILC3s operate in a pathogenic function in AD through the secretion of IL-17A and IL-22 (43, 74) (Figure 3). The percentage of IL-17$^+$ T cells in peripheral blood was significantly higher in AD patients compared with healthy controls and correlated with the severity of the disease (75). Furthermore, immunohistochemical results revealed a significant infiltration of IL-17$^+$ T cells in the dermis of acute AD lesions, indicating that IL-17 is the mediator of AD inflammation (75). Nakajima et al. found that IL-17A deficiency in mice alleviated the development of AD-like lesions and attenuated the expression levels of Th2 chemokines (76). IL-17A induces Th2-type immune responses in the AD mouse model, but drawing human conclusions from this model may be challenging.

Traditionally, Th17 cells are considered the primary source of IL-17, but recent studies have shown that IL-17 produced by ILC3s has a potentially important function in skin inflammation (11). Using *in-situ* mapping, Bruggen et al. discovered that skin lesions from AD patients had a significantly higher number of AHR$^+$ ILC3s than those of healthy human skin (43). Similarly, Kim et al. employed flow cytometry to uncover higher levels of ILC3s in the peripheral blood of AD patients compared with healthy controls and increased in HDM-treated C57BL/6 mice AD model (an allergen-induced mouse model with phenotypes similar to human AD) (77). These findings suggest the contribution of ILC3s to the development of AD. Kim et al. sorted ILC3s from skin-draining lymph nodes and spleens in HDM-induced AD mice and injected them subcutaneously into recipient mice (C57BL/6 mice). The results showed that the adoptive transfer of ILC3s in mice accelerated the development of AD inflammation, as evidenced by increased epidermal thickness and inflammatory granulocyte infiltration, implying that ILC3s alone are sufficient to exacerbate the symptoms of AD (77). Likewise, data from co-culture cell experiments indicate that IL-17A secreted by ILC3s triggers the synthesis of IL-33 by skin cells, promoting a type 2 response (77).

Healthy people's blood and skin contain NCR$^-$ ILC3s, which can develop into NCR$^+$ ILC3s and release IL-22 (78–80). Unlike psoriasis, IL-22 expression is more dominant than IL-17 in AD lesions (74). Clinical and animal studies have shown that IL-22 expression is significantly upregulated in AD-like skin lesions, with an important link between the skin barrier and adaptive immunity (81, 82). In a randomized, double-blind, placebo-controlled trial, fezakinumab (an anti-IL-22 monoclonal antibody) had good efficacy and safety in treating adult patients with moderate-to-severe AD, confirming IL-22 as a crucial driver of AD (83). In addition, ustekinumab, a monoclonal antibody that binds to the p40 subunit of IL-12 and IL-23 and limits the progression of the Th17 inflammatory immune response, is controversial in the clinical efficacy of AD patients (84). A patient with long-standing AD showed remarkable improvement following ustekinumab treatment (84). Contrarily, one case report indicated that AD was aggravated in a patient with psoriasis who had a history of childhood atopy while receiving ustekinumab medication, raising the possibility that ustekinumab treatment may be linked to AD relapse (85). These clinical trials indicate that biologics targeting ILC3-associated cytokines may be a new approach to treating AD, but caution and more trial data are still needed.

5 Possible ILC-related signaling pathways in atopic dermatitis

The Notch signaling pathway has been reported to be an important player in the biology of ILCs. Moreover, ILC2s and ILC3s are significantly elevated in skin lesions of AD patients compared with normal human skin. ILC2 cells express KLRG1 and CRTH2, and ILC3 cells express AHR. Therefore, four possible signaling pathways related to ILCs in AD are discussed below.

5.1 The Notch signaling pathway

Skin ILCs are bona fide tissue-resident immune cells that control barrier homeostasis and maintain a healthy microbial ecology (14). During homeostasis, epidermal and dermal ILCs inhibit sebocyte proliferation and enhance commensalism of

Gram-positive cocci by expressing TNF and lymphotoxin downregulating Notch signaling (14). ILCs may be upstream signals of the Notch signaling pathway that regulate mucosal barrier immunity and skin surface microbial homeostasis in AD (Figure 3).

Notch signaling is one of the typical pathways of epithelial differentiation and regulates the proliferation, differentiation, migration, and apoptosis of epidermal cells together with other cellular pathways *in vivo* (86). Notch signaling plays a pivotal role in ensuring normal skin development and differentiation and maintaining skin barrier function, and its abnormal disruption will induce the development of inflammatory skin diseases (86, 87). Adult mice lacking Notch signaling produce large amounts of TSLP, which caused an AD-like inflammatory response, suggesting that enhanced Notch signaling may suppress TSLP production in AD (88). Notch receptors were strongly expressed in skin tissues of psoriasis and lichen planus patients; however, they were significantly downregulated in skin lesions of AD patients as compared with healthy controls, implying that the regulation and signaling of Notch receptors are more closely related to AD than to psoriasis and lichen planus (88).

5.2 The AHR signaling pathway

Substantial amounts of AHR^+ ILC3s have been reported in skin lesions of AD patients, suggesting that AHR expression may play an important role in the pathogenesis of AD (43). AHR is a ligand-dependent transcription factor that senses environmental changes. AHR could be activated by a wide range of endogenous and exogenous molecules, regulate gene expression *in vivo*, maintain tissue barriers in barrier organs, and control commensal microbiota (89–91). Growing evidence suggests that AHR can control ILCs *in vivo* (Figure 3).

The maintenance, survival, and function of ILC3s depend on AHR expression, which is also crucial for the defense and homeostasis of the host intestinal tissues (92, 93). Studies have shown that AHR deficiency reduces the number of intestinal $ROR\gamma t^+$ ILCs, and AHR is necessary for their survival and the generation of IL-22 under homeostatic conditions (94). In addition, the amount of AHR protein and mRNA expressed in ILC2s in the mouse intestine is higher than both ILC progenitors and other mature ILCs (95). In contrast to promoting the maintenance of ILC3s, AHR inhibits the function of ILC2s, suggesting that the host regulates intestinal ILC2–ILC3 homeostasis by engaging in the AHR pathway (95). Craig et al. reported that multiple factors in the pathogenesis of AD involve dysbiosis of the gut flora and increased intestinal permeability (96), suggesting a communication mechanism between the skin and the gut in AD patients. There may be pathways in the gut of AD patients where AHR signaling regulates ILC homeostasis, and the details of the molecular mechanisms remain poorly understood.

AHR is also highly expressed on skin cells, especially in the stratum corneum, and can maintain skin homeostasis by regulating epidermal barrier protein genes (97). Diosmin is considered a potential AHR agonist from a natural product that restores the skin barrier of human keratin-forming cells by upregulating the AHR pathway to enhance the expression of skin barrier proteins such as filoproteins and loricrin and their upstream regulators (98). In addition, coal tar, an ancient topical treatment for dermatological disorders, induces keratin-forming cell-derived antimicrobial peptides by activating the AHR signaling pathway, which is beneficial in restoring the damaged skin barrier in AD patients (99). Tapinarof, a natural activator of AHR, has been considered safe and effective in clinical trials to improve symptoms in AD patients (100, 101). *Malassezia* generates cultured metabolites as AHR ligands and may activate the AHR pathway, causing aberrant keratinization and scaling frequently observed in dermatological conditions (97). *Malassezia* is known to be one of the most common fungi associated with AD (102), indicating that there may be a mechanism for *Malassezia* activation of AHR signaling in AD involved in skin barrier defects in patients.

Diosgenin, coal tar, and tapinarof have all been shown to alleviate skin lesions in AD patients, while *Malassezia* metabolites have been shown to worsen the skin barrier by stimulating the AHR pathway. Clarifying the cell-intrinsic function of AHR in ILCs is crucial to develop a potential therapeutic strategy for AD, given that AHR and ILCs are closely linked and affect how AD develops.

5.3 The ILC2–KLRG1–E-cadherin axis

The killer cell lectin-like receptor G1 (KLRG1) is an inhibitory receptor belonging to the C-type lectin family, mainly expressed in NK cells and T cells, and its main ligands are E-cadherin and N-cadherin (103). KLRG1 engagement inhibits protein kinase B (AKT) phosphorylation, leading to proliferative dysfunction of T cells and NK cells (104). Alkon et al. showed that most ILCs in the skin lesions of AD patients belonged to the $CRTH2^+$ ILC2 subgroup (41). ILC2s were enriched in the skin of AD acute lesions, and KLRG1 expression on these cells was markedly increased compared with ILC2s in healthy and unaffected skin (58). Also, KLRG1 expression was further upregulated by IL-33 or TSLP as activators of ILC2s (58), supporting the connection between the function of ILC2s and KLRG1 expression. It was shown that activated skin-resident ILC2s express high levels of KLRG1, which significantly inhibit the function of ILC2s upon interaction with E-cadherin, as evidenced by the downregulation of the expression of GATA3, as well as reduced production of IL-13, IL-5, and AREG (58). This indicated that downregulation of E-cadherin may interrupt this inhibitory signal, prompting ILC2s to release more type 2 cytokines through this new barrier-sensing mechanism and even unrestricted ILC2 proliferation and cytokine expression (Figure 3).

E-cadherin, as one of the important ligands of KLRG1, is a central adhesion molecule widely found in normal epithelial cells, keratinocytes, and Langerhans cells and is pivotal for maintaining epithelial cell integrity (105). E-cadherin has been reported to be reduced in damaged skin of individuals with AD disease (58, 106), indicating that the absence of this epidermal linker protein may enhance the generation of more type 2 cytokines by ILC2s in AD. After shRNA knockdown of the FLG gene, human keratin-forming cells produce less E-cadherin, demonstrating that FLG gene

abnormalities may be the reason for the decreased expression of E-cadherin in lesional skin of AD patients (58).

Furthermore, granzyme B (GzmB) abnormalities are an important factor in the decreased expression of E-cadherin in patients with AD. GzmB is a serine protease that cleaves E-cadherin, a key mediator of skin injury, inflammation, and repair (107, 108). Plasma GzmB concentrations were significantly higher in AD patients than in healthy controls and positively correlated with pruritus and dermatitis severity (109). In contrast to non-lesional AD and healthy skin, Turner et al. showed that cell-specific GzmB immunological positivity was enhanced in the lesional AD dermis and expressed primarily by mast cells (107). *GzmB* $^{-/-}$ mice exhibited fewer mast cells, less severe dermatitis, and better skin barrier function compared with wild controls in an oxazolone (OXA)-induced mouse dermatitis model (OXA was repeatedly applied as a hapten to the mouse ear to cause skin inflammation similar to that of human AD), indicating that GzmB may be a potential therapeutic target for AD (107). The findings further showed that E-cadherin was reduced in the epidermis of both *GzmB* $^{-/-}$ and WT mice with OXA dermatitis compared with control skin, and the reduction was more pronounced in WT mice compared to *GzmB* $^{-/-}$ mice (107). In addition, immunohistochemical results showed that E-cadherin in living human skin showed lower staining intensity with tissues incubated with GzmB, and preincubation of GzmB with VTI-1002, a potent and specific small-molecule inhibitor of GzmB, followed by exposure of *in-vitro* skin lessened the effect of GzmB on the detection of E-cadherin (107). The above experimental results suggest that high expression of GzmB in AD patients may lead to impaired barrier function in AD by cleaving E-cadherin.

The ILC2–KLRG1–E-cadherin axis is a novel skin barrier sensing mechanism that contributes to a fuller understanding of the pathogenesis of impaired skin barrier function in AD. Reducing the expression of GzmB and promoting the binding of E-cadherin to KLRG1 in AD patients may provide practical ideas for limiting the inflammation caused by ILC2s.

5.4 The PGD2–CRTH2–ILC2 pathway

Prostaglandin D2 (PGD2) is the predominant prostaglandin produced by activated mast cells. As reviewed by Honda et al., the skin of AD patients produces several prostaglandins, including PGD2 (110). Inagaki et al. reported that urinary levels of PGD2 metabolites in children AD patients were essentially the same as in healthy control children, suggesting that PGD2 metabolites may not be a useful clinical indicator for assessing AD (111). Additionally, cyclooxygenase inhibitors were unsuccessful in alleviating the symptoms of AD, implying a weak association of prostaglandins with AD pathogenesis (110). However, prostaglandin receptors, as mediators of inflammation, have recently been found to play a crucial regulatory function in AD development. PGD2 has two central receptors, the D-prostanoid receptor (DP) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2), which exert opposite regulatory functions at different stages of skin inflammation (112).

On the one hand, PGD2–DP signaling reduces early skin inflammation by promoting vascular endothelial barrier formation and inhibiting skin DC migration to draining LNs (112). On the other hand, CRTH2 was determined to be expressed on human ILCs and is more critical in allergic inflammation (28). PGD2–CRTH2 signaling exerts a pro-inflammatory effect in the late stages of the disease by effectively activating type 2 immune cells and the activation of ILC2s (110, 112) (Figure 3). The recruitment response of ILC2s to tissues is enhanced following PGD2–CRTH2 pathway activation, and their expression of the IL-33 receptor (ST2) and IL-25 receptor subunit (IL-17RA) is upregulated, promoting the production of type 2 cytokines as well as other inflammatory cytokines (113, 114). The relationship between mast cells and ILC2s in AD (110) suggests that the PGD2–CRTH2–ILC2 axis controls Th2 cell-associated inflammatory responses (113–115). In analogy to PGD2, cysteinyl leukotrienes (CysLTs) are another lipid inflammatory mediator secreted by IgE-mediated activated mast cells that exert biological functions by binding to the G protein-coupled cysteinyl leukotriene receptor 1 (CysLT1) and CysLT2 (116).

ILC2s have been demonstrated to express functional CysLT1 in both humans and animals, and CysLT1 levels in ILC2s isolated from AD patients were noticeably higher than those in healthy control subjects at both the protein and mRNA levels (117, 118). *In-vitro* experiments showed that CysLTs enhanced the activation of human ILC2s by PGD2 and epithelial cytokines, promoted the migration and survival of ILC2s, and induced the secretion of type 2 cytokines by ILC2s (118). This study further revealed that CysLTs, endogenously synthesized by human-activated mast cells, also induced IL-5 and IL-13 production by ILC2s, which was considerably but only partially inhibited by CysLT1 receptor antagonists such as montelukast (118).

Golub et al. summarized that the Notch signaling pathway could be regarded as one of the key future strategic targets for regulating the immune response of ILCs to inflammation (119). The Notch pathway has been identified as an important feature driving the KLRG1^{+} ILC2 subtype and a dominant pathway downstream of the AHR during NCR^{+} ILC3 generation (119). The development of novel AD therapeutic approaches may benefit from further research on the upstream signals that stimulate Notch receptor protein upregulation, the molecular mechanisms that activate the AHR signaling pathway to inhibit the response of ILC2s, strategies to effectively reduce the expression of GzmB or decrease the degradation of E-cadherin in AD patients, and the role of antagonizing the effect of CRTH2 on ILC2s.

6 Perspectives

ILCs are gradually recognized as modulators of tissue homeostasis and inflammation and will undoubtedly become an emerging key factor in AD belonging to Th2-type allergic diseases. The data suggest the presence of ILCs in the normal skin of mice and humans, and their expression varies with the skin layer. ILCs accumulate in the skin of AD patients and AD mouse models, and their function is related to the degree of inflammation. Currently,

ILC2s are considered the critical subtype of pro-inflammatory ILCs in AD, contributing mainly through the secretion of many pro-inflammatory factors and crosstalk with other immune cells. As discussed above, the part of ILC3s in AD is poorly explored, and ILC3s have great potential in skin barrier function and tissue repair. In addition, ILCs are pivotal in regulating the balance between the skin surface and gut microbial bacteria in AD. The AHR signaling pathway, a critical point in holding the balance of ILC2s and ILC3s *in vivo*, can potentially become a new therapeutic target for AD.

In the last decade, numerous studies have revealed the critical role of ILCs in lung and intestinal inflammation, but the understanding of the biology of ILCs in the skin is only the "tip of the iceberg." ILCs in skin inflammation are carried out to provide a way worthy of exploration. The following needs further study: 1) Due to the absence of cell-specific surface markers and the limited reagents available, it is still difficult to accurately differentiate ILCs from T cells. 2) It is unclear how the kind and concentration of cytokines in the microenvironment relate to the activity of ILCs and whether ILCs secrete mixed cytokines like Th cells in AD. 3) Although the upstream activation signals of ILCs are known to be associated with signaling pathways in inflammatory diseases, the precise mechanisms by which they interact with other immune or non-immune cells remain to be explored in depth. 4) Since ILCs contribute to wound healing and infection resistance, it remains unclear whether treatment targeting ILC depletion disrupts the mucosal homeostasis of the patient and the relationship between the microbiota and ILCs of the skin and gut.

Strategies to address the above issues may focus on the following areas. 1) To determine cell-specific surface markers and the tissue distribution of each human ILC subpopulation, emerging technologies such as mass spectrometry, flow cytometry, and single-cell analysis methods could be used to analyze the proteomic, transcriptional, and genomic changes in ILCs (120). 2) It will be easier to induce and maintain ILCs *in vitro* with a better understanding of their origin and maintenance, enabling the execution of pertinent cellular experiments to further explore the relationship between ILCs and cytokines. 3) The rational application of dynamic *in-vivo* real-time imaging tools to study the trafficking mechanisms of ILCs in various AD mouse models will improve our understanding of the immune networks and signaling pathways associated with human diseases (120). 4) To explore the adverse effects of targeting depleted ILCs to treat AD patients, detailed information on the mechanisms of ILCs in the skin and intestinal mucosa of AD patients should be studied, which requires numerous animal experiments and clinical trials to gather necessary scientific evidence.

Collectively, although much evidence suggests that alterations in the phenotype and function of ILCs are inextricably linked to the development of AD, the picture of the role of ILCs in AD remains unclear. Understanding the biological and regulatory mechanisms in the epithelial immune barrier of ILCs will pose a significant research challenge in the future. The current review will provide insight into the pathogenesis of AD and may help develop safe and effective treatment strategies for patients with refractory AD.

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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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