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Community series in the role of CD1- and MR1-restricted T cells in immunity and disease, volume II

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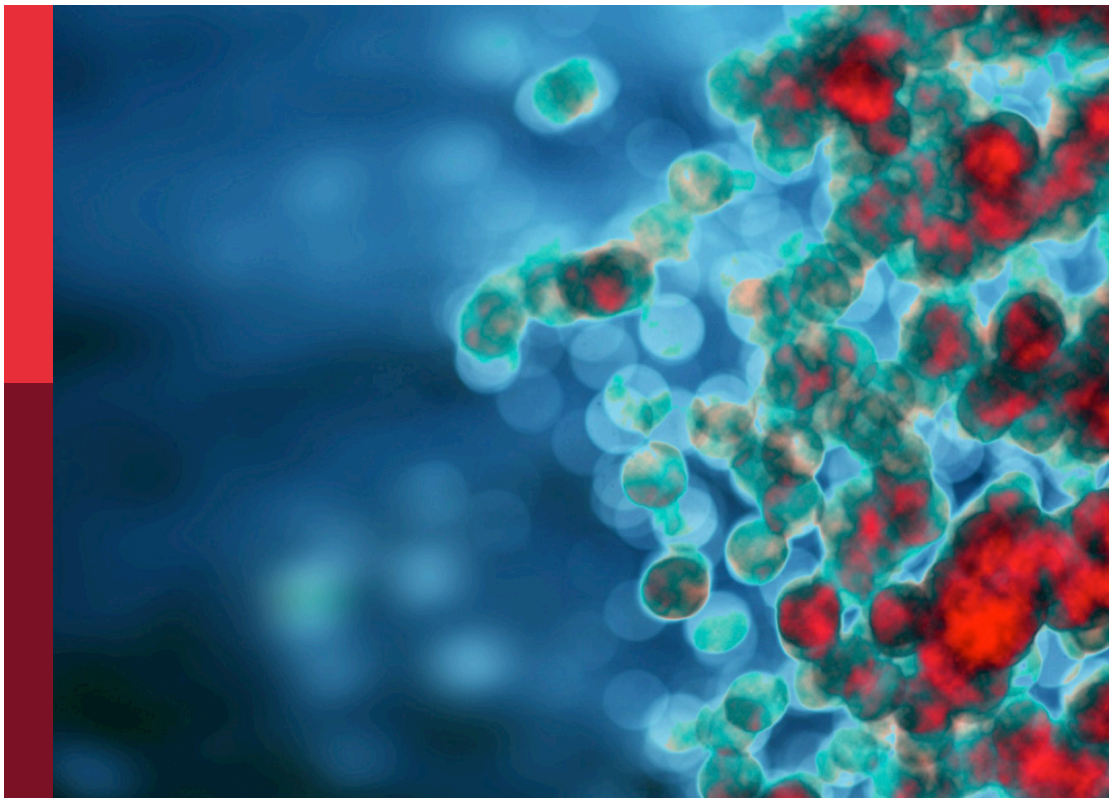
Published in
Frontiers in Immunology



ISSN 1664-8714
ISBN 978-2-8325-5483-8
DOI 10.3389/978-2-8325-5483-8

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Community series in the role of CD1- and MR1-restricted T cells in immunity and disease, volume II

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Citation

Van Kaer, L., Iwabuchi, K., eds. (2024). *Community series in the role of CD1- and MR1-restricted T cells in immunity and disease, volume II*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-5483-8

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frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 16 September 2024
DOI 10.3389/fimmu.2024.1490010

OPEN ACCESS

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RECEIVED 02 September 2024
ACCEPTED 04 September 2024
PUBLISHED 16 September 2024

CITATION
Iwabuchi K and Van Kaer L (2024) Editorial: Community series in the role of CD1- and MR1-restricted T cells in immunity and disease, volume II.
Front. Immunol. 15:1490010.
doi: 10.3389/fimmu.2024.1490010

Editorial: Community series in the role of CD1- and MR1-restricted T cells in immunity and disease, volume II

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KEYWORDS

CD1, MR1, natural killer T cells, mucosal-associated invariant T cells, nonpeptide antigens, immunity, immune-mediated disease, unconventional T cells

Editorial on the Research Topic
Community series in the role of CD1- and MR1-restricted T cells in immunity and disease, volume II

Cluster of differentiation 1 (CD1) and major histocompatibility complex (MHC)-related 1 (MR1) are MHC class I-related proteins that present non-peptidic antigens to subsets of T lymphocytes with innate-like features (1–6). Since publication of volume I of this Community Series, much progress has been made on the functions and therapeutic potential of CD1- and MR1-restricted T cells. Volume II includes five reviews, seven mini reviews, four original research articles, and one brief research article that provide examples of recent developments in this field.

CD1 has four isoforms, termed CD1a, -b, -c, and -d, that present lipid or glycolipid antigens to T cells (7). The primary research article by Szoke-Kovacs et al. provides new insight into the repertoire of lipids associated with these distinct CD1 isoforms, showing binding with a wide variety of lipids that closely mirror the cellular lipidome. Although all isoforms bind with a common set of lipids, each isoform also contains some unique lipid species, suggesting divergent roles in antigen presentation and immune responses. These findings should inform future studies on the capacity of self-lipids to activate CD1-restricted T cells, with potential therapeutic implications.

Many articles contained in volume II focus on CD1d, which presents glycolipid antigens to natural killer T (NKT) cells, most of which express semi-invariant T cell receptors (TCRs) and are thus called invariant NKT (iNKT) cells (5, 6, 8). The mini review by Hayashizaki et al. discusses the remarkable diversity of glycolipid antigens recognized by iNKT cells. They examine the molecular mechanisms by which the TCRs of iNKT cells can react with such a diverse set of glycolipids, how iNKT cells can generate nuanced immune responses against these antigens, and how the information gleaned from these studies can be employed for developing vaccines to augment cellular and humoral immune responses against microbial pathogens. While iNKT cells share their developmental origins with conventional MHC-restricted T cells, they require unique transcriptional programs that are critical for iNKT cell lineage commitment, acquisition of innate-like features, and

differentiation of distinct iNKT effector cell subsets (9). The primary research article by Guo et al. explores the mechanisms by which the epigenetic modifier polycomb repressive complex 2 (PRC2) modulates iNKT cell development, employing mice conditionally deficient for the core subunit of PRC2, termed embryonic ectoderm development (Eed), within the T cell lineage. These animals display various iNKT cell alterations, including reduced numbers, disrupted differentiation, increased cell death, a bias towards interleukin (IL)-4 cytokine production, and alterations in the expression of lineage-specific transcription factors. Remarkably, these animals are exquisitely sensitive to iNKT cell-mediated liver injury induced by acetaminophen, highlighting the critical impact of epigenetic regulators on iNKT cell effector cell differentiation and organ-specific disease. The review by Cui et al. discusses the heterogeneity of iNKT cells within different tissues. They review the intra- and extra-thymic mechanisms that result in the generation of distinct iNKT effector cell subsets and their heterogeneity within distinct tissues, such as the thymus, lung, liver, intestine and adipose tissue. They call attention to a circulating NKT cell subset that depends on IL-15 production by thymic epithelial cells for its development. They further explore the relevance of this heterogeneity in iNKT effector cell subsets to the development of cancer immunotherapies.

A number of articles focus on the role of iNKT cells in disease and the development of iNKT cell-based immunotherapies. The review by Rakjashekar et al. discusses the contribution of iNKT cells to protective immunity against viruses, which do not express enzymes for lipid synthesis but can activate iNKT cells by inducing cytokines and/or agonistic self-lipid antigens. They discuss the role of iNKT cells to protective immunity against important human viral infections and the mechanisms employed by some viruses to downregulate CD1d expression and evade iNKT cell activation. These findings are discussed in the context of prophylactic and therapeutic approaches for viral infections. The review by Kumar et al. focuses on the role of iNKT cells in the development of tissue fibrosis during chronic tissue inflammation, including idiopathic pulmonary fibrosis. They review human and mouse studies implicating iNKT cells in the initiation and progression of inflammatory cascades involving neutrophils, macrophages and fibroblasts that ultimately lead to fibrosis development. They conclude that blocking iNKT cell activation may provide a means to prevent fibrosis development. The mini review by Lee et al. discusses the role of CD1d and iNKT cells to the maintenance of intestinal health and the development of gastrointestinal inflammation and disease. They review studies providing evidence for protective or pathogenic roles of iNKT cells in the development of inflammatory bowel disease, the protective effects of glycolipid-mediated iNKT cell activation on murine colitis, and the contribution of intrinsic signaling by CD1d to these activities. It is concluded that NKT cells are promising targets for designing immune therapies for inflammatory bowel disease. The mini review by Satoh and Iwabuchi examines the contribution of NKT cells to obesity-associated tissue inflammation and its metabolic consequences. This type of research has been associated with divergent results obtained by different research groups, likely due to differences in experimental procedures, animal models, and intestinal microbiota of animal colonies (10). The authors discuss the complex interactions of distinct NKT cell subsets with a diverse set of CD1d-expressing cell types in the adipose tissue, and highlight the potential contribution of intrinsic CD1d signaling to the metabolic alterations observed. The primary research article by Yamasaki et al. explores the adjuvant properties of iNKT cells in licensing dendritic cells for antigen presentation to naïve, conventional MHC-restricted T cells. These investigators introduced prostate cancer antigens, together with mRNA for CD1d, into allogeneic cells, and then loaded these cells with the prototypical iNKT cell antigen α-galactosylceramide (α-GalCer), before injecting them into recipient animals that were then challenged with prostate cancer cells. Not only did they find efficient CD8 T cell priming against the introduced (primary) tumor antigens, but also against secondary antigens, via epitope spreading. These CD8 T cell responses displayed both prophylactic and therapeutic activities against prostate tumor progression. The primary research article by Hoo et al. explores the capacity of α-GalCer to modulate the induction of experimental sepsis in mice. They found that treatment of mice with α-GalCer one week prior to sepsis induction attenuates lethality, in a manner involving iNKT cell polarization towards IL-4 and IL-10 production, and expansion of IL-10-producing B cells. From these findings it is concluded that α-GalCer holds promise for immune prophylaxis of sepsis. The mini review by Takami and Motohashi examines the therapeutic potential of adoptively transferred iNKT cells against cancer. Since CD1d is largely monomorphic, both autologous and allogeneic sources of iNKT cells have been explored. The authors review the clinical trials that have employed such approaches, including iNKT cells modified with chimeric antigen receptors (CARs).

MR1 presents vitamin B metabolites and other small molecules to MR1-restricted T cells, most of which express semi-invariant TCRs and are called mucosal-associated invariant T (MAIT) cells (5, 6, 11–13). The mini review by Ito and Yamasaki discusses the regulation of MAIT cells by host-derived rather than microbial antigens. They focus on recent findings that bile acid-derived metabolites can bind MR1 and activate MAIT cells, which may influence their preferential localization and/or maintenance in the liver. Similar mechanisms might be at play to recruit and/or maintain MAIT cells in other tissue locations where they are prevalent. The mini review by Fukui et al. focuses on the functions of MAIT cells in eye diseases. The authors discuss the protective role of MAIT cells in the development of autoimmune uveitis, in a mechanism that likely involves TCR engagement. Since cognate antigen is able to ameliorate clinical symptoms and visual function, the authors conclude that MAIT cell antigens have therapeutic potential in autoimmune uveitis and possibly other inflammatory eye diseases. Yigit et al. review the potential utility of MAIT cells in cancer immunotherapy. They discuss the complex interactions of MAIT cells with the cancer microenvironment, and how these cells can display both tumor-promoting and -suppressing activities. Nevertheless, these cells hold great promise for the development of cancer immunotherapies, for example by programming them with CARs, with the potential of employing allogeneic MAIT cells as off-the-shelf cell therapies against cancer.

A few of the articles included in this Research Topic cover responses mediated by both CD1d- and MR1-restricted αβ T cells, as well as by another subset of unconventional T cells, γδ T cells, which

react with a variety of different ligands, including non-peptidic ones (14, 15). The brief research report by N'guessan et al. explores the potential association of human immunodeficiency virus (HIV)-1 vaccine efficacy with the activation of innate immune cells and unconventional T cells. Using human subjects from the Thai phase III HIV-1 vaccine trial (RV144), these investigators found increased iNKT, MAIT and γδ T cell activation following vaccine administration, suggesting that these cells may contribute to vaccine-induced humoral responses. The review paper by Lv et al. discusses the role of unconventional T cells in the central nervous system. They explore the contribution of iNKT, MAIT and γδ T cells in maintaining brain homeostasis, their roles during the development of acute (e.g., stroke and traumatic brain injury) and chronic (e.g., multiple sclerosis) brain injury, and their involvement in the development of neurodegenerative diseases such Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis. The mini review by Liu et al. examines the role of innate-like T cells, including NKT, MAIT and γδ T cells, in the pathogenesis of necrotizing enterocolitis (NEC), a life-threatening gastrointestinal disease associated with bacterial invasion that primarily affects preterm babies. They argue that accumulation of NKT and MAIT cells in preterm babies, possibly mediated by microbial dysbiosis, may contribute to NEC development. Thus, blocking the activation of these cells may provide an immunotherapeutic approach to NEC.

In summary, the articles contained in volume II of this Community Series provide a snapshot of current research on the biology of CD1- and MR1-restricted T cells. We hope this work will spur additional research that will ultimately contribute to the development of new immunotherapies.

Author contributions

KI: Writing – review & editing. LVK: Writing – review & editing, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. Work in the authors' labs was supported by the Japan Society for Promotion of Science (to KI), the Japan Agency for Medical Research and Development (to KI), the Japan Ministry of Education, Culture, Sports, Science and Technology (to KI), the National Institutes of Health (to LVK), and the American Heart Association (to LVK).

Acknowledgments

We are grateful to all colleagues who contributed and/or reviewed manuscripts for volume II of this Community Series.

Conflict of interest

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

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

References

1. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nat Immunol.* (2015) 16:1114–23. doi: 10.1038/ni.3298

2. Legoux F, Salou M, Lantz O. Unconventional or preset αβ T cells: evolutionarily conserved tissue-resident T cells recognizing nonpeptidic ligands. *Annu Rev Cell Dev Biol.* (2017) 33:511–35. doi: 10.1146/annurev-cellbio-100616-060725

3. Mayassi T, Barreiro LB, Rossjohn J, Jabri B. A multilayered immune system through the lens of unconventional T cells. *Nature.* (2021) 595:501–10. doi: 10.1038/s41586-021-03578-0

4. Van Kaer L, Postoak JL, Song W, Wu L. Innate and innate-like effector lymphocytes in health and disease. *J Immunol.* (2022) 209:199–207. doi: 10.4049/jimmunol.2200074

5. Mori L, Lepore M, De Libero G. The immunology of CD1- and MR1-restricted T cells. *Annu Rev Immunol.* (2016) 34:479–510. doi: 10.1146/annurev-immunol-032414-112008

6. Salio M, Silk JD, Jones EY, Cerundolo V. Biology of CD1- and MR1-restricted T cells. *Annu Rev Immunol.* (2014) 32:323–66. doi: 10.1146/annurev-immunol-032713-120243

7. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annu Rev Immunol.* (2004) 22:817–90. doi: 10.1146/annurev.immunol.22.012703.104608

8. Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nat Rev Immunol.* (2004) 4:231–7. doi: 10.1038/nri1309

9. Pellicci DG, Koay HF, Berzins SP. Thymic development of unconventional T cells: how NKT cells, MAIT cells and γδ T cells emerge. *Nat Rev Immunol.* (2020) 20:756–70. doi: 10.1038/s41577-020-0345-y

10. Wu L, Van Kaer L. Contribution of lipid-reactive natural killer T cells to obesity-associated inflammation and insulin resistance. *Adipocyte.* (2013) 2:12–6. doi: 10.4161/adip.22296

11. Godfrey DI, Koay HF, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. *Nat Immunol.* (2019) 20:1110–28. doi: 10.1038/s41590-019-0444-8

12. Toubal A, Nel I, Lotersztajn S, Lehuen A. Mucosal-associated invariant T cells and disease. *Nat Rev Immunol.* (2019) 19:643–57. doi: 10.1038/s41577-019-0191-y

13. Provine NM, Klenerman P. MAIT cells in health and disease. *Annu Rev Immunol.* (2020) 38:203–28. doi: 10.1146/annurev-immunol-080719-015428

14. Chien YH, Meyer C, Bonneville M. γδ T cells: first line of defense and beyond. *Annu Rev Immunol.* (2014) 32:121–55. doi: 10.1146/annurev-immunol-032713-120216

15. Ribot JC, Lopes N, Silva-Santos B. γδ T cells in tissue physiology and surveillance. *Nat Rev Immunol.* (2021) 21:221–32. doi: 10.1038/s41577-020-00452-4

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TYPE Review
PUBLISHED 25 September 2023
DOI 10.3389/fimmu.2023.1260503

OPEN ACCESS

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RECEIVED 17 July 2023
ACCEPTED 06 September 2023
PUBLISHED 25 September 2023

CITATION
Kumar V, Hertz M, Agro A and Byrne AJ (2023) Type 1 invariant natural killer T cells in chronic inflammation and tissue fibrosis. *Front. Immunol.* 14:1260503. doi: 10.3389/fimmu.2023.1260503

Type 1 invariant natural killer T cells in chronic inflammation and tissue fibrosis

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Chronic tissue inflammation often results in fibrosis characterized by the accumulation of extracellular matrix components remodeling normal tissue architecture and function. Recent studies have suggested common immune mechanisms despite the complexity of the interactions between tissue-specific fibroblasts, macrophages, and distinct immune cell populations that mediate fibrosis in various tissues. Natural killer T (NKT) cells recognizing lipid antigens bound to CD1d molecules have been shown to play an important role in chronic inflammation and fibrosis. Here we review recent data in both experimental models and in humans that suggest a key role of type 1 invariant NKT (iNKT) cell activation in the progression of inflammatory cascades leading to recruitment of neutrophils and activation of the inflammasome, macrophages, fibroblasts, and, ultimately, fibrosis. Emerging evidence suggests that iNKT-associated mechanisms contribute to type 1, type 2 and type 3 immune pathways mediating tissue fibrosis, including idiopathic pulmonary fibrosis (IPF). Thus, targeting a pathway upstream of these immune mechanisms, such as the inhibition of iNKT activation, may be important in modulating various fibrotic conditions.

KEYWORDS

CD1d, NKT cells, fibrosis, macrophages, fibroblasts, IPF, NASH

Introduction

Fibrosis is characterized by the deposition of extracellular matrix components, such as collagen and fibronectin. The fibrotic process can affect different tissues and represents an ultimate outcome of chronic inflammatory diseases (1). Although collagen deposition can be a reversible repair process in response to injury, repetitive insult and chronic inflammation can lead to a dysregulation of the wound-healing response and progression to fibrosis (1–4). The fibrotic niche is comprised of several immune cells that drive key steps in the fibrotic process, including neutrophils, mast cells, macrophages, fibroblasts, and NKT cells (5). Here we review some of the recent findings on how a particular NKT cell subset, iNKT cells, play a key role in chronic inflammation and fibrosis

in both lung and liver diseases. Although fibrosis is also a major feature of several chronic inflammatory diseases, here we will primarily focus on recent data supporting a key role of iNKT cell activation in the progression of fibrosis in IPF and non-alcoholic steatohepatitis (NASH). IPF is an interstitial lung disease characterized by progressive lung scarring with a median survival of 2-3 years after diagnosis (6). NASH is a leading cause of global liver disease fueled by increasing rates of obesity, diabetes, and metabolic syndrome and is characterized by inflammation, injury, and accumulation of fat in the liver (7). Currently, no therapeutic solution exists that halt disease progression of IPF or NASH and a better understanding of the cellular and molecular mechanisms involved in progressive fibrotic disease is necessary for the development of new therapeutic interventions. We will briefly review different NKT cell subsets, important parameters to consider when characterizing NKT cells in health and disease and highlight some of the key pathways that are impacted by iNKT activation leading to fibrosis. Finally, we will propose a model of complex cellular interactions of different iNKT subsets secreting type 1, type 2 and type 3 cytokines impacting TGF-β-associated macrophage activation, neutrophil infiltration, fibroblast transformation and fibrosis.

Two distinct subsets of CD1d-restricted NKT cells with opposite effects in chronic inflammation

NKT cells are a group of innate-like T cells expressing NK cell receptors and antigen T cell receptors (TCR) that recognize lipid antigens presented in the context of a class I MHC-like, non-polymorphic molecule CD1d (8–10). NKT cells become rapidly activated following either TCR recognition of the CD1d-bound lipid antigen or following inflammatory cytokine-associated signaling and produce large amounts of type 1, type 2 or type 3 cytokines. Accordingly, NKT1 predominantly secrete GM-CSF and IFN-γ, NKT2 secrete IL-4, IL-5, and IL-13, and NKT17 secrete IL-17A and IL-22. NKT cells have diverse functions bridging adaptive and innate immune responses. NKT cells are comprised of two main subsets, iNKT cells and type 2 NKT cells (10–12). While both NKT cell subsets are predominantly NK1.1$^+$ (mouse) or CD161$^+$/CD56$^+$ (human) and share common features in mice and in humans (13–15), they can be differentiated by their TCR alpha and beta-chain usage and antigen recognition. Since not all murine strains express the NK1.1 marker, and given its surface expression is modulated following iNKT activation, NK1.1 expression alone is not a good marker for NKT cell identification (see Box 1). iNKT cells mostly express semi-invariant germ line encoded TCR α-chain (75-88%), Vα14/Jα18 in mice and Vα24/Jα18 in humans, paired with a more diverse non-germ line Vβ genes, Vβ8.2, Vβ7 or Vβ2 in mice and Vβ11 in humans, and can be identified using α-galactosyl ceramide (αGalCer)-loaded CD1d-tetramers or a clonotypic TCR-specific mAb. In contrast, type 2 NKT cells express a relatively diverse TCR repertoire. Type 2 NKT cells are not reactive to αGalCer and a major subset recognizes a self-glycolipid 3-sulfated β-galactosyl ceramide (sulfatide) (16, 17). iNKT and type 2 NKT cells also display distinct modes of antigen recognition (18, 19). More importantly, these subsets can have opposite immune functions. iNKT cells promote inflammatory disease while type 2 NKT cells are protective in several chronic inflammatory diseases (20, 21). A protective role of appropriately activated type 2 NKT cells in inflammatory and autoimmune disease has recently been reviewed (22–24) and will not be included here.

iNKT cell subsets (NKT1, NKT2 and NKT17) with different cytokine profiles and functions

Based on the expression of transcription factors and cytokines, mouse and human iNKT cells can be divided into NKT1, NKT2 and NKT17 subsets, similar to CD4$^+$ helper T subsets, and accordingly

BOX 1 Challenges in characterizing iNKT cells.

•NKT cells can be categorized into two subsets based upon whether they express a semi-invariant or a diverse TCR. Murine and human iNKT cells, but not type 2 NKT cells, recognize the foreign marine sponge-derived glycolipid, α-galactosylceramide (αGalCer), and αGalCer/CD1d-tetramers can be used for the monitoring iNKT cells in both mice and humans (8, 32). Murine type 2 NKT cells, but not iNKT cells, recognize the self-glycolipid, 3-sulfated β-galactosyl ceramide (sulfatide), and the self-lysophospholipid, lysophosphatidylcholine (LPC) (16, 33) and sulfatide/CD1d-tetramers can be used for the monitoring of type 2 NKT cells in mice.

•αGalCer is a high affinity binder for CD1d and TCR and acts like a super antigen for iNKT cells that can activate most iNKT cells in a non-physiological manner. In contrast, self-lipids recognized by iNKT cells, are generally low affinity binders for CD1d or TCR, do not behave like αGalCer (34). αGalCer activation of iNKT cells may skew iNKT responses from a predominantly pathogenic Th1 response to a predominantly protective Th2 repsonse (35–38). Data using αGalCer should be cautiously interpreted regarding the physiological role of iNKT cells.

•A number of studies in humans have used CD3$^+$CD56$^+$ cells as a marker for NKT cells. It is clear from several studies that CD3$^+$CD56$^+$ cells are comprised of population of unconventional T cells, including iNKT cells, MAIT cells, γδ T cells, type 2 NKT cells and a subset of CD8$^+$ T cells (39). Data reporting CD3$^+$CD56$^+$ cells as NKT cells should be interpreted with this in mind.

•It is also important to use tetramer staining in combination with intracytoplasmic cytokine, chemokine staining, or transcription profiling to determine the function of iNKT cells. Absolute iNKT cell number alone, or cell surface expression of TCR, may not provide a complete picture of the specific *in vivo* role of iNKT cells subsets. At different times during a chronic inflammation different iNKT cell subset may be activated that have distinct roles in the inflammatory cascade (40, 41).

•Chronically activated iNKT cells downregulate their TCR surface expression, thus, reduced cell surface staining may not accurately reflect the absolute number of iNKT cells. A qPCR approach specific for the conserved VJ-region of the invariant TCR, in combination with the tetramer staining, may more accurately reveal their frequency as well as their state of activation (40).

•It should be emphasized that iNKT cells may have a protective role during initial acute inflammation (42–46) whereas chronic activation of iNKT cells may induce a more pathogenic role. Therefore, murine models that are generally acute in nature may not accurately reflect the role of iNKT cells in human chronic conditions such as lung or liver fibrosis.

affect distinct type 1, type 2 and type 3 immune pathways, respectively (25–28). NKT1 express T-bet and predominantly secrete GM-CSF and IFN-γ, NKT2 express GATA3, PLZF^{high} and secrete IL-4, IL-5, and IL-13, and NKT17 express RORγt and secrete IL-17A and IL-22 (25–30). It is clear that their innate-like features, including rapid response in hours, place them at the frontline of responses against infection, tumor, and tissue injury. Generally, iNKT cells are tissue-resident and enriched in peripheral tissues, such as lung and liver, and play an important role in tissue homeostasis and immunity (25, 26). Recently, another subset of iNKT cells has been identified in both mouse and in humans referred to as circulating iNKT cells that are distinct developmentally from the tissue-resident iNKT cells (31). These cells are circulating and are CD244$^+$CXCR6$^+$, whereas tissue resident cells are CD244$^-$CXCR6$^+$. It is interesting that tissue-resident iNKT cells are preferentially localized in lung parenchyma, as opposed to the blood vessels where most circulating iNKT cells as well as other lymphocytes typically reside (31). iNKT cell subsets with circulating properties can perhaps sense tissue injuries from chronic inflammation and orchestrate systemic immunity through the activation of a downstream cascade of other immune cells. Thus, circulating and tissue-resident iNKT cell subsets are analogous to the group 1 innate lymphoid cells (ILCs) with tissue resident ILCs and conventional circulating NK cells, and together they provide diverse systemic and tissue-specific immune regulation.

iNKT cell activation is a key upstream event promoting chronic inflammation and fibrosis

In several murine models of chronic inflammation and fibrosis, as well as in humans, it has been shown that iNKT cells are selectively activated and play a pathogenic role. For example, in pulmonary fibrosis, carbon tetrachloride (CCL4)-induced fibrosis, ischemia reperfusion injury, Con A-induced hepatitis, primary biliary cirrhosis, Lieber-DeCarli liquid alcohol diet, and choline-deficient amino acid enriched (CDAA) diet, iNKT cells become activated and are pathogenic (18–21, 41, 47, 48). In some tissues, iNKT cells accumulate significantly following injury (41, 47, 49) and the accumulation is dependent on chemokine receptor CXCR6 interactions (50). Thus, in Jα18$^{-/-}$ mice genetically deficient in iNKT cells, but not type 2 NKT cells, both inflammation and fibrosis are significantly ameliorated (41, 47, 51). Consistent with chronic activation, iNKT cells express FAS/FASL and downregulate or internalize their TCR surface expression as shown by reduced staining with CD1d-tetramers (52–54). It is important to mention that the activation of iNKT cells in tissue may be dependent on the presence of CD1d in parenchymal cells in lung, kidney, skin, and heart tissues commonly affected by fibrosis. For example, it has been shown that the CD1d expression in hepatocytes is necessary for the iNKT activation during inflammatory liver injury (55). It is likely that CD1d expression in tissue-resident cells, such as hepatocytes, is needed for either *in situ* lipid presentation or that CD1d recognition locally is required for full activation or maintenance of iNKT cells in chronic inflammatory conditions. Consistent with the experimental data, iNKT cells are chronically activated and secrete significantly higher levels of proinflammatory cytokines in NASH, severe alcoholic hepatitis, lupus nephritis and IPF patients in comparison to healthy volunteers (23, 40, 56). Importantly, the increase of IFN-γ$^+$iNKT cells correlates with the progression of NAFLD Activity Score (NAS) and fibrosis staging in patients (40, 56). These data are consistent with earlier reports in NASH patients that quantified T cell markers (CD56$^+$CD3$^+$) that are not specific for NKT cells and may include other T cell populations as described in Box 1 (51). Another important finding during fibrosis is a significant increase in adaptive T cells, such as CD8$^+$ T cells in both mice (40, 57) and in humans (58–60). Accordingly, inhibition of iNKT cells results in a decrease of infiltrating CD8$^+$ T cells into liver tissue (40, 41, 57). It is also important to mention that genetic deficiency or inhibition of iNKT cells also leads to significantly reduced macrophages, conventional T cell accumulation and activation, as well as a reduction in the levels of key inflammatory and pro-fibrogenic cytokines including IL-1β, IL-6, and TNFα in fibrotic tissues in mice (23, 40, 41, 47). Among other cells in the fibrotic niche, stellate cell activation has also been shown to be associated with iNKT cell activation (61). Consistent with a critical pathogenic role of iNKT cells, specific inhibition of iNKT cells with a clinically relevant RARβγ agonist, tazarotene, significantly protects mice from hepatic fibrosis (23, 40, 41, 57). One of the key features that provides tazarotene specificity for iNKT cells, and not type 2 NKT cells or conventional T cells, is the significant upregulation of RARγ receptors in iNKT cells compared to these other T cell populations (41).

Similarly, in the bleomycin-induced lung injury model of pulmonary fibrosis, inhibition or skewed activation of iNKT cells by sulfatide or αGalCer, respectively, leads to inhibition of vimentin concentration in lung tissues as well as reduction of fibrosis-promoting cytokines, including TGF-β, IL-5, and IL-13 (48, 49, 62). Inhibition of iNKT cells also results in dampening of arginase+ alveolar macrophages involved in fibrosis (48, 49, 62). Furthermore, manipulation of αGalCer-mediated iNKT-STAAT1-CXCL9 axis has been shown to contribute to vessel fibrosis in pulmonary hypertension caused by lung fibrosis (63). Thus IFN-γ secretion following αGalCer administration inhibits type 2 cytokine-mediated fibrotic responses. The bleomycin-induced model of lung fibrosis used as a model of IPF recapitulates several features but does not accurately replicate the human resolving disease, IPF. For example, the bleomycin model can be induced in recombination-activating gene (RAG) deficient mice that lack mature B and T cells (49) and therefore does not involve a full spectrum of chronic inflammatory components like that observed in the progression of IPF (2, 64–66). Nevertheless, in this model manipulation of the iNKT cytokine response with the super antigen-like ligand αGalCer or following type 2 NKT activation via sulfatide results in the inhibition of TGF-β dependent pathway, blunting fibrosis (48, 49, 67). Consistent with the iNKT involvement in experimental model of lung fibrosis, a significantly increased frequency of IFN-γ$^+$NKT cells in bronchial alveolar lavage (BAL) fluid relative to healthy volunteers also correlates with increased alveolar macrophages, a predictive

marker of disease progression in IPF patients. Collectively, data in experimental models as well as in NASH, alcoholic liver disease, and IPF patients implicate iNKT cells in the pathogenesis of fibrosis in humans.

iNKT cell subsets contribute to type 1, type 2 and type 3 cytokine pathways promoting fibrosis

Chronic inflammation plays a key role in promoting tissue fibrosis. Several studies have shown that inflammation associated with fibrosis in both lung and in liver tissues is comprised of a mixture of type 1, type 2 and type 3 cytokine-associated immune pathways (1, 2, 5, 65, 66, 68). Although not clearly understood, several immune cells likely participate or are influenced by the dynamic secretion of these cytokines in the fibrotic tissues eventually leading to macrophage activation and fibroblast transformation. The differential cytokines secreted by iNKT cell subsets can influence type 1 (GM-CSF and IFN-γ), type 2 (IL-4, IL-5, IL-13) and type 3 (IL-17A and IL-22) immune responses (69). The three defined iNKT cell subsets (NKT1, NKT2, NKT17) are present in chronic inflammatory tissues, including the lung and liver. It is likely that with the progression of fibrosis, one or more iNKT cell subset(s) become dominant: for example, NKT17 becomes less prevalent in comparison to NKT1 and NKT 2 subsets with the progression of experimental hepatic fibrosis (40). Thus, cytokines secreted by iNKT subsets can contribute to different pathways of tissue fibrosis at different stages of disease. For example, IFN-γ secreting pulmonary NKT1 cell activation exacerbates experimental lung injury (70). Type 2 cytokines, such as, IL-4, IL-5 and IL-13 are associated with both innate and adaptive immune cells, including NKT2, Th2, ILC2, eosinophils, and IL-13 activated macrophages that are normally involved in tissue repair (2). However, dysregulation or overt activation of the repair process contributes to infection and allergen-driven fibrosis in several organs, including the lung (71–73). In chronic inflammatory lung disease, pathogenic IL-13 producing macrophages are stimulated by a CD1d-dependent iNKT cell interaction both in experimental models as well as in chronic obstructive pulmonary disease (COPD) patients (62). Consistently, in IPF patients, high levels of IL-13 and IL-13R have been found in both blood and in the lungs (2, 74, 75). In a humanized severe combined immunodeficiency (SCID) mouse model of IPF, humanized anti-IL-13 antibody treatment results in a significant reduction in fibrosis and increased epithelial repair (76). Additionally, humanized monoclonal anti-IL-13 antibody treatment has shown some promise in a subset of severe asthma patients (77) but has failed to meet primary efficacy endpoints in IPF clinical trials (78, 79). It is important to emphasize that IL-13-driven fibrosis is both TGF-β-dependent (2, 72, 80) and TGF-β independent (81). Thus, IL-4 and IL-13 cytokines can activate arginase+ macrophages and fibroblasts driving the progression of fibrosis (2). Importantly, type 2 cytokine driven fibroblast activation is not only important but also necessary for the development of hepatic fibrosis (82). Thus, type 2 cytokines in a TGF-β-dependent and independent fashion can have significant impact on two key cell types, fibroblasts, and macrophages, driving the progression of tissue fibrosis. Additionally, chronic activation of iNKT cells secreting IL-4 and IL-5 cytokines results in lung disease where animals develop COPD-like symptoms including increased mucus, fibrosis, and emphysema (83). Notably, patients with COPD also have increased number of iNKT cells in PBMCs (84). Furthermore, OVA-induced airway hypersensitivity is significantly abrogated in iNKT cell deficient $J\alpha18^{-/-}$ mice with decreases in IL-4, IL-5, and IL-13 cytokines in BAL fluid (85). NKT2-mediated IL-4 signaling has also been shown to promote macrophage activation and fibroblast to myofibroblast transition in renal fibrosis (86). As detailed below, tissue resident NKT17 cells play a key role in neutrophil infiltration into injured tissues, and can subsequently influence other cells, such as macrophages, γδ T cells and conventional Th17 responses. Additionally, IL-17A produced by iNKT cells has been shown to promote liver fibrosis in patients with primary biliary cholangitis (87). Collectively, these data suggest that iNKT cells play an important role in influencing all three cytokine associated pathways involved in tissue fibrosis, including IPF.

iNKT-associated signaling is important for neutrophil infiltration into tissue and fibrosis

One of the hallmarks of inflammatory diseases leading to fibrosis is the infiltration of neutrophils into tissues that are critical regulators of both adaptive and innate immunity (88). We found that neutrophil accumulation into fibrotic liver tissue is dependent on the activation of iNKT cells (40, 41, 47). This is due to the inhibition of the upregulation of several cytokines and chemokines, including MIP-1, MIP-2, IL-6, and osteopontin that are involved in the neutrophil infiltration into tissues following injury. Notably, in non-inflamed lung NKT17 is predominant, whereas NKT1 predominates in the liver of naive mice (25, 89). However, following chronic inflammation and fibrosis, NKT17 replaces NKT1 as the dominant subset, and ultimately as the disease progresses NKT1 and NKT2 subsets dominate in mice as well as in humans (40, 90). In comparison to other lymphocytes, iNKT cells are also abundantly present in the lung vasculature and the interstitial tissue of both mice and humans (25, 91). Similar to circulating iNKT cells, NKT17 predominate within the interstitial tissues, whereas NKT1 and NKT2 are predominantly present in the vasculature. Injury or infections, such as streptococcus preumoniae, has been shown to induce expansion and vascular extravasation of iNKT cells in a CCL2-dependent fashion, and the iNKT cells are intimately connected to neutrophils infiltration (92). Notably, in asthma and airway hypersensitivity models, IL-17A-secreting NKT17 are required for the infiltration of neutrophils into lung tissues (93, 94). Accordingly, iNKT cell activation enhances inflammation in asthma models, and iNKT cell-deficient mice

have reduced airway hypersensitivity (25). Recent studies using scRNAseq data of macrophage populations in fibrotic tissues from both liver and lung tissues from NASH and IPF patients, respectively, have clarified the heterogeneity of "scar-associated macrophages" (SAMs) involved in fibrosis (65, 66). These SAMs are a subset of $CD9^{+}TREM2^{+}$ macrophages that express SPP1, GPNMB, FABP5 and CD63 and can differentiate from monocytes with type 3 cytokines GM-CSF, IL-17A and TGF-β (65). Furthermore, $MMP9^{+}$ neutrophils that participate in the activation of TGF-β and secrete type 3 cytokines (GM-CSF and IL-17A) are co-clustered with these SAMs in the fibrotic tissue. Accordingly, in murine models, blockade of GM-CSF, IL-17A and TGF-β significantly inhibited the expansion of these $FABP5^{+}$ SAMs and hepatic and pulmonary fibrosis. These studies indicate that IL-17A, GM-CSF and TGF-β can collaboratively induce monocytes-to-$FABP5^{+}$ SAM differentiation and promote pathogenic collagen deposition by mesenchymal cells in IPF and NASH patients (65). Collectively, IL-17A signaling associated with NKT17, γδ T cells and neutrophils in the fibrotic niche plays a critical role in fibrosis progression in both IPF and NASH patients.

Involvement of iNKT cells in inflammasome activation in fibrosis

Several lines of investigation indicate that the NLRP3 inflammasome activation is an important step that drives pro-fibrotic changes in tissue, and that this inflammatory complex could contribute significantly to both IPF and liver fibrosis. Thus, cell specific NLRP3 inflammasome activation in myeloid cells or in neutrophils resulted in extensive hepatic inflammation in parenchyma followed by fibrogenesis and fibrosis in murine NASH models (95–97). These data are consistent with the earlier findings that showed NLRP3 activation blockade leads to inhibition of chronic inflammation and fibrosis (98). Similarly, caspase 1 and NLRP3 knockout mice are protected against hepatitis and associated liver fibrosis (99). Notably, both inflammasome activation and infiltration of immune cells, including neutrophils, is dependent on the presence of activated iNKT cells in experimental models (40, 41, 47). Pro-inflammatory iNKT cells produces TNFα and IL-17A and it is likely that iNKT cell derived cytokines are required for priming of the NLRP3 inflammasome. Consistent with our hypothesis, it has been shown that iNKT cell-derived TNFα was required for the optimal secretion of IL-1α and IL-1β by myeloid cells in response to iNKT cell activation (100, 101). A critical role of TNFα and IL-17A as mediators of tissue fibrosis induced by constitutive NLRP3 activation in myeloid cells has recently been shown (102) (103). NLRP3 activation has also been implicated in interstitial kidney fibrosis involving Smad3 activation that promotes TGF-β signaling (103). Similarly, inflammation and wound healing repair response to injury in lung depend on NLRP3 activation that maintain a balance for MMPs and TIMPS involved in lung fibrosis (4). Collectively, inflammasome activation, an important step in tissue fibrosis, is also critically impacted by the activation of iNKT cells.

iNKT-associated mechanisms impacting key immune pathways in IPF

Mechanisms driving fibrosis following initial tissue injury are complex and involve key interactions among several immune cells, including macrophages and fibroblasts. As stated in the sections above, recent investigations in experimental models as well as in IPF and NASH patients suggest a key involvement of common or conserved immune pathways in fibrosis in different tissues. Alarmins (IL-25, TSLP) are the earliest cytokines secreted following tissue damage and they contribute to fibrosis indirectly with actions from type 2 cytokine-secreting immune cells, such as NKT2, ILC2, $CD4^{+}$Th2 cells, etc. Thus IL-25 and IL-13 secreting ILC2 are also found in IPF patients (104). Aside from the important role of TGF-β in fibrosis, type 1, type 2 and type 3 cytokine-associated immune responses are also key inducers of fibrosis (1, 2, 5, 65, 66, 68). iNKT cell derived cytokines, chemokines, and their interactions with other immune cells in the fibrotic milieu play an important role in the progression of tissue inflammation and fibrosis: **1)** iNKT subsets, NKT1, NKT2 and NKT17 have major influences on type 1, type 2 and type 3 immunity and are implicated in the progression fibrosis (23, 28, 40, 41, 47, 49, 56, 105); **2)** infiltration and the accumulation of neutrophils following tissue injury is inhibited in the absence iNKT cells (21, 23, 40, 41); **3)** adaptive Th1/Th2 skewing of adaptive immunity has been shown to be driven by an early activation of iNKT cells; **4)** NKT2 interactions with type 2 driven lymphocytes, including eosinophils and ILC2, should play an important role in type 2 cytokine immune responses (25, 27, 28, 30, 34, 106, 107); **5)** additionally, iNKT activation also has been shown to promote Hh-Wnt signaling that plays a key role in the transition of epithelial cells into myofibroblasts that deposit matrix proteins (28, 51). Based upon data in experimental models and in humans and the fact that iNKT cell activation is a common upstream pathway in the propagation of chronic inflammation driving fibrosis, we propose a mechanism highlighting key events in the progression of lung fibrosis in IPF (see Figure 1).

Alarmins, like IL-33, are constitutively expressed at high levels in epithelial barrier tissues and endothelial barriers and are immediately released following tissue injury. iNKT cells constitutively express the ST2 chain specific of the IL-33 receptor on their surface (108, 109), and IL-33 is a co-stimulatory signal for NKT1, NKT2, and NKT17 activation (108, 110). Following lung epithelial injury, tissue-resident NKT17 cells become activated and secrete IL-17A associated with the recruitment of neutrophils into lung tissue. It is likely that IL-17A secretion from both iNKT17 as well as from γδ T cells become involved in subsequent inflammatory events, including activation of macrophages. As the inflammation progresses other subsets of iNKT cells such as tissue resident NKT1 and NKT2 cells, as well as circulating NKT2 cells that are recruited into lung tissues during chronic immune response, play an important role in activating resident or recruited macrophages. The fibrotic response is either characterized by the predominance of a TGF-β-dependent fibrotic pathway associated with M1-like monocyte/macrophage phenotype driven by type 1 (IL-1β and IL-

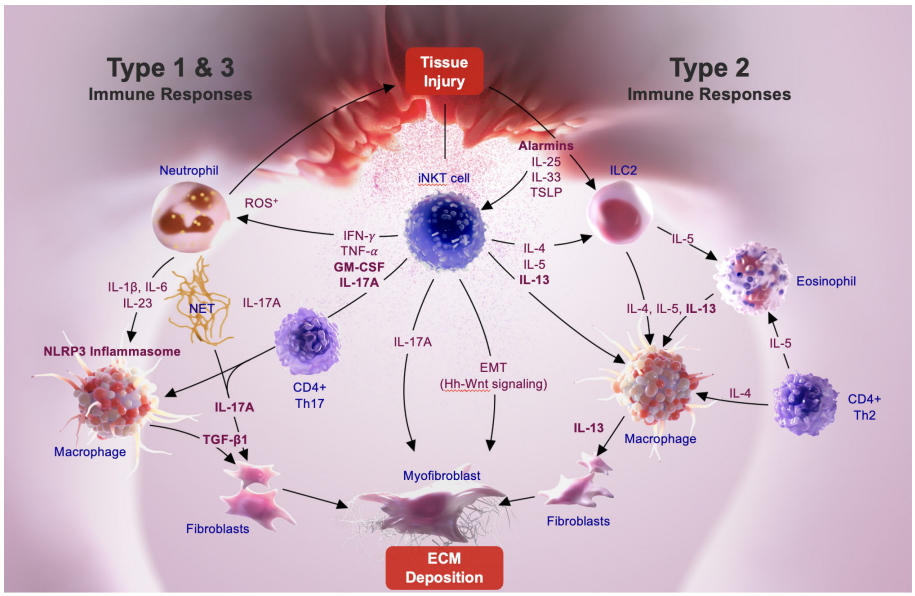

FIGURE 1
iNKT-mediated pathways driving pulmonary fibrosis.

6) or type 3, (GM-CSF and IL-17) cytokine pathway, or alternatively, by the predominance of TGF-β-independent pathway associated with IL-13-dependent M2-like macrophages driven by the type 2 (IL-4, IL-5, and IL-13) pathway. iNKT cell interactions with macrophages and/or neutrophils also results in inflammasome activation, a key component in fibrosis. Thus, depending on the stimuli or injury, upstream events leading to the activation of either inflammasome-IL-1β-IL-17A-TGF-β axis, or the GM-CSF-IL-17A-TGF-β axis is impacted by iNKT cell subset activation. Acute phase alarmins, such as IL-25, IL-33 and TSLP, function as central initiators of the type 2 immunity driven fibrosis that are triggered by IL-4 and IL-13 produced in association with innate lymphoid cells (ILC2), eosinophils, and Th2 cells in the fibrotic milieu. IL-4, IL-5, and IL-13 cytokine secretion by NKT2 cells, and other type 2 cells, result in IL-13 mediated activation of both macrophages and myofibroblasts that further contribute to fibrosis progression. Thus, iNKT cells are involved in the regulation of all three, type 1, type 2 and type 3 cytokine associated fibrotic pathways. The cross regulation of iNKT cell subsets as well as mechanisms that dictate whether type 1, type 2, or type 3 immunity predominates during the progression of lung fibrosis in IPF are not clear. However, inhibition of iNKT cells, including NKT1, NKT2 and NKT17 subsets, is likely to inhibit type 1, 2 and 3 key cytokine pathways driving fibrosis in IPF.

Future perspectives

As investigations continue to unravel the details of the cellular and molecular pathways in fibrosis that are common across tissues, such as the activation of iNKT cell subsets and their role in promoting fibrosis, it may be possible to develop novel biomarkers and therapeutic targets that differentiate stages of fibrosis progression. We have shown that activated iNKT cells in PBMCs from patients strongly correlate with fibrosis and disease progression (40). Development of cellular and molecular markers that predict the dominance of one or more specific pathways at a given time or subset of IPF patients may facilitate patient stratification and/or targeted therapies based upon these biomarkers and may improve outcomes. In complex diseases like IPF and NASH, targeting multiple molecular pathways may be required to address the complex mechanisms driving disease. Some of the disappointing outcomes in clinical trials blocking a single cytokine, for example IL-13 alone, may be a result of: **1)** mixed type 1, 2, and 3 driven responses in which a single cytokine response does not dominate; **2)** unintended consequences of cross-regulation of cytokine signaling in the fibrotic tissue; and/or **3)** disruption of the beneficial or protective aspects of some cytokines. Thus, rather than blocking a particular key cytokine that has many pleiotropic effects, targeting an immune pathway that can facilitate a re-balancing of downstream pathways, and restoring immune homeostasis, may be crucial for an effective treatment strategy for fibrosis. As covered in this brief review, the blocking of an earlier upstream pathway, such as iNKT cell activation, may dampen all three key cytokine-associated pathways and ultimately may lead to the development of novel therapeutic strategies in IPF. As mentioned earlier, it is likely that in patients different NKT cell subsets may promote fibrosis at different timepoints or stages of disease. In this scenario, an agent that blocks all iNKT cell subsets is highly desirable. We have found that an agonist of RARβγ inhibits cytokine secretion from all three iNKT cell subsets and leads to significant inhibition of fibrosis in murine models (40) Future studies are needed to evaluate whether inhibition of iNKT cells in a clinical setting can impact the progression of lung or liver fibrosis. Furthermore, a combination strategy, such as type 2 NKT cell activation leading to a powerful immunoregulatory mechanism

along with the inhibition of iNKT cell activation, may be needed as an effective therapeutic approach in tissue fibrosis.

Author contributions

VK: Conceptualization, Writing – original draft. MH: Writing – review & editing. AA: Writing – review & editing. AB: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Work in Kumar laboratory was supported by the National Institutes of Health grants: CA100660 and AA020864 to VK.

Conflict of interest

VK, MH, and AA are co-founders of GRU Bio and hold equity.

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

References

1. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat Med* (2012) 18(7):1028–40. doi: 10.1038/nm.2807

2. Gieseck RL 3rd, Wilson MS, Wynn TA. Type 2 immunity in tissue repair and fibrosis. *Nat Rev Immunol* (2018) 18(1):62–76. doi: 10.1038/nri.2017.90

3. Lupher ML Jr., Gallatin WM. Regulation of fibrosis by the immune system. *Adv Immunol* (2006) 89:245–88. doi: 10.1016/S0065-2776(05)89006-6

4. Pinar AA, Samuel CS. Immune mechanisms and related targets for the treatment of fibrosis in various organs. *Curr Mol Med* (2022) 22(3):240–9. doi: 10.2174/1566524022666220114122839

5. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration, and fibrosis. *Immunity* (2016) 44(3):450–62. doi: 10.1016/j.immuni.2016.02.015

6. Maher TM, Bendstrup E, Dron L, Langley J, Smith G, Khalid JM, et al. Global incidence and prevalence of idiopathic pulmonary fibrosis. *Respir Res* (2021) 22(1):197. doi: 10.1186/s12931-021-01791-z

7. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* (2018) 67(1):328–57. doi: 10.1002/hep.29367

8. Bendelac A, Rivera MN, Park SH, Roark JH. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol* (1997) 15:535–62. doi: 10.1146/annurev.immunol.15.1.535

9. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13 (2):101–17. doi: 10.1038/nri3369

10. Brigl M, Brenner MB. CD1: antigen presentation and T cell function. *Annu Rev Immunol* (2004) 22:817–90. doi: 10.1146/annurev.immunol.22.012703.104608

11. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi: 10.1146/annurev.immunol.25.022106.141711

12. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. *Nat Immunol* (2010) 11(3):197–206. doi: 10.1038/ni.1841

13. Exley M, Garcia J, Balk SP, Porcelli S. Requirements for CD1d recognition by human invariant Valpha24+ CD4-CD8- T cells. *J Exp Med* (1997) 186(1):109–20. doi: 10.1084/jem.186.1.109

14. Exley MA, Bigley NJ, Cheng O, Shaulov A, Tahir SM, Carter QL, et al. Innate immune response to encephalomyocarditis virus infection mediated by CD1d. *Immunology* (2003) 110(4):519–26. doi: 10.1111/j.1365-2567.2003.01779.x

15. Shaulov A, Yue S, Wang R, Joyce RM, Balk SP, Kim HT, et al. Peripheral blood progenitor cell product contains Th1-biased noninvariant CD1d-reactive natural killer T cells: implications for posttransplant survival. *Exp Hematol* (2008) 36(4):464–72. doi: 10.1016/j.exphem.2007.12.010

16. Arrenberg P, Halder R, Dai Y, Maricic I, Kumar V. Oligoclonality and innate-like features in the TCR repertoire of type II NKT cells reactive to a beta-linked self-glycolipid. *Proc Natl Acad Sci U S A* (2010) 107(24):10984–9. doi: 10.1073/pnas.1000576107

17. Jahng A, Maricic I, Aguilera C, Cardell S, Halder RC, Kumar V. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med* (2004) 199(7):947–57. doi: 10.1084/jem.20031389

18. Girardi E, Maricic I, Wang J, Mac TT, Iyer P, Kumar V, et al. Type II natural killer T cells use features of both innate-like and conventional T cells to recognize sulfatide self antigens. *Nat Immunol* (2012) 13(9):851–6. doi: 10.1038/ni.2371

19. Patel O, Pellicci DG, Gras S, Sandoval-Romero ML, Uldrich AP, Mallevaey T, et al. Recognition of CD1d-sulfatide mediated by a type II natural killer T cell antigen receptor. *Nat Immunol* (2012) 13(9):857–63. doi: 10.1038/ni.2372

20. Halder RC, Aguilera C, Maricic I, Kumar V. Type II NKT cell-mediated anergy induction in type I NKT cells prevents inflammatory liver disease. *J Clin Invest* (2007) 117(8):2302–12. doi: 10.1172/JCI31602

21. Arrenberg P, Maricic I, Kumar V. Sulfatide-mediated activation of type II natural killer T cells prevents hepatic ischemic reperfusion injury in mice. *Gastroenterology* (2011) 140(2):646–55. doi: 10.1053/j.gastro.2010.10.003

22. Dhodapkar MV, Kumar V. Type II NKT cells and their emerging role in health and disease. *J Immunol* (2017) 198(3):1015–21. doi: 10.4049/jimmunol.1601399

23. Marrero I, Maricic I, Feldstein AE, Loomba R, Schnabl B, Rivera-Nieves J, et al. Complex network of NKT cell subsets controls immune homeostasis in liver and gut. *Front Immunol* (2018) 9:2082. doi: 10.3389/fimmu.2018.02082

24. Marrero I, Ware R, Kumar V. Type II NKT cells in inflammation, autoimmunity, microbial immunity, and cancer. *Front Immunol* (2015) 6:316. doi: 10.3389/fimmu.2015.00316

25. Crosby CM, Kronenberg M. Tissue-specific functions of invariant natural killer T cells. *Nat Rev Immunol* (2018) 18(9):559–74. doi: 10.1038/s41577-018-0034-2

26. Godfrey DI, Le Nours J, Andrews DM, Uldrich AP, Rossjohn J. Unconventional T cell targets for cancer immunotherapy. *Immunity* (2018) 48(3):453–73. doi: 10.1016/j.immuni.2018.03.009

27. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. Corrigendum: The burgeoning family of unconventional T cells. *Nat Immunol* (2016) 17(4):469. doi: 10.1038/ni0416-469a

28. Kumar V. NKT-cell subsets: promoters and protectors in inflammatory liver disease. *J Hepatol* (2013) 59(3):618–20. doi: 10.1016/j.jhep.2013.02.032

29. Joyce S, Okoye GD, Van Kaer L. Natural killer T lymphocytes integrate innate sensory information and relay context to effector immune responses. *Crit Rev Immunol* (2021) 41(4):55–88. doi: 10.1615/CritRevImmunol.2021040076

30. Van Kaer L, Postoak JL, Song W, Wu L. Innate and innate-like effector lymphocytes in health and disease. *J Immunol* (2022) 209(2):199–207. doi: 10.4049/jimmunol.2200074

31. Cui G, Shimba A, Jin J, Ogawa T, Muramoto Y, Miyachi H, et al. A circulating subset of iNKT cells mediates antitumor and antiviral immunity. *Sci Immunol* (2022) 7 (76):eabj8760. doi: 10.1126/sciimmunol.abj8760

32. Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, et al. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is

highly conserved through mammalian evolution. *J Exp Med* (1998) 188(8):1521–8. doi: 10.1084/jem.188.8.1521

33. Maricic I, Girardi E, Zajonc DM, Kumar V. Recognition of lysophosphatidylcholine by type II NKT cells and protection from an inflammatory liver disease. *J Immunol* (2014) 193(9):4580–9. doi: 10.4049/jimmunol.1400699

34. Godfrey DI, Kronenberg M. Going both ways: immune regulation *via* CD1d-dependent NKT cells. *J Clin Invest* (2004) 114(10):1379–88. doi: 10.1172/JCI200423594

35. Cao Z, Yuan Y, Jeyabalan G, Du Q, Tsung A, Geller DA, et al. Preactivation of NKT cells with alpha-GalCer protects against hepatic ischemia-reperfusion injury in mouse by a mechanism involving IL-13 and adenosine A2A receptor. *Am J Physiol Gastrointest Liver Physiol* (2009) 297(2):G249–58. doi: 10.1152/ajpgi.00041.2009

36. Jahng AW, Maricic I, Pedersen B, Burdin N, Naidenko O, Kronenberg M, et al. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* (2001) 194(12):1789–99. doi: 10.1084/jem.194.12.1789

37. Singh AK, Wilson MT, Hong S, Olivares-Villagomez D, Du C, Stanic AK, et al. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* (2001) 194(12):1801–11. doi: 10.1084/jem.194.12.1801

38. Ferhat MH, Robin A, Barbier L, Thierry A, Gombert JM, Barbarin A, et al. The impact of invariant NKT cells in sterile inflammation: the possible contribution of the alarmin/cytokine IL-33. *Front Immunol* (2018) 9:2308. doi: 10.3389/fimmu.2018.02308

39. Koay HF, Gherardin NA, Nguyen THO, Zhang W, Habel JR, Seneviratna R, et al. Are NKT cells a useful predictor of COVID-19 severity? *Immunity* (2022) 55(2):185–7. doi: 10.1016/j.immuni.2022.01.005

40. Maricic I, Marrero I, Eguchi A, Nakamura R, Johnson CD, Dasgupta S, et al. Differential activation of hepatic invariant NKT cell subsets plays a key role in progression of nonalcoholic steatohepatitis. *J Immunol* (2018) 201(10):3017–35. doi: 10.4049/jimmunol.1800614

41. Maricic I, Sheng H, Marrero I, Seki E, Kisseleva T, Chaturvedi S, et al. Inhibition of type I natural killer T cells by retinoids or following sulfatide-mediated activation of type II natural killer T cells attenuates alcoholic liver disease in mice. *Hepatology* (2015) 61(4):1357–69. doi: 10.1002/hep.27632

42. Park O, Jeong WI, Wang L, Wang H, Lian ZX, Gershwin ME, et al. Diverse roles of invariant natural killer T cells in liver injury and fibrosis induced by carbon tetrachloride. *Hepatology* (2009) 49(5):1683–94. doi: 10.1002/hep.22813

43. Lisbonne M, L'Helgoualc'h A, Nauwelaers G, Turlin B, Lucas C, Herbelin A, et al. Invariant natural killer T-cell-deficient mice display increased CCl(4) -induced hepatitis associated with CXCL1 over-expression and neutrophil infiltration. *Eur J Immunol* (2011) 41(6):1720–32. doi: 10.1002/eji.201041006

44. Duwaerts CC, Sun EP, Cheng CW, van Rooijen N, Gregory SH. Cross-activating invariant NKT cells and kupffer cells suppress cholestatic liver injury in a mouse model of biliary obstruction. *PloS One* (2013) 8(11):e79702. doi: 10.1371/journal.pone.0079702

45. Wintermeyer P, Cheng CW, Gehring S, Hoffman BL, Holub M, Brossay L, et al. Invariant natural killer T cells suppress the neutrophil inflammatory response in a mouse model of cholestatic liver damage. *Gastroenterology* (2009) 136(3):1048–59. doi: 10.1053/j.gastro.2008.10.027

46. Shiga Y, Sugamata R, Iwamura C, Nagao T, Zao J, Kawakami K, et al. Effect of invariant natural killer T cells with IL-5 and activated IL-6 receptor in ventilator-associated lung injury in mice. *Exp Lung Res* (2014) 40(1):1–11. doi: 10.3109/01902148.2013.854518

47. Mathews S, Feng D, Maricic I, Ju C, Kumar V, Gao B. Invariant natural killer T cells contribute to chronic-plus-binge ethanol-mediated liver injury by promoting hepatic neutrophil infiltration. *Cell Mol Immunol* (2016) 13(2):206–16. doi: 10.1038/cmi.2015.06

48. Grabarz F, Aguiar CF, Correa-Costa M, Braga TT, Hyane MI, Andrade-Oliveira V, et al. Protective role of NKT cells and macrophage M2-driven phenotype in bleomycin-induced pulmonary fibrosis. *Inflammopharmacology* (2018) 26(2):491–504. doi: 10.1007/s10787-017-0383-7

49. Kim JH, Kim HY, Kim S, Chung JH, Park WS, Chung DH. Natural killer T (NKT) cells attenuate bleomycin-induced pulmonary fibrosis by producing interferon-gamma. *Am J Pathol* (2005) 167(5):1231–41. doi: 10.1016/S0002-9440(10)61211-4

50. Wehr A, Baeck C, Heymann F, Niemietz PM, Hammerich L, Martin C, et al. Chemokine receptor CXCR6-dependent hepatic NK T Cell accumulation promotes inflammation and liver fibrosis. *J Immunol* (2013) 190(10):5226–36. doi: 10.4049/jimmunol.1202909

51. Syn WK, Agboola KM, Swiderska M, Michelotti GA, Liaskou E, Pang H, et al. NKT-associated hedgehog and osteopontin drive fibrogenesis in non-alcoholic fatty liver disease. *Gut* (2012) 61(9):1323–9. doi: 10.1136/gutjnl-2011-301857

52. Bandyopadhyay K, Marrero I, Kumar V. NKT cell subsets as key participants in liver physiology and pathology. *Cell Mol Immunol* (2016) 13(3):337–46. doi: 10.1038/cmi.2015.115

53. Li Z, Soloski MJ, Diehl AM. Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. *Hepatology* (2005) 42(4):880–5. doi: 10.1002/hep.20826

54. Valenti L, Fracanzani AL, Fargion S. The immunopathogenesis of alcoholic and nonalcoholic steatohepatitis: two triggers for one disease? *Semin immunopathol* (2009) 31(3):359–69. doi: 10.1007/s00281-009-0152-9

55. Zeissig S, Peuker K, Iyer S, Gensollen T, Dougan SK, Olszak T, et al. CD1d-Restricted pathways in hepatocytes control local natural killer T cell homeostasis and hepatic inflammation. *Proc Natl Acad Sci U S A.* (2017) 114(39):10449–54. doi: 10.1073/pnas.1701428114

56. Marrero I, Maricic I, Morgan TR, Stolz AA, Schnabl B, Liu ZX, et al. Differential activation of unconventional T cells, including iNKT cells, in alcohol-related liver disease. *Alcohol Clin Exp Res* (2020) 44(5):1061–74. doi: 10.1111/acer.14323

57. Bhattacharjee J, Kirby M, Softic S, Miles L, Salazar-Gonzalez RM, Shivakumar P, et al. Hepatic natural killer T-cell and CD8+ T-cell signatures in mice with nonalcoholic steatohepatitis. *Hepatol Commun* (2017) 1(4):299–310. doi: 10.1002/hep4.1041

58. Ma C, Kesarwala AH, Eggert T, Medina-Echeverz J, Kleiner DE, Jin P, et al. NAFLD causes selective CD4(+) T lymphocyte loss and promotes hepatocarcinogenesis. *Nature* (2016) 531(7593):253–7. doi: 10.1038/nature16969

59. Sutti S, Bruzzi S, Albano E. The role of immune mechanisms in alcoholic and nonalcoholic steatohepatitis: a 2015 update. *Expert Rev Gastroenterol hepatol* (2016) 10(2):243–53. doi: 10.1586/17474124.2016.1111758

60. Wolf MJ, Adili A, Piotrowitz K, Abdullah Z, Boege Y, Stemmer K, et al. Metabolic activation of intrahepatic CD8+ T cells and NKT cells causes nonalcoholic steatohepatitis and liver cancer *via* cross-talk with hepatocytes. *Cancer Cell* (2014) 26(4):549–64. doi: 10.1016/j.ccell.2014.09.003

61. Liu Y, Munker S, Mullenbach R, Weng HL. IL-13 signaling in liver fibrogenesis. *Front Immunol* (2012) 3:116. doi: 10.3389/fimmu.2012.00116

62. Kim EY, Battaile JT, Patel AC, You Y, Agapov E, Grayson MH, et al. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. *Nat Med* (2008) 14(6):633–40. doi: 10.1038/nm1770

63. Jandl K, Marsh LM, Mutgan AC, Crnkovic S, Valzano F, Zabini D, et al. Impairment of the NKT-STAT1-CXCL9 axis contributes to vessel fibrosis in pulmonary hypertension caused by lung fibrosis. *Am J Respir Crit Care Med* (2022) 206(8):981–98. doi: 10.1164/rccm.202201-0142OC

64. Eming SA, Wynn TA, Martin P. Inflammation and metabolism in tissue repair and regeneration. *Science* (2017) 356(6342):1026–30. doi: 10.1126/science.aam7928

65. Fabre T, Barron AMS, Christensen SM, Asano S, Bound K, Lech MP, et al. Identification of a broadly fibrogenic macrophage subset induced by type 3 inflammation. *Sci Immunol* (2023) 8(82):eadd8945. doi: 10.1126/sciimmunol.add8945

66. Fabre T, Molina MF, Soucy G, Goulet JP, Willems B, Villeneuve JP, et al. Type 3 cytokines IL-17A and IL-22 drive TGF-beta-dependent liver fibrosis. *Sci Immunol* (2018) 3(28):eaar7754. doi: 10.1126/sciimmunol.aar7754

67. Kimura T, Ishii Y, Morishima Y, Shibuya A, Shibuya K, Taniguchi M, et al. Treatment with alpha-galactosylceramide attenuates the development of bleomycin-induced pulmonary fibrosis. *J Immunol* (2004) 172(9):5782–9. doi: 10.4049/jimmunol.172.9.5782

68. Hart KM, Fabre T, Sciurba JC, Gieseck RL 3rd, Borthwick LA, Vannella KM, et al. Type 2 immunity is protective in metabolic disease but exacerbates NAFLD collaboratively with TGF-beta. *Sci Transl Med* (2017) 9(396):eaal3694. doi: 10.1126/scitranslmed.aal3694

69. Nilsson J, Hornberg M, Schmidt-Christensen A, Linde K, Nilsson M, Carlus M, et al. NKT cells promote both type 1 and type 2 inflammatory responses in a mouse model of liver fibrosis. *Sci Rep* (2020) 10(1):21778. doi: 10.1038/s41598-020-78688-2

70. Aoyagi T, Yamamoto N, Hatta M, Tanno D, Miyazato A, Ishii K, et al. Activation of pulmonary invariant NKT cells leads to exacerbation of acute lung injury caused by LPS through local production of IFN-gamma and TNF-alpha by Gr-1+ monocytes. *Int Immunol* (2011) 23(2):97–108. doi: 10.1093/intimm/dxq460

71. Fuschiotti P. Role of IL-13 in systemic sclerosis. *Cytokine* (2011) 56(3):544–9. doi: 10.1016/j.cyto.2011.08.030

72. Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, et al. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med* (2001) 194(6):809–21. doi: 10.1084/jem.194.6.809

73. Weng HL, Liu Y, Chen JL, Huang T, Xu LJ, Godoy P, et al. The etiology of liver damage imparts cytokines transforming growth factor beta1 or interleukin-13 as driving forces in fibrogenesis. *Hepatology* (2009) 50(1):230–43. doi: 10.1002/hep.22934

74. Murray LA, Argentieri RL, Farrell FX, Bracht M, Sheng H, Whitaker B, et al. Hyper-responsiveness of IPF/UIP fibroblasts: interplay between TGFbeta1, IL-13 and CCL2. *Int J Biochem Cell Biol* (2008) 40(10):2174–82. doi: 10.1016/j.biocel.2008.02.016

75. Park SW, Ahn MH, Jang HK, Jang AS, Kim DJ, Koh ES, et al. Interleukin-13 and its receptors in idiopathic interstitial pneumonia: clinical implications for lung function. *J Korean Med Sci* (2009) 24(4):614–20. doi: 10.3346/jkms.2009.24.4.614

76. Murray LA, Zhang H, Oak SR, Coelho AL, Herath A, Flaherty KR, et al. Targeting interleukin-13 with tralokinumab attenuates lung fibrosis and epithelial damage in a humanized SCID idiopathic pulmonary fibrosis model. *Am J Respir Cell Mol Biol* (2014) 50(5):985–94. doi: 10.1165/rcmb.2013-0342OC

77. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, et al. Lebrikizumab treatment in adults with asthma. *N Engl J Med* (2011) 365(12):1088–98. doi: 10.1056/NEJMoa1106469

78. Parker JM, Glaspole IN, Lancaster LH, Haddad TJ, She D, Roseti SL, et al. A phase 2 randomized controlled study of tralokinumab in subjects with idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* (2018) 197(1):94–103. doi: 10.1164/rccm.201704-0784OC

79. Wijsenbeek MS, Kool M, Cottin V. Targeting interleukin-13 in idiopathic pulmonary fibrosis: from promising path to dead end. *Eur Respir J* (2018) 52 (6):1802111. doi: 10.1183/13993003.02111-2018

80. Fichtner-, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat Med* (2006) 12(1):99–106. doi: 10.1038/nm1332

81. Kaviratne M, Hesse M, Leusink M, Cheever AW, Davies SJ, McKerrow JH, et al. IL-13 activates a mechanism of tissue fibrosis that is completely TGF-beta independent. *J Immunol* (2004) 173(6):4020–9. doi: 10.4049/jimmunol.173.6.4020

82. Gieseck RL 3rd, Ramalingam TR, Hart KM, Vannella KM, Cantu DA, Lu WY, et al. Interleukin-13 activates distinct cellular pathways leading to ductular reaction, steatosis, and fibrosis. *Immunity* (2016) 45(1):145–58. doi: 10.1016/j.immuni.2016.06.009

83. Tsao CC, Tsao PN, Chen YG, Chuang YH. Repeated activation of lung invariant NKT cells results in chronic obstructive pulmonary disease-like symptoms. *PloS One* (2016) 11(1):e0147710. doi: 10.1371/journal.pone.0147710

84. Pichavant M, Remy G, Bekaert S, Le Rouzic O, Kervoaze G, Vilain E, et al. Oxidative stress-mediated iNKT-cell activation is involved in COPD pathogenesis. *Mucosal Immunol* (2014) 7(3):568–78. doi: 10.1038/mi.2013.75

85. Akbari O, Faul JL, Hoyte EG, Berry GJ, Wahlstrom J, Kronenberg M, et al. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *N Engl J Med* (2006) 354(11):1117–29. doi: 10.1056/NEJMoa053614

86. Liu B, Jiang J, Liang H, Xiao P, Lai X, Nie J, et al. Natural killer T cell/IL-4 signaling promotes bone marrow-derived fibroblast activation and M2 macrophage-to-myofibroblast transition in renal fibrosis. *Int Immunopharmacol* (2021) 98:107907. doi: 10.1016/j.intimp.2021.107907

87. Jia H, Chen J, Zhang X, Bi K, Zhou H, Liu T, et al. IL-17A produced by invariant natural killer T cells and CD3(+) CD56(+) alphaGalcer-CD1d tetramer(-) T cells promote liver fibrosis in patients with primary biliary cholangitis. *J Leukoc Biol* (2022) 112(5):1079–87. doi: 10.1002/JLB.2A0622-586RRRR

88. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* (2011) 11(8):519–31. doi: 10.1038/nri3024

89. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-specific distribution of iNKT cells impacts their cytokine response. *Immunity* (2015) 43 (3):566–78. doi: 10.1016/j.immuni.2015.06.025

90. Heinrichs D, Brandt EF, Fischer P, Kohncke J, Wirtz TH, Guldiken N, et al. Unexpected pro-fibrotic effect of MIF in non-alcoholic steatohepatitis is linked to a shift in NKT cell populations. *Cells* (2021) 10(2):252. doi: 10.3390/cells10020252

91. Scanlon ST, Thomas SY, Ferreira CM, Bai L, Krausz T, Savage PB, et al. Airborne lipid antigens mobilize resident intravascular NKT cells to induce allergic airway inflammation. *J Exp Med* (2011) 208(10):2113–24. doi: 10.1084/jem.20110522

92. Thanabalasuriar A, Neupane AS, Wang J, Krummel MF, Kubes P. iNKT cell emigration out of the lung vasculature requires neutrophils and monocyte-derived dendritic cells in inflammation. *Cell Rep* (2016) 16(12):3260–72. doi: 10.1016/j.celrep.2016.07.052

93. Lee KA, Kang MH, Lee YS, Kim YJ, Kim DH, Ko HJ, et al. A distinct subset of natural killer T cells produces IL-17, contributing to airway infiltration of neutrophils but not to airway hyperreactivity. *Cell Immunol* (2008) 251(1):50–5. doi: 10.1016/j.cellimm.2008.03.004

94. Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, et al. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J Exp Med* (2007) 204(5):995–1001. doi: 10.1084/jem.20061551

95. Frising UC, Ribo S, Doglio MG, Malissen B, van Loo G, Wullaert A. Nlrp3 inflammasome activation in macrophages suffices for inducing autoinflammation in mice. *EMBO Rep* (2022) 23(7):e54339. doi: 10.15252/embr.202154339

96. Kaufmann B, Kui L, Reca A, Leszczynska A, Kim AD, Booshehri LM, et al. Cell-specific deletion of NLRP3 inflammasome identifies myeloid cells as key drivers of liver inflammation and fibrosis in murine steatohepatitis. *Cell Mol Gastroenterol Hepatol* (2022) 14(4):751–67. doi: 10.1016/j.jcmgh.2022.06.007

97. Kaufmann B, Leszczynska A, Reca A, Booshehri LM, Onyuru J, Tan Z, et al. NLRP3 activation in neutrophils induces lethal autoinflammation, liver inflammation, and fibrosis. *EMBO Rep* (2022) 23(11):e54446. doi: 10.15252/embr.202154446

98. Mridha AR, Wree A, Robertson AAB, Yeh MM, Johnson CD, Van Rooyen DM, et al. NLRP3 inflammasome blockade reduces liver inflammation and fibrosis in experimental NASH in mice. *J Hepatol* (2017) 66(5):1037–46. doi: 10.1016/j.jhep.2017.01.022

99. Dixon LJ, Berk M, Thapaliya S, PapouChado BG, Feldstein AE. Caspase-1-mediated regulation of fibrogenesis in diet-induced steatohepatitis. *Lab Invest* (2012) 92 (5):713–23. doi: 10.1038/labinvest.2012.45

100. Chow MT, Duret H, Andrews DM, Faveeuw C, Moller A, Smyth MJ, et al. Type I NKT-cell-mediated TNF-alpha is a positive regulator of NLRP3 inflammasome priming. *Eur J Immunol* (2014) 44(7):2111–20. doi: 10.1002/eji.201344329

101. Franchi L, Eigenbrod T, Nunez G. Cutting edge: TNF-alpha mediates sensitization to ATP and silica *via* the NLRP3 inflammasome in the absence of microbial stimulation. *J Immunol* (2009) 183(2):792–6. doi: 10.4049/jimmunol.0900173

102. Wree A, McGeough MD, Inzaugarat ME, Eguchi A, Schuster S, Johnson CD, et al. NLRP3 inflammasome driven liver injury and fibrosis: Roles of IL-17 and TNF in mice. *Hepatology* (2018) 67(2):736–49. doi: 10.1002/hep.29523

103. Wang W, Wang X, Chun J, Vilaysane A, Clark S, French G, et al. Inflammasome-independent NLRP3 augments TGF-beta signaling in kidney epithelium. *J Immunol* (2013) 190(3):1239–49. doi: 10.4049/jimmunol.1201959

104. Hams E, Armstrong ME, Barlow JL, Saunders SP, Schwartz C, Cooke G, et al. IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis. *Proc Natl Acad Sci U S A.* (2014) 111(1):367–72. doi: 10.1073/pnas.1315854111

105. Kim JO, Kim DH, Chang WS, Hong C, Park SH, Kim S, et al. Asthma is induced by intranasal coadministration of allergen and natural killer T-cell ligand in a mouse model. *J Allergy Clin Immunol* (2004) 114(6):1332–8. doi: 10.1016/j.jaci.2004.09.004

106. Lexmond WS, Neves JF, Nurko S, Olszak T, Exley MA, Blumberg RS, et al. Involvement of the iNKT cell pathway is associated with early-onset eosinophilic esophagitis and response to allergen avoidance therapy. *Am J Gastroenterol* (2014) 109 (5):646–57. doi: 10.1038/ajg.2014.12

107. Jyonouchi S, Smith CL, Saretta F, Abraham V, Ruymann KR, Modayur-Chandramouleeswaran P, et al. Invariant natural killer T cells in children with eosinophilic esophagitis. *Clin Exp Allergy* (2014) 44(1):58–68. doi: 10.1111/cea.12201

108. Bourgeois E, Van LP, Samson M, Diem S, Barra A, Roga S, et al. The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. *Eur J Immunol* (2009) 39(4):1046–55. doi: 10.1002/eji.200838575

109. Smithgall MD, Comeau MR, Yoon BR, Kaufman D, Armitage R, Smith DE. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int Immunol* (2008) 20 (8):1019–30. doi: 10.1093/intimm/dxn060

110. Ferhat M, Robin A, Giraud S, Sena S, Goujon JM, Touchard G, et al. Endogenous IL-33 contributes to kidney ischemia-reperfusion injury as an alarmin. *J Am Soc Nephrol* (2018) 29(4):1272–88. doi: 10.1681/ASN.2017060650

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 03 October 2023
DOI 10.3389/fimmu.2023.1273459

OPEN ACCESS

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RECEIVED 06 August 2023
ACCEPTED 20 September 2023
PUBLISHED 03 October 2023

CITATION
Lv M, Zhang Z and Cui Y (2023) Unconventional T cells in brain homeostasis, injury and neurodegeneration.
Front. Immunol. 14:1273459.
doi: 10.3389/fimmu.2023.1273459

Unconventional T cells in brain homeostasis, injury and neurodegeneration

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The interaction between peripheral immune cells and the brain is an important component of the neuroimmune axis. Unconventional T cells, which include natural killer T (NKT) cells, mucosal-associated invariant T (MAIT) cells, γδ T cells, and other poorly defined subsets, are a special group of T lymphocytes that recognize a wide range of nonpolymorphic ligands and are the connection between adaptive and innate immunity. Recently, an increasing number of complex functions of these unconventional T cells in brain homeostasis and various brain disorders have been revealed. In this review, we describe the classification and effector function of unconventional T cells, review the evidence for the involvement of unconventional T cells in the regulation of brain homeostasis, summarize the roles and mechanisms of unconventional T cells in the regulation of brain injury and neurodegeneration, and discuss immunotherapeutic potential as well as future research goals. Insight of these processes can shed light on the regulation of T cell immunity on brain homeostasis and diseases and provide new clues for therapeutic approaches targeting brain injury and neurodegeneration.

KEYWORDS

NKT cell, γδ T cell, MAIT cell, meninges, brain injury, ischemic stroke, multiple sclerosis, neurodegeneration

Introduction

Recently, the communication between peripheral immune cells and the brain has attracted the attention of an increasing number of researchers with the discovery of meningeal lymphatic vessels, a way for peripheral immune cells to infiltrate into the brain parenchyma and communicate with brain resident cells (1, 2). It is well known that the blood-brain barrier (BBB) is damaged upon brain injury, and peripheral immune cells enter the brain and release various cytokines or immune mediators to regulate the outcome of brain injury (3). Notably, under physiological conditions, some interfaces, including the meninges, choroid plexus and cerebrospinal fluid have been found to harbor various types of immune cells (4), which are involved in cognitive function (5), synaptic plasticity (6, 7) and neurogenesis (6). Thus, the immune system can communicate with the brain in both

physiological and pathological conditions to modulate brain homeostasis and the progression of neurological diseases.

Unlike conventional T cells, which express an αβ TCR to recognize peptide antigens presented by major histocompatibility complex (MHC) molecules, unconventional T cells are a special group of T lymphocytes that mainly recognize a variety of self and non-self-antigens presented by nonclassical MHC molecules (8, 9). These unconventional T cells include natural killer T (NKT) cells, γδ T cells, mucosal-associated invariant T (MAIT) cells, and other rare subsets (10). Despite the limited number of unconventional T cells in organs of the body, defects or deficiencies of these T cells play important roles in various diseases, such as cancer, autoimmune diseases, and infectious diseases (11, 12). Notably, unconventional T cells were reported to be involved in both brain homeostasis (13, 14) and a variety of neurological diseases (15). Therefore, understanding the functions and molecular mechanisms of unconventional T cells in brain homeostasis and neurological diseases may provide clues for developing new therapies to maintain brain health.

In this review, we describe the classification and effector functions of unconventional T cells, including NKT cells, MAIT cells and γδ T cells; summarize the current reports on the functions of unconventional T cells in brain homeostasis, brain injuries caused by cerebral ischemic stroke, traumatic brain injury (TBI) and autoimmune-related multiple sclerosis, and neurodegenerative diseases; and discuss the immunotherapeutic potential of these cells as well as future goals of studies.

Classification and function of unconventional T cells

Unlike conventional T cells which recognize peptide antigens presented by classical MHC molecules, unconventional T cells recognize diverse nonpeptide antigens through nonclassical MHC molecules. These unconventional T cells mainly include MAIT cells, NKT cells, and γδ T cells, which are generated from the thymus. Although far less numerous than conventional αβ T cells, unconventional T cells have unique effector and regulatory roles and are often described as a bridge between innate and adaptive immunity (Figure 1).

Compared with conventional T cells, unconventional T cells recognize a broader spectrum of antigens, including self-antigens and non-self antigens, such as peptides recognized by conventional T cells, conserved lipids and metabolites derived from bacteria. The recognition of these diverse antigens enables them to regulate multiple levels of cellular immune responses (16). In addition, unconventional T cells often localize in tissues, especially mucosal interfaces, which help maintain tissue homeostasis and repair (11, 17). Moreover, unconventional T cells, such as NKT cells and MAIT

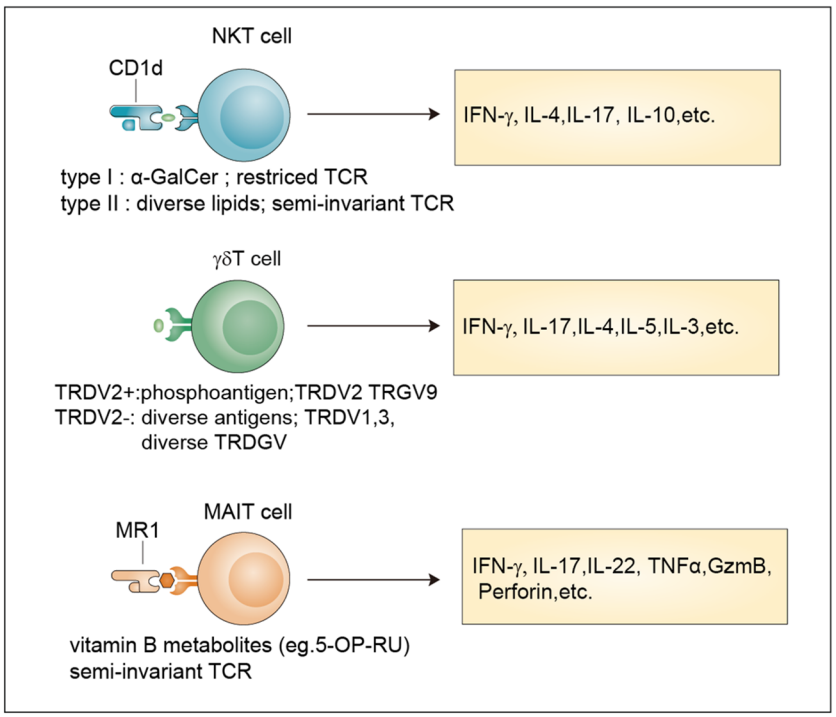

FIGURE 1
Classification and effector functions of unconventional T cells. Antigens, TCR properties and released-cytokines of NKT cells, γδ T cells and MAIT cells. NKT cells recognize antigens presented by Cd1d through an invariant αβ TCR. Type I NKT cells recognize the lipid antigen α-GalCer, while Type II NKT cells are reactive to more diverse lipid antigens. TRDV2^{+} cells and TRDV2^{-} γδ T cells have been defined based on their TCR δ-chain V region usage. TRDV2^{+} γδ T cells recognize phosphoantigens and TRDV2^{-} γδ T cells recognize more diverse antigens. MAIT cells are reactive to a limited array of microbe-derived vitamin B metabolites with a semi-invariant TCR. All these unconventional T cells release multiple cytokines after activation. α-GalCer, α-galactosylceramide.

cells, acquire their effector functions in the thymus and can be quickly activated in a similar way to innate immune cells after encountering antigens (9). These properties endow unconventional T cells with diverse functions that expand and complement the function of traditional innate and adaptive immune cells in protective immunity, barrier function, and tissue healing (Table 1).

Classification

NKT cells are generated from the $CD4^+CD8^+$ double-positive (DP) cells of the thymus, which represent a specialized subset of T cells that simultaneously express TCR and NK cell lineage markers. Two broad subsets, namely, type I NKT cells and type II NKT cells, have been defined (9). Typical type I NKT cells, also known as invariant NKT cells (iNKT cells), harbor an invariant TCRα chain (Vα14-Jα18 in mice and Vα24-Jα18 in human) coupled to a limited array of TCRβ chains that reactive to lipid antigens (α-GalCer) presented by CD1d (18). Currently, the endogenous ligands of iNKT cells remain unknown and need to be identified. In contrast, type II NKT cells have a more diverse repertoire than iNKT cells and can recognize a broader range of antigens, such as glycolipids, phospholipids, and hydrophobic antigens (19–21). The brain has abundant lipid content (22) indicating their involvement in NKT cell activation.

Similar to NKT cells, MAIT cells are also selected by DP cortical thymocytes and show limited TCR diversity that consists of an invariant α-chain (Vα 7.2-Jα 33 in humans; Vα 19-Jα 33 in mice) pairing with limited β chains (β2 or Vβ13 chain in humans; Vβ8 or Vβ6 chain in mice) (9). Accordingly, antigens recognized by MAIT cells are limited and are mainly vitamin B metabolites including 6-formylpterin, folic acid and several ribityllumazines and pyrimidine-based intermediates presented by MR1, a β2-microglobulin-associated antigen-presenting molecule (23).

Different from MAIT cells and NKT cells, γδ T cell subsets are generated from $CD4^-CD8^-$ double negative (DN) thymocytes and express a TCR γ-chain paring with a TCR δ-chain that exhibits limited TRGV and TRDV gene-segment usage (9). A unique feature of murine γδ T cells is that different tissues express distinct Vγ segments regarding the Vγ chains (24). In humans, two categories of γδ T cells including $TRDV2^+$ cells and $TRDV2^-$ cells have been broadly defined according to their TCR δ-chain V region usage, with the former coexpressing TRGV9 and the latter pairing with an array of TRGV genes (25). For many years, many researchers have tried to identify new γδ TCR ligands, and the involvement of butyrophilins or the recognition of MHC-like molecules has been revealed (26, 27). However, considering their important roles in both health and diseases (28, 29), large-scale screening methods based on current technologies such as the CRISPR/Cas9 system for further ligand identification are still needed.

TABLE 1 Comparison between innate immune cells, conventional T cells and unconventional T cells.

	Innate immune cells	Conventional T cells	Unconventional T cells
Cells types	monocytes, macrophages, dendritic cells, neutrophils, basophils, eosinophils, NK cells, mast cells, and ILCs	αβ T cells	Mainly NKT cells, MAIT cells and γδ T cells
Recognition	Non-self structures (PAMPs), or danger or damage-associated molecules (DAMPs) are recognized by PRRs	Peptide antigens presented by MHC-I or MHC-II on APCs are recognized by TCRs	Self-antigens and microbial extended self and non-self-antigens are recognized by TCRs
Activation	PRRs activation dependent, very quick	TCR activation dependent, relatively slow	Innate-like activation modes, very quick
Function	primary line of defense against pathogens	adaptive protective immunity against pathogens	Tissue homeostasis, primary line of defense

ILCs, Innate lymphoid cells; PAMPs, pathogen associated molecular patterns; DAMPs, damage or danger associated molecular patterns; PRRs, pattern recognition receptors.

Effector functions

After TCR-mediated activation, unconventional T cells are activated to secrete cytokines, exert cytotoxic effector functions, and undergo proliferative expansion. Unlike conventional T cells, unconventional T cells can rapidly become effector cells and produce cytokines within minutes to hours upon antigen activation, which is an innate-like property (9). More importantly, unconventional T cells including NKT cells and MAIT cells acquire effector functions in the thymus during early development which requires the expression of transcription factor PLZF and costimulatory signals mediated by signaling lymphocyte activation molecules (SLAM) (9, 30). This feature confers unconventional T cells the ability to migrate to peripheral tissue early in life and rapidly respond to stimuli (11).

In response to antigen-mediated activation, NKT cells quickly secrete large amounts of proinflammatory cytokines, such as interleukin 17 (IL-17) and interferon-γ (IFN-γ), and anti-inflammatory cytokines, such as IL-4 and IL-10 (31). By comparing with the classification methods of conventional $CD4^+$ T cells, researchers divide NKT cells into different subsets namely Th1-like NKT cell (NKT1), Th2-like NKT cell (NKT2), Th17-like NKT cell (NKT17) and Tfh-like NKT cell (NKTfh) based on the transcription factors and cytokines co-expressed by NKT cells (32, 33). Their differentiation potential is determined by the activation mechanism, which, in turn, determines the effects on the progression of diseases such as cancer, tissue injuries, autoimmune disease, and infection (34).

MAIT cells can be activated in two different ways: TCR-dependent and TCR-independent ways (35). TCR-dependent activation requires the presentation of microbe-derived intermediates by MR1 to TCR in cooperation with costimulators such as CD28 or some cytokines (36). After activation, MAIT cells secrete proinflammatory cytokines, mainly IL-17. TCR-independent activation is induced by IL-18 in combination with

type I interferons, IL-12, or IL-15 and potentiated by TL1A, a membra of the TNF-superfamily. This form of activation stimulates a modest cytokine production dominated by IFN-γ instead of IL-17 (35, 37). They are involved in responses to infections, cancer, and tissue repair (35).

Upon activation by extracellular viruses, bacteria, fungi, or intracellular pathogens, γδ T cells can quickly produce various cytokines, such as tumor necrosis factor (TNF), IL-17, IFNγ, IL-4, IL-13 and IL-5 to protect the host against pathogens (38). In addition, they also exert cytotoxicity against transformed or infected cells by enhancing the expression of death-inducing receptors such as CD95 and TNF-related apoptosis-inducing ligand receptors (TRAILR) or releasing cytotoxic effector molecules, such as granzymes and perforin (39). Moreover, some immunosuppressive cytokines such as IL-10 and transforming growth factor-β (TGFβ), can be produced by γδ T cells to manipulate innate and adaptive immunity (38).

After brain injury and neurodegeneration, many damage-associated molecular patterns (DAMPs) are released and these unconventional T cells can be recruited into the brain and activated to secrete cytokines and growth factors that may regulate innate and adaptive immune cells or directly interact with neurons to regulate the outcome of the disease. However, some interesting questions still exist. For example, what types of brain-specific antigens do these unconventional T cells recognize? Are specific subsets of these unconventional T cells generated during brain homeostasis or after disease progression? How do these unconventional T cells interact with brain-resident cells?

Unconventional T cells in brain homeostasis

Compared with other organs, the brain has a unique response to environmental stimuli owing to the existence of the BBB and the inability of neurons to self-renew (40, 41). Both intrinsic neuronal properties and external environmental factors determine the neuronal excitability and homeostasis of the brain (42). As an external factor, peripheral immune cells contribute to the homeostasis of many tissues (43), whereas their contribution to brain homeostasis and function has been largely ignored for years owing to the sporadic presence of peripheral immune cells in the brain parenchyma under homeostatic conditions.

Unlike the relatively immune-privileged brain parenchyma, the surrounding meninges are populated by many resident immune cells and serve as an important interface with the peripheral immune system. Recent studies have updated our knowledge of meningeal immunity regarding how the complicated immune niche of the meninges affects brain functions and plasticity in a disease-free context (44) as well as in brain disorders. It is well known that conventional T cells play key roles in maintaining brain functions, including spatial learning (5, 45), social behaviors (46) and sensory responses (47). Given the importance of unconventional T cells, their regulation is recognized as a factor in the maintenance of brain homeostasis (Figure 2).

Early studies used polymerase chain reaction (PCR) analysis to show that diverse types of γδ TCRs are expressed in normal brain tissue (48). Currently, it is widely agreed that a large number of γδ T cells exist in the meninges instead of the normal brain parenchyma. Studies showed that γδ T cells derived from the fetus infiltrate the meninges after birth through the CXCR6–CXCL16 axis and are maintained by slow self-renewal (7, 49). Interestingly, these γδ T cells display sufficient IL-17-producing capability that is independent of inflammatory signals and are more activated than peripheral cells in the spleen. Ribeiro et al. proposed that T-cell receptor engagement in combination with signals derived from commensal microbes contributes to IL-17A production. Functional studies using IL-17-knockout mice and γδ TCR-knockout mice demonstrated that meningeal IL-17-producing γδ T cells are required for short-term memory maintenance and plasticity of glutamatergic synapses. IL-17 promotes the secretion of brain-derived neurotrophic factor (BDNF) from glial cells in the mouse hippocampus, which facilitates the synaptic plasticity of neurons to maintain short-term memory (7). Meanwhile, another study demonstrated that the release of IL-17 promotes anxiety-like behaviors by contributing to the proper release of neurotransmitters from the excitatory synapses of medial prefrontal cortex (mPFC) neurons in an IL-17R-dependent manner (49). These two studies found that IL-17-producing γδ T cells promote brain homeostasis, although some questions remain to be resolved. For example, how do cytokines of meningeal origin function in the parenchyma? How do meningeal γδ T cells sustain the production of IL-17? Does IL-17 act in an IL-17R-dependent manner?

Recently, a study showed that MAIT cells can be found in the meninges but are absent in brain parenchymal regions, and they exhibit high expression of antioxidant proteins that promote the maintenance of meningeal MAIT cells. MAIT cell deficiency results in reactive oxygen species (ROS) accumulation and barrier leakage, which results in inflammation of the brain parenchyma and cognitive decline in the brains of 7-month-old mice (50). Thus, MAIT cells contribute to the maintenance of physiological neural function by preserving the integrity of the meningeal barrier as well as meningeal homeostasis (14, 50, 51). These reports indicate that both γδ T cells and MAIT cells are involved in maintaining brain homeostasis. Whether NKT cells also exist in the meninges and contribute to brain homeostasis is a matter that needs to be investigated in the future.

Unconventional T cells in brain injury

Both acute brain injuries, including cerebral ischemic stroke, hemorrhagic stroke, and brain traumatic injury, and chronic brain injuries resulting from autoimmune-related attacks, such as multiple sclerosis, entail a high risk of mortality and disability. The recognition of DAMPs released from damaged brains initiates strong inflammatory responses in the brain parenchyma, which promotes the recruitment of peripheral immune cells, such as dendritic cells, neutrophils, and T cells to the region of injury (3, 52). Many reports have demonstrated that immune responses-

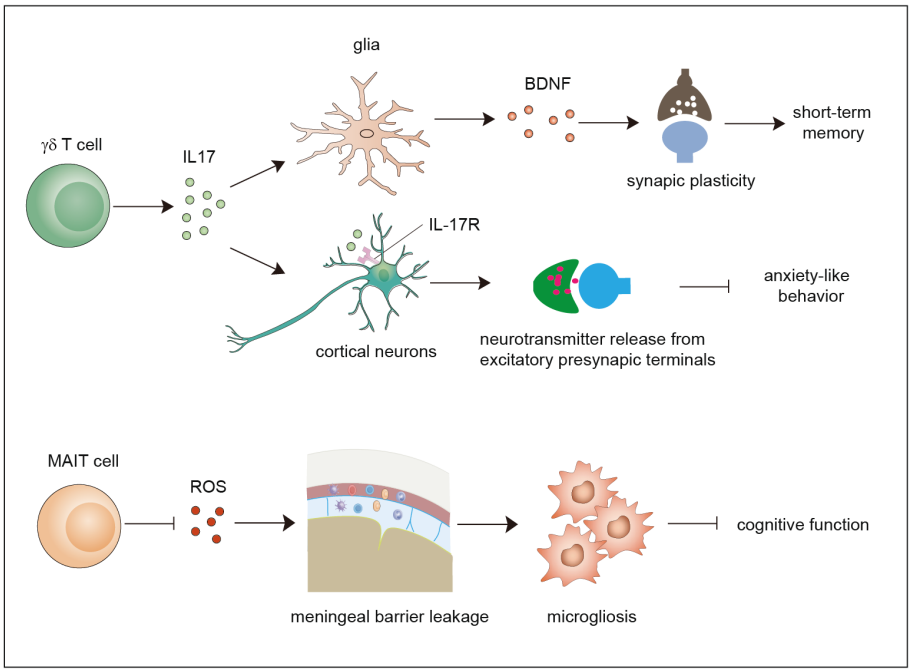

FIGURE 2
Functions of γδ T cells and MAIT cells in brain homeostasis. Meningeal γδ T cells mainly produce IL-17. The released IL-17 cytokine can on one hand act on glial cells to trigger BDNF expression to improve synaptic plasticity which potentiates short-term memory, and on the other hand directly act on neurons through IL-17R to enhance the release of neurotransmitters from excitatory presynaptic terminals to avoid anxiety-like behavior. MAIT cells express antioxidant molecules to preserve meningeal barrier homeostasis and integrity and protect the brain from cognitive decline. IL-17, interleukin 17; BDNF, brain-derived neurotrophic factor.

induced by peripheral immune cells regulate the pathogenesis of brain injury as well as long-term functional recovery (3, 53). Therefore, the functional properties of unconventional T cells in acute and chronic cerebral injury cannot be ignored.

Ischemic stroke

Ischemic stroke is a devastating brain injury with high mortality and morbidity worldwide. Early reperfusion by intravenous alteplase and thrombectomy remains an effective treatment but leads to secondary brain damage owing to ischemia/reperfusion injury. Therefore, there is an urgent need to understand the mechanism and alleviate brain damage (54). Multiple studies have elucidated the roles of peripheral T cells in various stages of ischemic stroke (55–57), which may provide novel perspectives on immunotherapies for ischemic stroke. Previous studies have revealed the infiltration of unconventional T cells in the brain parenchyma (15, 58) and alternations in the number of unconventional T cells in the peripheral blood (59), indicating the involvement of unconventional T cells in ischemic cerebral injury (Table 2).

As a bridging system between innate and adaptive immunity, iNKT cells accumulate in the ischemic hemisphere or peripheral blood in mice or rats subjected to middle cerebral artery occlusion (MCAO) or patients suffering stroke. In addition, these infiltrating iNKT cells primarily produce Th2-like cytokines, including IL-10 and IL-5, and few Th1-like cytokines, such as IL-12 and IFN-γ (15, 69–71). The influence of iNKT cells on the severity of cerebral ischemic stroke depends on the study methods and mouse model used. Administration of the specific iNKT cell-activating ligand α-GalCer, compared with a vehicle control, increases infarct volume and improves neurological deficiency and brain edema after pMCAO (62). Nevertheless, depletion of iNKT cells by using $Cd1d^{-/-}$ mice fails to alter infarct volume after tMCAO (60, 61) but the mice showed greater pulmonary damage, decreased survival rate, and more infiltration of neutrophils, a characteristic of poststroke pulmonary infections. Within the currently feasible conditions of clinical treatment, stroke-induced immunosuppression is detrimental to the human body, as it increases the risk of poststroke infections, especially infections of the lung and urinary tract (55, 56, 72). Several studies provide a mechanistic explanation of this phenomenon, and T lymphocytes are involved in modulating poststroke-induced immunosuppression as well as the subsequent infections (73). Interestingly, upon ischemia, iNKT cells exhibit restricted crawling and produce more Th2-like cytokines and fewer Th1-like cytokines, a set of conditions that are present in patients with acute ischemic stroke (60, 71). Selective immunomodulation of iNKT cells with α-GalCer or propranolol, a nonspecific β-adrenergic receptor blocker, promotes the production of proinflammatory cytokines and restricts pulmonary infection after ischemic stroke (60). Therefore, iNKT cells are involved in poststroke infections, and modulating iNKT cell function with the corresponding ligand may provide a potential way to alleviate stroke-associated infections. Thus far, it remains unknown how

TABLE 2 Direct evidence of unconventional T cells in ischemic stroke.

Ischemic stroke models	Study methods	Cell type	Effects on disease progression	Reference
tMCAO	$CD1d^{-/-}$ mice	type I and II NKT cell	Fail to influence infarct volume, facilitate post-stroke immune suppression in $CD1d^{-/-}$ mice	60, 61
tMCAO	α-Galcer injection	type I NKT cell	α-Galcer injection inhibits post-stroke infection	60
pMCAO	α-Galcer or its analog injection	type I NKT cell	α-Galcer injection increases infarct volume and aggregate brain edema	62
tMCAO	$MR1^{-/-}$ mice	MAIT cell	MAIT cell deficiency leads to a reduction of infarction and an improvement of neurological dysfunction.	63
tMCAO	DNT cell transfer to $Rag1^{-/-}$ mice	DNT cell	DNTs enhance neuroinflammatory responses and exacerbate ischemic brain injury by modulating the FasL/PTPN2/TNF-α signaling axis.	64
tMCAO	TCRγδ KO mice	γδ T cell	γδ T cell ameliorated the I/R injury	65
tMCAO	IL-17A blocking antibody	IL-17-producing γδ T cell	IL-17 neutralization reduces neutrophil invasion and protects mice from ischemic injury	66
tMCAO	$CCR6^{-/-}$ mice	IL-17-producing γδ T cell	CCR6 triggers the infiltration of IL-17-producing γδ T cells to exacerbate brain injury	67
tMCAO	TCRγδ KO mice	γδ T cell	γδ T cells-deficiency alleviates motor function injury and reduces the volume of brain infarction	68

tMCAO, transient middle cerebral artery occlusion; pMACO, permanent middle cerebral artery occlusion.

iNKT cells are activated after ischemia, considering their restricted recognition of lipid antigens.

γδ T cells also accumulate in the peripheral blood or brain parenchyma of stroke patients as well as MCAO-subjected mice as early sensors of ischemic brain injury (65–67, 74). Notably, brain-infiltrating γδ T cells are the main sources of IL-17, one of the main neuroinflammatory cytokines released after ischemia (65). The migration of IL-17-producing γδ T cells into the ischemic hemisphere relies on C-C chemokine receptor type 6 (CCR6) (67). Genetic depletion or antibody blockade of γδ T cells or genetic blockade of $IL\text{-}17^+$ γδ T-cells infiltration leads to improved neurological outcomes by regulating neutrophil recruitment and BBB integrity (65–68). Interestingly, alterations in the intestinal flora-induced by antibiotic treatment substantially alleviated ischemic brain damage in mice. The priming of intestinal dendritic cells (DC) by altered commensal bacteria results in local Treg cell expansion and $IL\text{-}17^+$ γδ T cells suppression, which further leads to decreased migration of intestinal IL-17-producing γδ T cells into the brain meninges and protects the brain against ischemic injury (75). This study confirms the vital regulation of the microbiota–gut–brain axis in response to cerebral ischemic stroke. Clinical and experimental studies indicate that gut microbial composition is altered after ischemia (76–79); stroke risks and stroke outcomes are influenced by the composition of the gut microbiota (75, 77, 80). For instance, antibiotic treatment or fecal matter transplantation from young mice into aged mice all improve stroke outcomes by changing gut microbiota (81). Although long-term regulation is mainly mediated by the vagus nerve and by metabolites, immune cells such as γδ T cells also contribute to the modulatory effect of microbiota on brain injury (75, 82).

Likewise, the infiltration of MAIT cells is increased early after ischemic stroke. MR1 deficiency or suppression of MAIT cell activation using a suppressive MR1 ligand decreases infarct volume, improves neurological deficits, and reduces microglial activation as well as the production of proinflammatory cytokines (63), which indicates that the use of MAIT cells may serve as a new method for the treatment of acute ischemic stroke by regulating neuroinflammation.

Apart from iNKT cells, MAIT cells and γδ T cells, DNT ($CD3^+CD4^-CD8^-$) cells are another unconventional T-cell subset that shows positive expression of T lymphocyte antigen receptors but negative expression of CD4 and CD8 markers (83). One report showed that DNT cells are involved in poststroke neuroinflammation in patients and animal models (64). After stroke, DNT cells accumulate in peripheral blood as well as in the ischemic penumbra. The infiltrating DNTs in the brain parenchyma exacerbate brain injury and promote neuroinflammatory responses via the FasL/PTPN2/TNF-α signaling axis. Blockade of the above pathway restricts DNT-mediated neuroinflammation and improves the outcomes of stroke (64). Many studies have observed and reported functional subsets of NKT cells, γδ T cells or MAIT cells based on CD4 and CD8 expression, including DP or DN phenotypes; thus, CD4/CD8 DP or DN cells are likely to be a mixture of many cell subpopulations with this phenotype, in which some functional subsets of NKT, γδ T cells or MAIT cells might be

included (84–89). Therefore, whether these DNT cells include NKT cells, γδ T cells or MAIT cells is a matter that still needs further investigation.

Hemorrhagic stroke

Hemorrhagic stroke, which accounts for 10-20% of all strokes has considerable morbidity and mortality, and the most common type of hemorrhagic stroke is intracerebral hemorrhage (ICH) (90). Recent reports have shown that neuroinflammation and host immune responses contribute to the pathophysiology of hemorrhagic stroke (91, 92). As the BBB is also damaged during hemorrhagic stroke, the role of brain parenchyma-infiltrating peripheral immune cells has been studied (93), although their role is slightly more limited in hemorrhagic stroke than ischemic stroke. Previous studies demonstrated that both $CD4^+$ T and $CD8^+$ T lymphocyte numbers are increased in murine models of intracerebral hemorrhage, which are associated with inflammatory damage during hemorrhagic stroke (94, 95). Regulatory T (Treg) cell, an immunosuppressive T-cell subset, exerts neuroprotective effects in ICH and subarachnoid hemorrhage (SAH) (96, 97). At present, the known role of unconventional T cells in hemorrhagic stroke is very limited. However, considering the regulatory roles of unconventional T cells in ischemic stroke, the function of unconventional T cells in hemorrhagic stroke is also worth investigating in the future.

Traumatic brain injury

Traumatic brain injury (TBI) is another class of brain injury that involves complex neurological processes including acute molecular alternations and chronic neurocognitive decline (98, 99). In peripheral blood from trauma patients, circulating NKT cell and MAIT cell numbers are reduced (100), and NKT cells show reduced proliferative capacity and IFNγ production in response to α-GalCer stimulation, which may affect disease outcomes (100). Mice with γδ T cell-deficiency exhibit alleviated inflammation in the acute phase of experimental TBI and show improved cognitive function in the chronic phase of TBI. In addition, different subsets of γδ T cells play distinct roles in TBI-induced brain injury. Vγ1 γδ T cells infiltrate the brain parenchyma and produce IFN-γ and IL-17 to trigger microglia-mediated neuroinflammation, whereas Vγ4 γδ T-cell subsets secrete TGF-β to maintain microglial homeostasis and reduce TBI-induced brain injury (101). The roles of different unconventional T-cell subsets in TBI or other forms of brain injury and their mechanism of action remain unknown.

Autoimmune-induced chronic brain injury

Multiple sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) that causes irreversible neurological injuries by attacking the protective myelin sheaths of neurons (102, 103). Autoreactive T-cells mediated demyelination has been intensively investigated and is currently regarded as the main attack on the CNS in MS, but the initial cause of the disease needs investigation (104, 105). Understanding the mechanism of neuron-specific T-cell generation can provide an important target for effective treatment. Experimental autoimmune encephalomyelitis (EAE), a widely accepted animal model of MS used by researchers, shares some pathological features with clinical MS, including strong neuroinflammatory responses, massive demyelination and axonal loss (106). Therefore, studies based on EAE models can provide important clues for the clinical treatment of MS.

Several studies examined circulating NKT cell numbers and functions in MS patients. NKT cell numbers change differently in the blood of MS patients from different studies (107–110). In addition, distinct cytokine production profiles have been observed in NKT cells from MS patients. $CD4^+$ NKT cells produce more IL-4 in RRMS (relapsing-remitting) patients than in patients with progressive MS or in healthy controls (111). However, NKT cells in secondary progressive MS patients display a proinflammatory response (112). Different types of MS or distinct stages of its progression may account for these conflicting findings.

The implication of NKT cells in EAE is elusive, although it has been investigated by many studies (Figure 3). Elimination of NKT cells by using mice from distinct backgrounds or EAE models exhibits conflicting effects. Some studies have observed no obvious effects (113, 114), whereas some studies have observed disease exacerbation in CD1d-knockout mice (115) as well as in Jα18-deficient mice (114, 116, 117). Similarly, administration of α-GalCer or its analog to activate NKT cells also exhibits contrasting effects on the outcome of EAE. Most studies have shown that iNKT cells activation alleviate the Th1 response, improve the Th2 response (113, 114, 118) or potentiate MDSC (119) and M2 macrophage differentiation (116) to improve EAE outcomes (113, 114, 116, 118, 119). Surprisingly, high doses of α-GalCer selectively elevate Th1 and Th17 responses by interacting with CD1d expressed on T cells but not type I NKT cells to exacerbate EAE progression (120). Furthermore, immunization of α-GalCer in combination with myelin antigens potentiates EAE in B10.PL mice by promoting the Th1 response and preventing EAE in C57BL/6 mice by increasing IL-4 production (121). Therefore, the outcome of EAE relies on the downstream targets that NKT cells activate. In addition to α-GalCer, sulfatide, a major component of the myelin sheath, can activate type II NKT cells, which suppress IFN-γ and IL-4 production by pathogenetic antigen-reactive T cells and protect mice against EAE in a CD1d-dependent manner (122). Notably, the first-in-human study using the iNKT cell stimulatory drug (2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-N-tetracosanoyl-2-amino-1,3,4-nonanetriol (OCH) based on previous mouse studies (118, 123) demonstrated the immunomodulatory effects of iNKT cells on MS patients, providing valuable information for the clinical application of OCH as a potential therapy for MS (124). Moreover, some currently available drugs targeting MS alter the properties of NKT cells (15).

γδ T cells accumulate to a significant extent in the brains of MS patients (125), and their frequency is also increased in the peripheral blood and cerebrospinal fluid of MS patients (126–

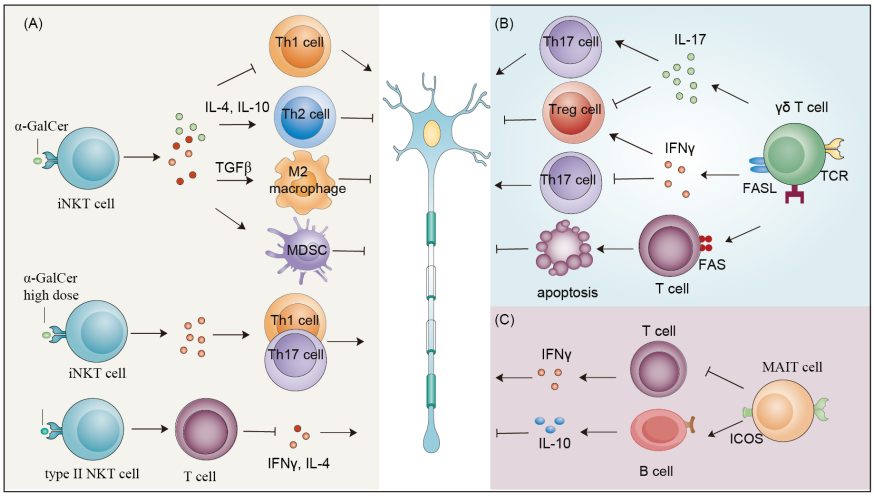

FIGURE 3
Effects of NKT cells, γδ T cells and MAIT cells on EAE. **(A)** Stimulating iNKT cells with α-GalCer or its analog can either reduce Th1 response or improve Th2 response, potentiate MDSC or M2 macrophage differentiation to improve EAE outcomes. High doses of α-GalCer selectively increase Th1 and Th17 responses to exacerbate EAE progression. Sulfatide activates type II NKT cells, which suppress IFN-γ and IL-4 production by pathogenetic antigen-reactive T cells and protect mice from EAE. **(B)** IL-17-producing γδ T cells exacerbate EAE progression by increasing inflammation by not only promoting Th17 cell function but also restraining the generation of Tregs. IFNγ-producing γδ T cells suppress the activity of Th17 cells and release chemokines to recruit Treg cells to reduce inflammatory signals. Moreover, γδ T cells can induce the apoptosis of encephalitogenic T cells through Fas/Fas ligand signaling and facilitate recovery from EAE. **(C)** MAIT cells alleviate the progression of EAE by decreasing inflammatory mediators and increasing IL-10 production. MDSC, myeloid-derived suppressor cell; EAE, experimental autoimmune encephalomyelitis; Treg, regulatory T cells.

128). Further phenotypical and functional analysis showed that circulating γδ T cells in relapsing-remitting multiple sclerosis (RRMS) patients have a reduction in the circulating $CCR5^+$ γδ T-cell subset with decreased EOMES and granzyme B mRNA abundance and increased production of IFN-γ (129). To better characterize the infiltrated γδ T cells, TCR repertoire analysis was performed, and oligoclonal expansion of γδ T cells was observed, suggesting their responses to common antigens (127, 130, 131). Accordingly, common antigens recognized by γδ T cells were reported. A subpopulation of γδ T cells in MS lesions respond to heat-shock proteins, including HSP70 (132) and HSP65 (133). In addition, sulfatide, a myelin-derived antigen, can also be recognized by γδ T cells in the MS brain indicating that lipid antigens can be presented to γδ T cells after MS (134). Thus, the number, specificity, and function of γδ T cells altered in MS patients, although the variation may be distinct in different types and progression stages of MS.

The specific subsets and effector function of γδ T cells in EAE have been investigated extensively (Figure 3). Several studies have suggested that diverse γδ T subsets are present in the brain in EAE. During the early stage of EAE, brain parenchyma-infiltrating γδ T cells harbor a restricted TCR repertoire, including Vδ1, Vδ5, Vδ4, Vγ1–3, and Vγ6, while during later phases, Vγ and Vδ are widely distributed in the brain (135, 136). During disease progression, peripheral γδ T cells can be induced to produce cytokines, especially IL-17, in response to stimulation with IL-23 and IL-1β, which are released by dendritic cells and microglia in EAE (137, 138). IL-23 or IL-15-activated γδ T cells exacerbate EAE progression by increasing inflammation by not only promoting Th17 cell function but also restraining the generation of Tregs (139–141). However, IFN-γ-producing γδ T cells suppress the activity of Th17 cells and release chemokines to recruit Treg cells to reduce inflammatory signals (139). Moreover, γδ T cells can induce the apoptosis of encephalitogenic T cells through the Fas/FasL signaling pathway and potentiate functional recovery after EAE (142). Notably, encouraging data have been obtained in several clinical trials targeting IL-17 in relapsing-remitting MS patients (143, 144). As an important cytokine released from T cells, IL-17 can, on one hand, directly inhibit oligodendrocyte progenitor cell (OPC) differentiation and exert toxic effects on OPCs and oligodendrocytes (145–147), altering myelin stability and regeneration after MS, and, on the other hand, regulate immune cell activation which indirectly affects myelination. Therefore, in the early stage of EAE, γδ T cells are more pathogenic than protective given that γδ T cells abundantly and predominantly release IL-17 in the brain.

As with NKT cells and γδ T cells, many studies have confirmed the infiltration of MAIT cells especially $CD8^+$ MAIT cells, into the brain upon activation by IL-18 or IL-23 triggered by enriched fungal content in the gut as observed through immunofluorescence staining or TCR repertoire analysis (148–152). Their number and function in the peripheral blood remain controversial, as one study observed no obvious difference between relapsing-remitting MS patients and healthy volunteers (153), while others showed a reduced frequency in relapsing-remitting MS patients (149, 154, 155). Deficiency of MAIT cells in mice exacerbates EAE progression by increasing inflammatory mediators and reducing the production of anti-inflammatory cytokine IL-10 (156) (Figure 3). Future studies are needed to investigate the roles of MAIT cells during different stages of multiple sclerosis.

In addition to MS, another autoimmune condition of interest is autoimmune encephalitis, a group of diseases with subacute or progressive inflammation of the brain that results in various neurological or psychiatric symptoms owing to immune cell-produced antibodies against neuronal cell surface or synaptic antigens (157, 158). Previous studies have implicated that autoimmune encephalitis usually occurs when viruses or tumors cause neuronal proteins to be exposed to the immune system. Conventional T-cells and B-cells-mediated immune responses are responsible for leading to this disease, although the released antigens or the first encountered immune cells determine which immune cells dominate in a certain type of autoimmune encephalitis (159–162). Currently, the role of unconventional T cells in autoimmune encephalitis remains unknown. Considering their recognition of diverse antigens and their rapid response after activation, their roles in autoimmune encephalitis are worth investigating.

Unconventional T cells in neurodegeneration

Neurodegenerative diseases (NDDs) which include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) represent a major threat to human health. Identifying the initial causes and molecular mechanisms underlying each disease is required for the effective treatment of neurodegeneration (163, 164). Emerging evidence has shown that peripheral immune cells infiltrate the brain and impact neurodegeneration (165). T cells are present in brain tissues of patients with neurodegeneration, although they are less abundant than the lymphocytes that infiltrate the brain in multiple sclerosis (166–168). Currently, it is believed that peripheral immune cells can impact the progression of NDDs, either after infiltrating into the brain or while staying in the periphery (165), and the roles of unconventional T cells in NDDs are under investigation (Table 3).

The number of γδ T cells in the peripheral blood varies, with one study showing an increased number (176) and one study showing a reduced number (174). The variation may result from the different controls the authors chose as the former study used the blood from patients with neurological diseases other than PD, while the latter used healthy subjects. $CD4^+$ helper type 17 (Th17) and γδ (γδ17) T cells are two major classes of IL-17-producing cells, and have emerged as key players in the progression of diseases (75, 140). Elevated production of IL-17 in patients with PD and AD was observed (173, 177). Accordingly, Th17 cells have been revealed to exacerbate neuroinflammation and neurodegeneration in both PD and AD models (175, 178). Concomitant with the onset of cognitive decline, Brigas et al. observed that the meninges and brains of female mice but not male mice exhibit an accumulation of IL-17-producing γδ T cells by using the 3xTg-AD mouse model. More importantly, in the early stages of the disease, neutralization of IL-17 markedly prevents synaptic dysfunction independent of tau or Aβ pathology or BBB disruption to maintain short-term cognitive

TABLE 3 Evidence of unconventional T cells in neurodegeneration.

Neurological Diseases (Model or Sample)		Study tools	Cell subsets	Effects on NDDs	Reference
AD	5XFAD mice	MR1-deficient mice (IF)	MAIT cell	MR1-deficient 5XFAD mice exhibit a reduced plaque burden than WT mice.	169
	3xTg-AD mice	IL-17 blockage (flow cytometry)	γδ T cell	IL-17-producing γδ T cells were observed to increase in the brain and the meninges of female, but not male mice, by using the 3xTg-AD mice model; Blocking IL-17 prevents short-term cognitive deficits at early stages of the disease by improving synaptic dysfunction.	170
ALS	ALS patients' blood	flow cytometry	NKT cell	NKT cell ($CD3^+CD16^+CD56^+$) number was significantly increased.	171
	mSOD1 mice model	α-Galcer injection (flow cytometry)	type I NKT cell	NKT cell number (NK1.1 Cd1d-tetramer) increases dramatically in ALS mice; Immunomodulation of NKT cells using α-GalCer prolongs the life span of SOD1 mice.	172
PD	PD patient's blood	flow cytometry	γδ T cell	γδ T cell number was increased in the blood and CSF of the blood of PD patients compared with patients with other neurological diseases.	173
	PD patient's blood	flow cytometry	γδ T cell	γδ T cell number was reduced in patients with Parkinson's disease compared with age-matched health controls.	174, 175
	PD patient's blood	flow cytometry	iNKT cell	The frequency and absolute number of iNKT cells are reduced in the blood of PD patients compared with healthy controls.	170

AD, Alzheimer's Disease; PD, Parkins's Disease; NDDs, Neurodegenerative diseases.

function (170). Several reports have shown that γδ T cells can be stimulated by heat shock proteins (HSPs) and when cells are subjected to environmental stress, they express increased amounts of intracellular HSPs (179, 180) indicating antigenic involvement in NDDs. ELISAs of the CSF of PD patients revealed increased levels of HSP65 and HSP70 compared with corresponding controls, while no difference was observed in the serum (181). Whether HSPs can act as antigens for γδ T cells in other NDDs needs further investigation. As the ligands of γδ T cells are more diverse than other unconventional T cells, it will be interesting to test whether the aggregated proteins in NDD can directly activate or repress γδ T- cell activation.

Considering the implication of neuroinflammation in neurodegeneration, the evidence of NKT cells in neurodegenerative disease is limited (182). Zhou et al. showed that the frequency and number of iNKT cells are decreased in the peripheral blood of PD patients compared with healthy control subjects (174). In addition, one study showed obvious accumulation of NKT cells in the peripheral blood of ALS patients (171). Moreover, the number of iNKT cells was observed to increase in the liver, spleen, and spinal cord of transgenic mouse models with ALS (172). Furthermore, α-GalCer-induced NKT cell activation was found to suppress astrogliosis and alleviate motor neuronal death in the spinal cord. Modulation of NKT cells initiates a cytokine shift in the liver and facilitates T cell recruitment into the spinal cord which extends the lifespan of mSOD1 mice (172). For NKT cells, it is technically difficult to directly test their existence *in situ* owing to the limited methods available to specifically label this subset. In addition, it remains unknown whether NKT cell deficiency affects NDD progression. Considering the high content of glycolipids in the brain tissue (183), more research is needed to gain better insight into NKT cells in other NDDs.

Similar to NKT cells and γδ T cells, MAIT cells play a limited role in NDDs is also limited. One study revealed an increase in MAIT cell numbers and elevation of activation in the brains of 5XFAD mice. More importantly, a significantly reduced plaque burden was observed in MR1-deficient 5XFAD mice compared with WT mice indicating the contributing role of MAIT cells in the development of AD pathology (169), although the detailed molecular mechanism is unknown.

Potential clinical applications of unconventional T cells

The above-summarized roles of unconventional T cells indicate that modulating unconventional T cells in the following ways may have some clinical applications. First, the alternations of unconventional T cell subsets may be used as a diagnostic strategy. The frequency and number of NKT cells, MAIT cells and γδ T cells in the peripheral blood of patients suffering from brain injury (71, 107, 108, 153) and neurodegeneration (174, 176) significantly change. Monitoring the alternations in these cells during different stages of disease progression may provide an auxiliary way to diagnose disease progression. In addition, some drugs can be developed to block the function of these unconventional T cells. For example, abrogation of MAIT cell response can be achieved by administering MR1 blocking antibodies (184), while antibodies blocking BTN2 or BTN3 can be used to inhibit Vγ9Vδ2 T cell responses (185). Moreover, targeted immunotherapies are appreciated to manipulate unconventional T cells to modulate disease progression. On one hand, agonists of NKT cells, MAIT cells and γδ T cells, such as α-GalCer, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU) and aminobisphosphonates can activate the immune response under immune-suppressive conditions (25, 186, 187), and on the other hand, adoptive transfer of neuroprotective subsets of unconventional cells is another promising therapeutic strategy. With the progress that has been made in our understanding of the roles and mechanisms of unconventional T cells in brain injury and neurodegeneration, future clinical applications are within reach.

Conclusion and perspectives

T lymphocytes play critical roles in both brain homeostasis and neurological diseases. Unconventional T cells, a special group of T lymphocytes, are beginning to have their roles in the brain characterized. In this review, we present evidence of the crosstalk between unconventional T cells and the brain in the maintenance of brain homeostasis, brain injury and neurodegeneration. It is conceivable that unconventional T cells in the meninges facilitate homeostatic regulation of the brain. Nevertheless, owing to different investigation methods and tools, conflicting evidence about the roles of these unconventional T-cell subsets in brain injury has been reported. Therefore, by better understanding the regulation and detailed mechanisms of detrimental effects versus beneficial effects in regulating brain injury and neurodegeneration, we believe effective and novel therapeutic targets will be discovered in the future.

Questions to be resolved in the future:

(1) How do meningeal unconventional T cells communicate with glial cells and neurons in the brain parenchyma under physiological and pathological conditions?

(2) Aside from MAIT and γδ T cells, do other unconventional T cells exist in meninges and regulate brain homeostasis?

(3) After injury, does the brain harbor some antigens that activate specific unconventional T-cell subsets?

(4) Do unconventional T cells directly interact with glial cells and neurons after infiltration during brain injury and neurodegeneration?

(5) How can single-cell sequencing techniques be used to define unconventional T-cell subset specificity in the brain under either physiological or pathological conditions?

(6) How can *in-situ* detection methods for unconventional T cells in the brain be improved?

(7) How can the ratio and function of these unconventional T cells be calibrated to maintain brain homeostasis and modulate disease progression?

Author contributions

ML: Data curation, Investigation, Methodology, Software, Writing – original draft. ZZ: Data curation, Funding acquisition, Investigation, Methodology, Writing – original draft. YC: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Funding

The authors declare financial support was received for the research, authorship, and/or publication of this article. This work is supported by National Natural Science Foundation of China (82101380) and Natural Science Foundation of Shandong Province (ZR202102280316) to Zhaolong Zhang; Science and Technology Support Plan for Youth Innovation of Colleges and Universities of Shandong Province of China (2022KJ146) to YC.

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

References

1. Ahn JH, Cho H, Kim JH, Kim SH, Ham JS, Park I, et al. Meningeal lymphatic vessels at the skull base drain cerebrospinal fluid. *Nature* (2019) 572(7767):62–6. doi: 10.1038/s41586-019-1419-5

2. Da Mesquita S, Fu Z, Kipnis J. The meningeal lymphatic system: A new player in neurophysiology. *Neuron* (2018) 100(2):375–88. doi: 10.1016/j.neuron.2018.09.022

3. Zhang Z, Lv M, Zhou X, Cui Y. Roles of peripheral immune cells in the recovery of neurological function after ischemic stroke. *Front Cell Neurosci* (2022) 16:1013905. doi: 10.3389/fncel.2022.1013905

4. Croese T, Castellani G, Schwartz M. Immune cell compartmentalization for brain surveillance and protection. *Nat Immunol* (2021) 22(9):1083–92. doi: 10.1038/s41590-021-00994-2

5. Kipnis J, Cohen H, Cardon M, Ziv Y, Schwartz M. T cell deficiency leads to cognitive dysfunction: implications for therapeutic vaccination for schizophrenia and other psychiatric conditions. *Proc Natl Acad Sci USA* (2004) 101(21):8180–5. doi: 10.1073/pnas.0402268101

6. Ziv Y, Ron N, Butovsky O, Landa G, Sudai E, Greenberg N, et al. Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. *Nat Neurosci* (2006) 9(2):268–75. doi: 10.1038/nn1629

7. Ribeiro M, Brigas HC, Temido-Ferreira M, Pousinha PA, Regen T, Santa C, et al. Meningeal gammadelta T cell-derived IL-17 controls synaptic plasticity and short-term memory. *Sci Immunol* (2019) 4(40). doi: 10.1126/sciimmunol.aay5199

8. Kaminski H, Couzi L, Eberl M. Unconventional T cells and kidney disease. *Nat Rev Nephrol* (2021) 17(12):795–813. doi: 10.1038/s41581-021-00466-8

9. Pellicci DG, Koay HF, Berzins SP. Thymic development of unconventional T cells: how NKT cells, MAIT cells and gammadelta T cells emerge. *Nat Rev Immunol* (2020) 20(12):756–70. doi: 10.1038/s41577-020-0345-y

10. Constantinides MG, Belkaid Y. Early-life imprinting of unconventional T cells and tissue homeostasis. *Science* (2021) 374(6573):eabf0095. doi: 10.1126/science.abf0095

11. Mayassi T, Barreiro LB, Rossjohn J, Jabri B. A multilayered immune system through the lens of unconventional T cells. *Nature* (2021) 595(7868):501–10. doi: 10.1038/s41586-021-03578-0

12. Iwabuchi K, Van Kaer L. Editorial: role of CD1- and MR1-restricted T cells in immunity and disease. *Front Immunol* (2019) 10:1837. doi: 10.3389/fimmu.2019.01837

13. Park JH, Kang I, Lee HK. gammadelta T cells in brain homeostasis and diseases. *Front Immunol* (2022) 13:886397. doi: 10.3389/fimmu.2022.886397

14. Bird L. Meningeal MAIT cells preserve brain homeostasis. *Nat Rev Immunol* (2023) 23(1):4. doi: 10.1038/s41577-022-00824-y

15. Cui Y, Wan Q. NKT cells in neurological diseases. *Front Cell Neurosci* (2019) 13:245. doi: 10.3389/fncel.2019.00245

16. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nat Immunol* (2015) 16(11):1114–23. doi: 10.1038/ni.3298

17. Legoux F, Lantz O. Introduction to the special issue on "unconventional T cells". *Semin Immunol* (2022) 61-64:101666. doi: 10.1016/j.smim.2022.101666

18. Baranek T, de Amat Herbozo C, Mallevaey T, Paget C. Deconstructing iNKT cell development at single-cell resolution. *Trends Immunol* (2022) 43(7):503–12. doi: 10.1016/j.it.2022.04.012

19. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi: 10.1146/annurev.immunol.25.022106.141711

20. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. *Nat Immunol* (2010) 11(3):197–206. doi: 10.1038/ni.1841

21. Nishioka Y, Masuda S, Tomaru U, Ishizu A. CD1d-restricted type II NKT cells reactive with endogenous hydrophobic peptides. *Front Immunol* (2018) 9:548. doi: 10.3389/fimmu.2018.00548

22. Yoon JH, Seo Y, Jo YS, Lee S, Cho E, Cazenave-Gassiot A, et al. Brain lipidomics: From functional landscape to clinical significance. *Sci Adv* (2022) 8(37):eadc9317. doi: 10.1126/sciadv.adc9317

23. Godfrey DI, Koay HF, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. *Nat Immunol* (2019) 20(9):1110–28. doi: 10.1038/s41590-019-0444-8

24. Ribot JC, Lopes N, Silva-Santos B. gammadelta T cells in tissue physiology and surveillance. *Nat Rev Immunol* (2021) 21(4):221–32. doi: 10.1038/s41577-020-00452-4

25. Godfrey DI, Le Nours J, Andrews DM, Uldrich AP, Rossjohn J. Unconventional T cell targets for cancer immunotherapy. *Immunity* (2018) 48(3):453–73. doi: 10.1016/j.immuni.2018.03.009

26. Deseke M, Prinz I. Ligand recognition by the gammadelta TCR and discrimination between homeostasis and stress conditions. *Cell Mol Immunol* (2020) 17(9):914–24. doi: 10.1038/s41423-020-0503-y

27. Willcox BE, Willcox CR. gammadelta TCR ligands: the quest to solve a 500-million-year-old mystery. *Nat Immunol* (2019) 20(2):121–8. doi: 10.1038/s41590-018-0304-y

28. Fichtner AS, Ravens S, Prinz I. Human gammadelta TCR repertoires in health and disease. *Cells* (2020) 9(4). doi: 10.3390/cells9040800

29. Papotto PH, Yilmaz B, Silva-Santos B. Crosstalk between gammadelta T cells and the microbiota. *Nat Microbiol* (2021) 6(9):1110–7. doi: 10.1038/s41564-021-00948-2

30. Mao AP, Constantinides MG, Mathew R, Zuo Z, Chen X, Weirauch MT, et al. Multiple layers of transcriptional regulation by PLZF in NKT-cell development. *Proc Natl Acad Sci USA* (2016) 113(27):7602–7. doi: 10.1073/pnas.1601504113

31. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13(2):101–17. doi: 10.1038/nri3369

32. Chang PP, Barral P, Fitch J, Pratama A, Ma CS, Kallies A, et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol* (2011) 13(1):35–43. doi: 10.1038/ni.2166

33. Engel I, Seumois G, Chavez L, Samaniego-Castruita D, White B, Chawla A, et al. Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat Immunol* (2016) 17(6):728–39. doi: 10.1038/ni.3437

34. Dhodapkar MV, Kumar V. Type II NKT cells and their emerging role in health and disease. *J Immunol* (2017) 198(3):1015–21. doi: 10.4049/jimmunol.1601399

35. Hinks TSC, Zhang XW. MAIT cell activation and functions. *Front Immunol* (2020) 11:1014. doi: 10.3389/fimmu.2020.01014

36. Chen Z, Wang H, D'Souza C, Sun S, Kostenko L, Eckle SB, et al. Mucosal-associated invariant T-cell activation and accumulation after in *vivo* infection depends on microbial riboflavin synthesis and co-stimulatory signals. *Mucosal Immunol* (2017) 10(1):58–68. doi: 10.1038/mi.2016.39

37. van Wilgenburg B, Loh L, Chen Z, Pediongco TJ, Wang H, Shi M, et al. MAIT cells contribute to protection against lethal influenza infection in vivo. *Nat Commun* (2018) 9(1):4706. doi: 10.1038/s41467-018-07207-9

38. Bonneville M, O'Brien RL, Born WK. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* (2010) 10 (7):467–78. doi: 10.1038/nri2781

39. Dieli F, Troye-Blomberg M, Ivanyi J, Fournie JJ, Krensky AM, Bonneville M, et al. Granulysin-dependent killing of intracellular and extracellular Mycobacterium tuberculosis by Vgamma9/Vdelta2 T lymphocytes. *J Infect Dis* (2001) 184(8):1082–5. doi: 10.1086/323600

40. Turrigiano G. Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function. *Cold Spring Harb Perspect Biol* (2012) 4(1):a005736. doi: 10.1101/cshperspect.a005736

41. Segarra M, Aburto MR, Acker-Palmer A. Blood-brain barrier dynamics to maintain brain homeostasis. *Trends Neurosci* (2021) 44(5):393–405. doi: 10.1016/j.tins.2020.12.002

42. Pluvinage JV, Wyss-Coray T. Systemic factors as mediators of brain homeostasis, ageing and neurodegeneration. *Nat Rev Neurosci* (2020) 21(2):93–102. doi: 10.1038/s41583-019-0255-9

43. Meizlish ML, Franklin RA, Zhou X, Medzhitov R. Tissue homeostasis and inflammation. *Annu Rev Immunol* (2021) 39:557–81. doi: 10.1146/annurev-immunol-061020-053734

44. Alves de Lima K, Rustenhoven J, Kipnis J. Meningeal immunity and its function in maintenance of the central nervous system in health and disease. *Annu Rev Immunol* (2020) 38:597–620. doi: 10.1146/annurev-immunol-102319-103410

45. Brombacher TM, Nono JK, De Gouveia KS, Makena N, Darby M, Womersley J, et al. IL-13-mediated regulation of learning and memory. *J Immunol* (2017) 198 (7):2681–8. doi: 10.4049/jimmunol.1601546

46. Filiano AJ, Xu Y, Tustison NJ, Marsh RL, Baker W, Smirnov I, et al. Unexpected role of interferon-gamma in regulating neuronal connectivity and social behaviour. *Nature* (2016) 535(7612):425–9. doi: 10.1038/nature18626

47. Oetjen LK, Mack MR, Feng J, Whelan TM, Niu H, Guo CJ, et al. Sensory neurons co-opt classical immune signaling pathways to mediate chronic itch. *Cell* (2017) 171 (1):217–228 e13. doi: 10.1016/j.cell.2017.08.006

48. Szymanska B, Rajan AJ, Gao YL, Tronczynska E, Brosnan CF, Selmaj K. Evidence for gammadelta T cells with a restricted Vgamma6 junctional region in the normal mouse central nervous system. *J Neuroimmunol* (1999) 100(1-2):260–5. doi: 10.1016/s0165-5728(99)00204-0

49. Alves de Lima K, Rustenhoven J, Da Mesquita S, Wall M, Salvador AF, Smirnov I, et al. Meningeal gammadelta T cells regulate anxiety-like behavior via IL-17a signaling in neurons. *Nat Immunol* (2020) 21(11):1421–9. doi: 10.1038/s41590-020-0776-4

50. Zhang Y, Bailey JT, Xu E, Singh K, Lavaert M, Link VM, et al. Mucosal-associated invariant T cells restrict reactive oxidative damage and preserve meningeal barrier integrity and cognitive function. *Nat Immunol* (2022) 23(12):1714–25. doi: 10.1038/s41590-022-01349-1

51. Klein RS. Meningeal MAIT cells maintain meningeal and brain function. *Nat Immunol* (2022) 23(12):1659–61. doi: 10.1038/s41590-022-01368-y

52. Zhang W, Zhao J, Wang R, Jiang M, Ye Q, Smith AD, et al. Macrophages reprogram after ischemic stroke and promote efferocytosis and inflammation resolution in the mouse brain. *CNS Neurosci Ther* (2019) 25(12):1329–42. doi: 10.1111/cns.13256

53. Bao W, Lin Y, Chen Z. The Peripheral Immune System and Traumatic Brain Injury: Insight into the role of T-helper cells. *Int J Med Sci* (2021) 18(16):3644–51. doi: 10.7150/ijms.46834

54. Pan J, Konstas AA, Bateman B, Ortolano GA, Pile-Spellman J. Reperfusion injury following cerebral ischemia: pathophysiology, MR imaging, and potential therapies. *Neuroradiology* (2007) 49(2):93–102. doi: 10.1007/s00234-006-0183-z

55. Chamorro A, Meisel A, Planas AM, Urra X, van de Beek D, Veltkamp R. The immunology of acute stroke. *Nat Rev Neurol* (2012) 8(7):401–10. doi: 10.1038/nrneurol.2012.98

56. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. *Nat Med* (2011) 17(7):796–808. doi: 10.1038/nm.2399

57. Shi L, Sun Z, Su W, Xu F, Xie D, Zhang Q, et al. Treg cell-derived osteopontin promotes microglia-mediated white matter repair after ischemic stroke. *Immunity* (2021) 54(7):1527–1542 e8. doi: 10.1016/j.immuni.2021.04.022

58. Wang L, Yao C, Chen J, Ge Y, Wang C, Wang Y, et al. gammadelta T cell in cerebral ischemic stroke: characteristic, immunity-inflammatory role, and therapy. *Front Neurol* (2022) 13:842212. doi: 10.3389/fneur.2022.842212

59. Zhou C, Rao W, Zhou X, He D, Li Z, Dashtsoodol N, et al. Alteration of circulating unconventional T cells in cerebral ischemia: an observational study. *Sci Rep* (2022) 12(1):10078. doi: 10.1038/s41598-022-14343-2

60. Wong CH, Jenne CN, Lee WY, Leger C, Kubes P. Functional innervation of hepatic iNKT cells is immunosuppressive following stroke. *Science* (2011) 334 (6052):101–5. doi: 10.1126/science.1210301

61. Kleinschnitz C, Schwab N, Kraft P, Hagedorn I, Dreykluft A, Schwarz T, et al. Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood* (2010) 115(18):3835–42. doi: 10.1182/blood-2009-10-249078

62. Wang ZK, Xue L, Wang T, Wang XJ, Su ZQ. Infiltration of invariant natural killer T cells occur and accelerate brain infarction in permanent ischemic stroke in mice. *Neurosci Lett* (2016) 633:62–8. doi: 10.1016/j.neulet.2016.09.010

63. Nakajima S, Tanaka R, Yamashiro K, Chiba A, Noto D, Inaba T, et al. Mucosal-associated invariant T cells are involved in acute ischemic stroke by regulating neuroinflammation. *J Am Heart Assoc* (2021) 10(7):e018803. doi: 10.1161/JAHA.120.018803

64. Meng H, Zhao H, Cao X, Hao J, Zhang H, Liu Y, et al. Double-negative T cells remarkably promote neuroinflammation after ischemic stroke. *Proc Natl Acad Sci USA* (2019) 116(12):5558–63. doi: 10.1073/pnas.1814394116

65. Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, et al. Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. *Nat Med* (2009) 15(8):946–50. doi: 10.1038/nm.1999

66. Gelderblom M, Weymar A, Bernreuther C, Velden J, Arunachalam P, Steinbach K, et al. Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. *Blood* (2012) 120(18):3793–802. doi: 10.1182/blood-2012-02-412726

67. Arunachalam P, Ludewig P, Melich P, Arumugam TV, Gerloff C, Prinz I, et al. CCR6 (CC chemokine receptor 6) is essential for the migration of detrimental natural interleukin-17-producing gammadelta T cells in stroke. *Stroke* (2017) 48(7):1957–65. doi: 10.1161/STROKEAHA.117.016753

68. Dong X, Zhang X, Li C, Chen J, Xia S, Bao X, et al. gammadelta T cells aggravate blood-brain-barrier injury via IL-17A in experimental ischemic stroke. *Neurosci Lett* (2022) 776:136563. doi: 10.1016/j.neulet.2022.136563

69. Gelderblom M, Leypoldt F, Steinbach K, Behrens D, Choe CU, Siler DA, et al. Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. *Stroke* (2009) 40(5):1849–57. doi: 10.1161/STROKEAHA.108.534503

70. Lehmann J, Hartig W, Seidel A, Fuldner C, Hobohm C, Grosche J, et al. Inflammatory cell recruitment after experimental thromboembolic stroke in rats. *Neuroscience* (2014) 279:139–54. doi: 10.1016/j.neuroscience.2014.08.023

71. Wong CH, Jenne CN, Tam PP, Leger C, Venegas A, Ryckborst K, et al. Prolonged activation of invariant natural killer T cells and TH2-skewed immunity in stroke patients. *Front Neurol* (2017) 8:6. doi: 10.3389/fneur.2017.00006

72. Meisel C, Schwab JM, Prass K, Meisel A, Dirnagl U. Central nervous system injury-induced immune deficiency syndrome. *Nat Rev Neurosci* (2005) 6(10):775–86. doi: 10.1038/nrn1765

73. Prass K, Meisel C, Hoflich C, Braun J, Halle E, Wolf T, et al. Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J Exp Med* (2003) 198(5):725–36. doi: 10.1084/jem.20021098

74. He Y, Ma N, Xing C, Wang X, Xiao H, Zheng M, et al. Novel IL-6-secreting gammadeltaT cells increased in patients with atherosclerotic cerebral infarction. *Mol Med Rep* (2015) 11(2):1497–503. doi: 10.3892/mmr.2014.2796

75. Benakis C, Brea D, Caballero S, Faraco G, Moore J, Murphy M, et al. Commensal microbiota affects ischemic stroke outcome by regulating intestinal gammadelta T cells. *Nat Med* (2016) 22(5):516–23. doi: 10.1038/nm.4068

76. Liu Y, Luo S, Kou L, Tang C, Huang R, Pei Z, et al. Ischemic stroke damages the intestinal mucosa and induces alteration of the intestinal lymphocytes and CCL19 mRNA in rats. *Neurosci Lett* (2017) 658:165–70. doi: 10.1016/j.neulet.2017.08.061

77. Singh V, Roth S, Llovera G, Sadler R, Garzetti D, Stecher B, et al. Microbiota dysbiosis controls the neuroinflammatory response after stroke. *J Neurosci* (2016) 36 (28):7428–40. doi: 10.1523/JNEUROSCI.1114-16.2016

78. Houlden A, Goldrick M, Brough D, Vizi ES, Lenart N, Martinecz B, et al. Brain injury induces specific changes in the caecal microbiota of mice via altered autonomic activity and mucoprotein production. *Brain Behav Immun* (2016) 57:10–20. doi: 10.1016/j.bbi.2016.04.003

79. Stanley D, Moore RJ, Wong CHY. An insight into intestinal mucosal microbiota disruption after stroke. *Sci Rep* (2018) 8(1):568. doi: 10.1038/s41598-017-18904-8

80. Yamashiro K, Tanaka R, Urabe T, Ueno Y, Yamashiro Y, Nomoto K, et al. Gut dysbiosis is associated with metabolism and systemic inflammation in patients with ischemic stroke. *PloS One* (2017) 12(2):e0171521. doi: 10.1371/journal.pone.0171521

81. Spychala MS, Venna VR, Jandzinski M, Doran SJ, Durgan DJ, Ganesh BP, et al. Age-related changes in the gut microbiota influence systemic inflammation and stroke outcome. *Ann Neurol* (2018) 84(1):23–36. doi: 10.1002/ana.25250

82. Benakis C, Martin-Gallausiaux C, Trezzi JP, Melton P, Liesz A, Wilmes P. The microbiome-gut-brain axis in acute and chronic brain diseases. *Curr Opin Neurobiol* (2019) 61:1–9. doi: 10.1016/j.conb.2019.11.009

83. Li Y, Dong K, Fan X, Xie J, Wang M, Fu S, et al. DNT cell-based immunotherapy: progress and applications. *J Cancer* (2020) 11(13):3717–24. doi: 10.7150/jca.39717

84. Dashtsoodol N, Shigeura T, Aihara M, Ozawa R, Kojo S, Harada M, et al. Alternative pathway for the development of V(alpha)14(+) NKT cells directly from CD4(-)CD8(-) thymocytes that bypasses the CD4(+)CD8(+) stage. *Nat Immunol* (2017) 18(3):274–82. doi: 10.1038/ni.3668

85. Rout N, Else JG, Yue S, Connole M, Exley MA, Kaur A. Heterogeneity in phenotype and function of CD8+ and CD4/CD8 double-negative Natural Killer T cell

subsets in sooty mangabeys. *J Med Primatol* (2010) 39(4):224–34. doi: 10.1111/j.1600-0684.2010.00431.x

86. Van Coppernolle S, Vanhee S, Verstichel G, Snauwaert S, van der Spek A, Velghe I, et al. Notch induces human T-cell receptor gammadelta+ thymocytes to differentiate along a parallel, highly proliferative and bipotent CD4 CD8 double-positive pathway. *Leukemia* (2012) 26(1):127–38. doi: 10.1038/leu.2011.324

87. Gherardin NA, Waldeck K, Caneborg A, Martelotto LG, Balachander S, Zethoven M, et al. gammadelta T cells in merkel cell carcinomas have a proinflammatory profile prognostic of patient survival. *Cancer Immunol Res* (2021) 9 (6):612–23. doi: 10.1158/2326-6066.CIR-20-0817

88. Passos LSA, Magalhaes LMD, Soares RP, Marques AF, Nunes M, Gollob KJ, et al. Specific activation of CD4(-) CD8(-) double-negative T cells by Trypanosoma cruzi-derived glycolipids induces a proinflammatory profile associated with cardiomyopathy in Chagas patients. *Clin Exp Immunol* (2017) 190(1):122–32. doi: 10.1111/cei.12992

89. Dias J, Boulouis C, Gorin JB, Lal KG, Gibbs A, Loh L, et al. The CD4(-)CD8(-) MAIT cell subpopulation is a functionally distinct subset developmentally related to the main CD8(+) MAIT cell pool. *Proc Natl Acad Sci USA* (2018) 115(49):E11513–22. doi: 10.1073/pnas.1812273115

90. Montano A, Hanley DF, Hemphill JC. 3rd, hemorrhagic stroke. *Handb Clin Neurol* (2021) 176:229–48. doi: 10.1016/B978-0-444-64034-5.00019-5

91. Nasa P. Outcome of hemorrhagic stroke: host immune response can be a prediction tool! *Indian J Crit Care Med* (2022) 26(1):2–4. doi: 10.5005/jp-journals-10071-24095

92. Ohashi SN, DeLong JH, Kozberg MG, Mazur-Hart DJ, van Veluw SJ, Alkayed NJ, et al. Role of inflammatory processes in hemorrhagic stroke. *Stroke* (2023) 54 (2):605–19. doi: 10.1161/STROKEAHA.122.037155

93. Li X, Chen G. CNS-peripheral immune interactions in hemorrhagic stroke. *J Cereb Blood Flow Metab* (2023) 43(2):185–97. doi: 10.1177/0271678X221145089

94. Mracsko E, Javidi E, Na SY, Kahn A, Liesz A, Veltkamp R. Leukocyte invasion of the brain after experimental intracerebral hemorrhage in mice. *Stroke* (2014) 45 (7):2107–14. doi: 10.1161/STROKEAHA.114.005801

95. Loftspring MC, McDole J, Lu A, Clark JF, Johnson AJ. Intracerebral hemorrhage leads to infiltration of several leukocyte populations with concomitant pathophysiological changes. *J Cereb Blood Flow Metab* (2009) 29(1):137–43. doi: 10.1038/jcbfm.2008.114

96. Mao LL, Yuan H, Wang WW, Wang YJ, Yang MF, Sun BL, et al. Adoptive regulatory T-cell therapy attenuates perihematomal inflammation in a mouse model of experimental intracerebral hemorrhage. *Cell Mol Neurobiol* (2017) 37(5):919–29. doi: 10.1007/s10571-016-0429-1

97. Zhou K, Zhong Q, Wang YC, Xiong XY, Meng ZY, Zhao T, et al. Regulatory T cells ameliorate intracerebral hemorrhage-induced inflammatory injury by modulating microglia/macrophage polarization through the IL-10/GSK3beta/PTEN axis. *J Cereb Blood Flow Metab* (2017) 37(3):967–79. doi: 10.1177/0271678X16648712

98. Prince C, Bruhns ME. Evaluation and treatment of mild traumatic brain injury: the role of neuropsychology. *Brain Sci* (2017) 7(8). doi: 10.3390/brainsci7080105

99. Van Dijk D. Long-term cognitive impairment after critical illness. *N Engl J Med* (2014) 370(2):185. doi: 10.1056/NEJMc1313886

100. Jo YG, Kim JC, Jin HM, Cho YN, Kee SJ, Park YW. Natural killer T cells are numerically and functionally deficient in patients with trauma. *J Innate Immun* (2020) 12(4):344–54. doi: 10.1159/000504324

101. Abou-El-Hassan H, Rezende RM, Izzy S, Gabriely G, Yahya T, Tatematsu BK, et al. Vgamma1 and Vgamma4 gamma-delta T cells play opposing roles in the immunopathology of traumatic brain injury in males. *Nat Commun* (2023) 14 (1):4286. doi: 10.1038/s41467-023-39857-9

102. Frohman EM, Racke MK, Raine CS. Multiple sclerosis–the plaque and its pathogenesis. *N Engl J Med* (2006) 354(9):942–55. doi: 10.1056/NEJMra052130

103. Bjelobaba I, Savic D, Lavrnja I. Multiple sclerosis and neuroinflammation: the overview of current and prospective therapies. *Curr Pharm Des* (2017) 23(5):693–730. doi: 10.2174/1381612822666161214153108

104. Herz J, Filiano AJ, Wiltbank AT, Yogev N, Kipnis J. Myeloid cells in the central nervous system. *Immunity* (2017) 46(6):943–56. doi: 10.1016/j.immuni.2017.06.007

105. Kurschus FC. T cell mediated pathogenesis in EAE: Molecular mechanisms. *BioMed J* (2015) 38(3):183–93. doi: 10.4103/2319-4170.155590

106. Constantinescu CS, Farooqi N, O'Brien K, Gran B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br J Pharmacol* (2011) 164(4):1079–106. doi: 10.1111/j.1476-5381.2011.01302.x

107. Demoulins T, Gachelin G, Bequet D, Dormont D. A biased Valpha24+ T-cell repertoire leads to circulating NKT-cell defects in a multiple sclerosis patient at the onset of his disease. *Immunol Lett* (2003) 90(2-3):223–8. doi: 10.1016/j.imlet.2003.09.014

108. Gausling R, Trollmo C, Hafler DA. Decreases in interleukin-4 secretion by invariant CD4(-)CD8(-)V alpha 24J alpha Q T cells in peripheral blood of patientswith relapsing-remitting multiple sclerosis. *Clin Immunol* (2001) 98(1):11–7. doi: 10.1006/clim.2000.4942

109. Illes Z, Kondo T, Newcombe J, Oka N, Tabira T, Yamamura T. Differential expression of NK T cell V alpha 24J alpha Q invariant TCR chain in the lesions of multiple sclerosis and chronic inflammatory demyelinating polyneuropathy. *J Immunol* (2000) 164(8):4375–81. doi: 10.4049/jimmunol.164.8.4375

110. O'Keeffe J, Gately CM, Counihan T, Hennessy M, Leahy T, Moran AP, et al. T-cells expressing natural killer (NK) receptors are altered in multiple sclerosis and responses to alpha-galactosylceramide are impaired. *J Neurol Sci* (2008) 275(1-2):22–8. doi: 10.1016/j.jns.2008.07.007

111. Araki M, Kondo T, Gumperz JE, Brenner MB, Miyake S, Yamamura T. Th2 bias of CD4+ NKT cells derived from multiple sclerosis in remission. *Int Immunol* (2003) 15(2):279–88. doi: 10.1093/intimm/dxg029

112. De Biasi S, Simone AM, Nasi M, Bianchini E, Ferraro D, Vitetta F, et al. iNKT cells in secondary progressive multiple sclerosis patients display pro-inflammatory profiles. *Front Immunol* (2016) 7:555. doi: 10.3389/fimmu.2016.00555

113. Singh AK, Wilson MT, Hong S, Olivares-Villagomez D, Du C, Stanic AK, et al. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* (2001) 194(12):1801–11. doi: 10.1084/jem.194.12.1801

114. Furlan R, Bergami A, Cantarella D, Brambilla E, Taniguchi M, Dellabona P, et al. Activation of invariant NKT cells by alphaGalCer administration protects mice from MOG35-55-induced EAE: critical roles for administration route and IFN-gamma. *Eur J Immunol* (2003) 33(7):1830–8. doi: 10.1002/eji.200323885

115. Teige A, Teige I, Lavasani S, Bockermann R, Mondoc E, Holmdahl R, et al. CD1-dependent regulation of chronic central nervous system inflammation in experimental autoimmune encephalomyelitis. *J Immunol* (2004) 172(1):186–94. doi: 10.4049/jimmunol.172.1.186

116. Denney L, Kok WL, Cole SL, Sanderson S, McMichael AJ, Ho LP. Activation of invariant NKT cells in early phase of experimental autoimmune encephalomyelitis results in differentiation of Ly6Chi inflammatory monocyte to M2 macrophages and improved outcome. *J Immunol* (2012) 189(2):551–7. doi: 10.4049/jimmunol.1103608

117. Oh SJ, Chung DH. Invariant NKT cells producing IL-4 or IL-10, but not IFN-gamma, inhibit the Th1 response in experimental autoimmune encephalomyelitis, whereas none of these cells inhibits the Th17 response. *J Immunol* (2011) 186 (12):6815–21. doi: 10.4049/jimmunol.1003916

118. Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* (2001) 413(6855):531–4. doi: 10.1038/35097097

119. Kojo S, Seino K, Harada M, Watarai H, Wakao H, Uchida T, et al. Induction of regulatory properties in dendritic cells by Valpha14 NKT cells. *J Immunol* (2005) 175 (6):3648–55. doi: 10.4049/jimmunol.175.6.3648

120. Qian G, Qin X, Zang YQ, Ge B, Guo TB, Wan B, et al. High doses of alpha-galactosylceramide potentiate experimental autoimmune encephalomyelitis by directly enhancing Th17 response. *Cell Res* (2010) 20(4):480–91. doi: 10.1038/cr.2010.6

121. Jahng AW, Maricic I, Pedersen B, Burdin N, Naidenko O, Kronenberg M, et al. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* (2001) 194(12):1789–99. doi: 10.1084/jem.194.12.1789

122. Jahng A, Maricic I, Aguilera C, Cardell S, Halder RC, Kumar V. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med* (2004) 199(7):947–57. doi: 10.1084/jem.20031389

123. Oki S, Chiba A, Yamamura T, Miyake S. The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. *J Clin Invest* (2004) 113(11):1631–40. doi: 10.1172/JCI20862

124. Sato W, Noto D, Araki M, Okamoto T, Lin Y, Yamaguchi H, et al. First-in-human clinical trial of the NKT cell-stimulatory glycolipid OCH in multiple sclerosis. *Ther Adv Neurol Disord* (2023) 16:17562864231162153. doi: 10.1177/17562864231162153

125. Wucherpfennig KW, Newcombe J, Li H, Keddy C, Cuzner ML, Hafler DA. Gamma delta T-cell receptor repertoire in acute multiple sclerosis lesions. *Proc Natl Acad Sci USA* (1992) 89(10):4588–92. doi: 10.1073/pnas.89.10.4588

126. Schirmer L, Rothhammer V, Hemmer B, Korn T. Enriched CD161high CCR6+ gammadelta T cells in the cerebrospinal fluid of patients with multiple sclerosis. *JAMA Neurol* (2013) 70(3):345–51. doi: 10.1001/2013.jamaneurol.409

127. Shimonkevitz R, Colburn C, Burnham JA, Murray RS, Kotzin BL. Clonal expansions of activated gamma/delta T cells in recent-onset multiple sclerosis. *Proc Natl Acad Sci USA* (1993) 90(3):923–7. doi: 10.1073/pnas.90.3.923

128. Stinissen P, Vandevyver C, Medaer R, Vandegaer L, Nies J, Tuyls L, et al. Increased frequency of gamma delta T cells in cerebrospinal fluid and peripheral blood of patients with multiple sclerosis. Reactivity, cytotoxicity, and T cell receptor V gene rearrangements. *J Immunol* (1995) 154(9):4883–94.

129. Monteiro A, Cruto C, Rosado P, Martinho A, Rosado L, Fonseca M, et al. Characterization of circulating gamma-delta T cells in relapsing vs remission multiple sclerosis. *J Neuroimmunol* (2018) 318:65–71. doi: 10.1016/j.jneuroim.2018.02.009

130. Liedtke W, Meyer G, Faustmann PM, Warnatz H, Raine CS. Clonal expansion and decreased occurrence of peripheral blood gamma delta T cells of the V delta 2J delta 3 lineage in multiple sclerosis patients. *Int Immunol* (1997) 9(7):1031–41. doi: 10.1093/intimm/9.7.1031

131. Bieganowski P, Bieganowska K, Zaborski J, Czlonkowska A. Oligoclonal expansion of gamma delta T cells in cerebrospinal fluid of multiple sclerosis patients. *Mult Scler* (1996) 2(2):78–82. doi: 10.1177/135245859600200203

132. Battistini L, Salvetti M, Ristori G, Falcone M, Raine CS, Brosnan CF. Gamma delta T cell receptor analysis supports a role for HSP 70 selection of lymphocytes in multiple sclerosis lesions. *Mol Med* (1995) 1(5):554–62. doi: 10.1007/BF03401592

133. Selmaj K, Brosnan CF, Raine CS. Colocalization of lymphocytes bearing gamma delta T-cell receptor and heat shock protein hsp65+ oligodendrocytes in multiple sclerosis. *Proc Natl Acad Sci USA* (1991) 88(15):6452–6. doi: 10.1073/pnas.88.15.6452

134. Bai L, Picard D, Anderson B, Chaudhary V, Luoma A, Jabri B, et al. The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vdelta1 TCR. *Eur J Immunol* (2012) 42(9):2505–10. doi: 10.1002/eji.201242531

135. Olive C. Gamma delta T cell receptor variable region usage during the development of experimental allergic encephalomyelitis. *J Neuroimmunol* (1995) 62 (1):1–7. doi: 10.1016/0165-5728(95)00081-c

136. Wei YL, Han A, Glanville J, Fang F, Zuniga LA, Lee JS, et al. A highly focused antigen receptor repertoire characterizes gammadelta T cells that are poised to make IL-17 rapidly in naive animals. *Front Immunol* (2015) 6:118. doi: 10.3389/fimmu.2015.00118

137. Papotto PH, Goncalves-Sousa N, Schmolka N, Iseppon A, Mensurado S, Stockinger B, et al. IL-23 drives differentiation of peripheral gammadelta17 T cells from adult bone marrow-derived precursors. *EMBO Rep* (2017) 18(11):1957–67. doi: 10.15252/embr.201744200

138. Lukens JR, Barr MJ, Chaplin DD, Chi H, Kanneganti TD. Inflammasome-derived IL-1beta regulates the production of GM-CSF by CD4(+) T cells and gammadelta T cells. *J Immunol* (2012) 188(7):3107–15. doi: 10.4049/jimmunol.1103308

139. Ponomarev ED, Novikova M, Yassai M, Szczepanik M, Gorski J, Dittel BN. Gamma delta T cell regulation of IFN-gamma production by central nervous system-infiltrating encephalitogenic T cells: correlation with recovery from experimental autoimmune encephalomyelitis. *J Immunol* (2004) 173(3):1587–95. doi: 10.4049/jimmunol.173.3.1587

140. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KH. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* (2009) 31(2):331–41. doi: 10.1016/j.immuni.2009.08.001

141. Petermann F, Rothhammer V, Claussen MC, Haas JD, Blanco LR, Heink S, et al. gammadelta T cells enhance autoimmunity by restraining regulatory T cell responses via an interleukin-23-dependent mechanism. *Immunity* (2010) 33(3):351–63. doi: 10.1016/j.immuni.2010.08.013

142. Ponomarev ED, Dittel BN. Gamma delta T cells regulate the extent and duration of inflammation in the central nervous system by a Fas ligand-dependent mechanism. *J Immunol* (2005) 174(8):4678–87. doi: 10.4049/jimmunol.174.8.4678

143. Havrdova E, Belova A, Goloborodko A, Tisserant A, Wright A, Wallstroem E, et al. Activity of secukinumab, an anti-IL-17A antibody, on brain lesions in RRMS: results from a randomized, proof-of-concept study. *J Neurol* (2016) 263(7):1287–95. doi: 10.1007/s00415-016-8128-x

144. Orthmann-Murphy JL, Calabresi PA. Therapeutic application of monoclonal antibodies in multiple sclerosis. *Clin Pharmacol Ther* (2017) 101(1):52–64. doi: 10.1002/cpt.547

145. Paintlia MK, Paintlia AS, Singh AK, Singh I. Synergistic activity of interleukin-17 and tumor necrosis factor-alpha enhances oxidative stress-mediated oligodendrocyte apoptosis. *J Neurochem* (2011) 116(4):508–21. doi: 10.1111/j.1471-4159.2010.07136.x

146. Kang Z, Wang C, Zepp J, Wu L, Sun K, Zhao J, et al. Act1 mediates IL-17-induced EAE pathogenesis selectively in NG2+ glial cells. *Nat Neurosci* (2013) 16 (10):1401–8. doi: 10.1038/nn.3505

147. Liu H, Yang X, Yang J, Yuan Y, Wang Y, Zhang R, et al. IL-17 inhibits oligodendrocyte progenitor cell proliferation and differentiation by increasing K(+) channel kv1.3. *Front Cell Neurosci* (2021) 15:679413. doi: 10.3389/fncel.2021.679413

148. Gargano F, Guerrera G, Piras E, Serafini B, Di Paola M, Rizzetto L, et al. Proinflammatory mucosal-associated invariant CD8+ T cells react to gut flora yeasts and infiltrate multiple sclerosis brain. *Front Immunol* (2022) 13:890298. doi: 10.3389/fimmu.2022.890298

149. Willing A, Leach OA, Ufer F, Attfield KE, Steinbach K, Kursawe N, et al. CD8 (+) MAIT cells infiltrate into the CNS and alterations in their blood frequencies correlate with IL-18 serum levels in multiple sclerosis. *Eur J Immunol* (2014) 44 (10):3119–28. doi: 10.1002/eji.201344160

150. Held K, Bhonsle-Deeng L, Siewert K, Sato W, Beltran E, Schmidt S, et al. alphabeta T-cell receptors from multiple sclerosis brain lesions show MAIT cell-related features. *Neurol Neuroimmunol Neuroinflamm* (2015) 2(4):e107. doi: 10.1212/NXI.0000000000000107

151. Abrahamsson SV, Angelini DF, Dubinsky AN, Morel E, Oh U, Jones JL, et al. Non-myeloablative autologous haematopoietic stem cell transplantation expands regulatory cells and depletes IL-17 producing mucosal-associated invariant T cells in multiple sclerosis. *Brain* (2013) 136(Pt 9):2888–903. doi: 10.1093/brain/awt182

152. Annibali V, Ristori G, Angelini DF, Serafini B, Mechelli R, Cannoni S, et al. CD161(high)CD8+T cells bear pathogenetic potential in multiple sclerosis. *Brain* (2011) 134(Pt 2):542–54. doi: 10.1093/brain/awq354

153. Salou M, Nicol B, Garcia A, Baron D, Michel L, Elong-Ngono A, et al. Neuropathologic, phenotypic and functional analyses of Mucosal Associated Invariant T cells in Multiple Sclerosis. *Clin Immunol* (2016) 166-167:1–11. doi: 10.1016/j.clim.2016.03.014

154. Carnero Contentti E, Farez MF, Correale J. Mucosal-associated invariant T cell features and TCR repertoire characteristics during the course of multiple sclerosis. *Front Immunol* (2019) 10:2690. doi: 10.3389/fimmu.2019.02690

155. Miyazaki Y, Miyake S, Chiba A, Lantz O, Yamamura T. Mucosal-associated invariant T cells regulate Th1 response in multiple sclerosis. *Int Immunol* (2011) 23 (9):529–35. doi: 10.1093/intimm/dxr047

156. Croxford JL, Miyake S, Huang YY, Shimamura M, Yamamura T. Invariant V (alpha)19i T cells regulate autoimmune inflammation. *Nat Immunol* (2006) 7(9):987–94. doi: 10.1038/ni1370

157. Shin YW, Lee ST, Park KI, Jung KH, Jung KY, Lee SK, et al. Treatment strategies for autoimmune encephalitis. *Ther Adv Neurol Disord* (2018) 11:1756285617722347. doi: 10.1177/1756285617722347

158. Nissen MS, Ryding M, Meyer M, Blaabjerg M. Autoimmune encephalitis: current knowledge on subtypes, disease mechanisms and treatment. *CNS Neurol Disord Drug Targets* (2020) 19(8):584–98. doi: 10.2174/1871527319666200708133103

159. Bien CG, Vincent A, Barnett MH, Becker AJ, Blumcke I, Graus F, et al. Immunopathology of autoantibody-associated encephalitides: clues for pathogenesis. *Brain* (2012) 135(Pt 5):1622–38. doi: 10.1093/brain/aws082

160. Zeng C, Li L, Chen L, Li P, Chen M, Wu X, et al. Th17 cells regulate the progress of anti-NMDAR encephalitis. *Am J Transl Res* (2022) 14(9):6268–76.

161. Golombeck KS, Bonte K, Monig C, van Loo KM, Hartwig M, Schwindt W, et al. Evidence of a pathogenic role for CD8(+) T cells in anti-GABAB receptor limbic encephalitis. *Neurol Neuroimmunol Neuroinflamm* (2016) 3(3):e232. doi: 10.1212/NXI.0000000000000232

162. Dalmau J, Graus F. Antibody-mediated encephalitis. *N Engl J Med* (2018) 378 (9):840–51. doi: 10.1056/NEJMra1708712

163. Gitler AD, Dhillon P, Shorter J. Neurodegenerative disease: models, mechanisms, and a new hope. *Dis Model Mech* (2017) 10(5):499–502. doi: 10.1242/dmm.030205

164. Heemels MT. Neurodegenerative diseases. *Nature* (2016) 539(7628):179. doi: 10.1038/539179a

165. Berriat F, Lobsiger CS, Boillee S. The contribution of the peripheral immune system to neurodegeneration. *Nat Neurosci* (2023) 26(6):942–54. doi: 10.1038/s41593-023-01323-6

166. Beers DR, Henkel JS, Zhao W, Wang J, Appel SH. CD4+ T cells support glial neuroprotection, slow disease progression, and modify glial morphology in an animal model of inherited ALS. *Proc Natl Acad Sci USA* (2008) 105(40):15558–63. doi: 10.1073/pnas.0807419105

167. Weiss F, Labrador-Garrido A, Dzamko N, Halliday G. Immune responses in the Parkinson's disease brain. *Neurobiol Dis* (2022) 168:105700. doi: 10.1016/j.nbd.2022.105700

168. Evans FL, Dittmer M, de la Fuente AG, Fitzgerald DC. Protective and regenerative roles of T cells in central nervous system disorders. *Front Immunol* (2019) 10:2171. doi: 10.3389/fimmu.2019.02171

169. Wyatt-Johnson SK, Kersey HN, Codocedo JF, Newell KL, Landreth GE, Lamb BT, et al. Control of the temporal development of Alzheimer's disease pathology by the MR1/MAIT cell axis. *J Neuroinflamm* (2023) 20(1):78. doi: 10.1186/s12974-023-02761-6

170. Brigas HC, Ribeiro M, Coelho JE, Gomes R, Gomez-Murcia V, Carvalho K, et al. IL-17 triggers the onset of cognitive and synaptic deficits in early stages of Alzheimer's disease. *Cell Rep* (2021) 36(9):109574. doi: 10.1016/j.celrep.2021.109574

171. Rentzos M, Evangelopoulos E, Sereti E, Zouvelou V, Marmara S, Alexakis T, et al. Alterations of T cell subsets in ALS: a systemic immune activation? *Acta Neurol Scand* (2012) 125(4):260–4. doi: 10.1111/j.1600-0404.2011.01528.x

172. Finkelstein A, Kunis G, Seksenyan A, Ronen A, Berkutzki T, Azoulay D, et al. Abnormal changes in NKT cells, the IGF-1 axis, and liver pathology in an animal model of ALS. *PloS One* (2011) 6(8):e22374. doi: 10.1371/journal.pone.0022374

173. Sommer A, Marxreiter F, Krach F, Fadler T, Grosch J, Maroni M, et al. Th17 lymphocytes induce neuronal cell death in a human iPSC-based model of parkinson's disease. *Cell Stem Cell* (2019) 24(6):1006. doi: 10.1016/j.stem.2019.04.019

174. Zhou C, Zhou X, He D, Li Z, Xie X, Ren Y. Reduction of Peripheral Blood iNKT and gammadeltaT Cells in Patients With Parkinson's Disease: An Observational Study. *Front Immunol* (2020) 11:1329. doi: 10.3389/fimmu.2020.01329

175. Liu Z, Qiu AW, Huang Y, Yang Y, Chen JN, Gu TT, et al. IL-17A exacerbates neuroinflammation and neurodegeneration by activating microglia in rodent models of Parkinson's disease. *Brain Behav Immun* (2019) 81:630–45. doi: 10.1016/j.bbi.2019.07.026

176. Fiszer U, Mix E, Fredrikson S, Kostulas V, Olsson T, Link H. gamma delta+ T cells are increased in patients with Parkinson's disease. *J Neurol Sci* (1994) 121(1):39–45. doi: 10.1016/0022-510x(94)90154-6

177. Chen JM, Jiang GX, Li QW, Zhou ZM, Cheng Q. Increased serum levels of interleukin-18, -23 and -17 in Chinese patients with Alzheimer's disease. *Dement Geriatr Cognit Disord* (2014) 38(5-6):321–9. doi: 10.1159/000360606

178. Yang J, Kou J, Lalonde R, Fukuchi KI. Intracranial IL-17A overexpression decreases cerebral amyloid angiopathy by upregulation of ABCA1 in an animal model of Alzheimer's disease. *Brain Behav Immun* (2017) 65:262–73. doi: 10.1016/j.bbi.2017.05.012

179. Hirsh MI, Junger WG. Roles of heat shock proteins and gamma delta T cells in inflammation. *Am J Respir Cell Mol Biol* (2008) 39(5):509–13. doi: 10.1165/rcmb.2008-0090TR

180. Doherty PC, Allan W, Eichelberger M, Carding SR. Heat-shock proteins and the gamma delta T cell response in virus infections: implications for autoimmunity. *Springer Semin Immunopathol* (1991) 13(1):11–24. doi: 10.1007/BF01225275

181. Fiszer U, Fredrikson S, Czlonkowska A. Humoral response to hsp 65 and hsp 70 in cerebrospinal fluid in Parkinson's disease. *J Neurol Sci* (1996) 139(1):66–70. doi: 10.1016/0022-510X(96)00002-0

182. Chen WW, Zhang X, Huang WJ. Role of neuroinflammation in neurodegenerative diseases (Review). *Mol Med Rep* (2016) 13(4):3391–6. doi: 10.3892/mmr.2016.4948

183. Hirabayashi Y. A world of sphingolipids and glycolipids in the brain -Novel functions of simple lipids modified with glucose. *Proc Japan Acad Ser B-Physical Biol Sci* (2012) 88(4):129–43. doi: 10.2183/pjab.88.129

184. Gold MC, Cerri S, Smyk-Pearson S, Cansler ME, Vogt TM, Delepine J, et al. Human mucosal associated invariant T cells detect bacterially infected cells. *PloS Biol* (2010) 8(6):e1000407. doi: 10.1371/journal.pbio.1000407

185. Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, Ruan Z, et al. Butyrophilin 2A1 is essential for phosphoantigen reactivity by gammadelta T cells. *Science* (2020) 367(6478). doi: 10.1126/science.aay5516

186. Corbett AJ, Awad W, Wang H, Chen Z. Antigen recognition by MR1-reactive T cells; MAIT cells, metabolites, and remaining mysteries. *Front Immunol* (2020) 11:1961. doi: 10.3389/fimmu.2020.01961

187. Silva-Santos B, Mensurado S, Coffelt SB. gammadelta T cells: pleiotropic immune effectors with therapeutic potential in cancer. *Nat Rev Cancer* (2019) 19 (7):392–404. doi: 10.1038/s41568-019-0153-5

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 11 January 2024
DOI 10.3389/fimmu.2023.1343718

OPEN ACCESS

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RECEIVED 24 November 2023
ACCEPTED 26 December 2023
PUBLISHED 11 January 2024

CITATION
Lee SW, Park HJ, Van Kaer L and Hong S (2024) Role of CD1d and iNKT cells in regulating intestinal inflammation. *Front. Immunol.* 14:1343718. doi: 10.3389/fimmu.2023.1343718

Role of CD1d and iNKT cells in regulating intestinal inflammation

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Invariant natural killer T (iNKT) cells, a subset of unconventional T cells that recognize glycolipid antigens in a CD1d-dependent manner, are crucial in regulating diverse immune responses such as autoimmunity. By engaging with CD1d-expressing non-immune cells (such as intestinal epithelial cells and enterochromaffin cells) and immune cells (such as type 3 innate lymphoid cells, B cells, monocytes and macrophages), iNKT cells contribute to the maintenance of immune homeostasis in the intestine. In this review, we discuss the impact of iNKT cells and CD1d in the regulation of intestinal inflammation, examining both cellular and molecular factors with the potential to influence the functions of iNKT cells in inflammatory bowel diseases such as Crohn's disease and ulcerative colitis.

KEYWORDS

invariant NKT cells, inflammatory bowel diseases, CD1d, glycolipid antigens, commensal bacteria, short-chain fatty acids

1 Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the intestine stemming from an imbalanced immune response to gut microflora rather than infectious causes. IBD manifests primarily as two forms: Crohn's disease (CD) and ulcerative colitis (UC). Whereas CD affects the entire digestive tract, UC primarily involves inflammation in the colon and rectum (1, 2). Environmental factors such as a high-fat diet (HFD) and hormonal substances contribute to the increasing incidence of IBD in developed countries (1, 2). Although various immune cells have been implicated in the pathogenesis of IBD, we focus here on natural killer T (NKT) cells, a subset of innate-like T cells that play a crucial role in maintaining intestinal integrity. Unlike conventional T cells, NKT cells co-expressing NK cell receptors and T cell receptors (TCRs) respond to glycolipid antigens (Ags) presented by the major histocompatibility complex class I-related protein CD1d.

CD1d-restricted NKT cells mainly consist of type I invariant NKT (iNKT) and type II NKT cells that react to glycolipids, α-galactosylceramide (α-GalCer) and sulfatide, respectively (3–5). Human iNKT cells include mainly $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ double-negative (DN) subsets, whereas murine iNKT cells consist of $CD4^+$ and DN subsets. Moreover, peripheral blood iNKT cells occupy 0.1-0.2% and 1-2% of T cells in humans and mice, respectively. Type I iNKT cells are less abundant than type II NKT cells in human liver, but opposite relative frequencies are observed in mouse liver (6, 7).

NKT cells can produce various cytokines including IFN-γ, IL-4, IL-10, IL-13, and IL-17 (8–10). Moreover, iNKT cells are functionally categorized based on their preferential expression of transcription factors: T-bet for iNKT1, GATA3 and PLZF for iNKT2, RORγt for iNKT17, and E4BP4 for iNKT10 cells (11, 12). iNKT1 cells are predominant in the spleen and the liver, whereas iNKT2 cells predominantly localize to mesenteric lymph nodes (mLNs) (5, 11). IL-10-expressing iNKT10 cells are predominant in the visceral adipose tissue (VAT) as well as in the colonic lamina propria (LP) (13, 14). Whereas B6 mice contain a prominent population of iNKT1 cells in the thymus, Balb/c mice contain higher frequencies of iNKT2 cells in the thymus and mLNs (5). It was recently reported that non-immune cells (i.e., intestinal epithelial cells (IECs) and serotonin-secreting enterochromaffin cells) constitutively express CD1d molecules that can present glycolipid Ags (15–17). Although the canonical role of CD1d in lipid Ag presentation is well-characterized in immune cells such as dendritic cells (DCs), increasing evidence also shows non-canonical CD1d functions in CD1d-expressing mononuclear cells, including type 3 innate lymphoid cells (ILC3s) and IECs. Besides their capacity to present lipid Ag, CD1d molecules can intrinsically signal cells to become activated and produce cytokines (17–19). Emerging evidence indicates that intrinsic CD1d signaling in intestinal cells (i.e., tuft cells, Paneth cells, enterocytes, goblet cells, and neuroendocrine cells) has clinical implications in IBD. The quantity and phenotype of iNKT cells are altered in IBD patients compared with healthy individuals (13, 20). Moreover, since iNKT cells can produce pro- and anti-inflammatory cytokines, iNKT cell-elicited immune responses may be protective or harmful. This mini-review will focus on the immunomodulatory roles of CD1d and iNKT cells in two primary forms of IBD, CD and UC.

2 NKT cells in IBD

In CD, exaggerated T helper (Th)1 and Th17 immune responses characterize pathogenic lesions across the gastrointestinal tract (1, 2). CD patients exhibit a significant reduction in iNKT cell numbers in peripheral blood and intestinal tissues (20). Likewise, the acute mouse IBD model induced with dextran sulfate sodium (DSS) is characterized by increased Th1 and Th17 immune responses (21). iNKT cell-deficient mice display increased colitis severity in experimental models, indicating a regulatory role for colonic iNKT cells, particularly those producing anti-inflammatory cytokines like IL-9 and IL-10 (13, 22). Recently, we have demonstrated that IL-4- and IL-9-producing iNKT cells are significantly increased in the mLNs during IFN-γ-mediated intestinal inflammation after DSS treatment (23). Furthermore, consistent with our report, the prevalence of anti-inflammatory iNKT10 cells is elevated in CD patients compared to healthy individuals, suggesting the existence of regulatory pathways that compensate for the production of harmful pro-inflammatory cytokines in the intestine of CD patients (13).

In contrast to CD patients, IL-13-producing cell populations in the LP of UC patients contain CD1d-restricted type II NKT cells but not iNKT cells (24). Overexpression of CD1d in a type II NKT TCR transgenic (Tg) mouse model ($CD1d^{Tg}/24\alpha\beta^{Tg}$) forces the negative selection of type II NKT cells, consequently reducing the frequency of type II NKT cells (25). Thus, type II NKT cell-deficient $CD1d^{Tg}/24\alpha\beta^{Tg}$ mice spontaneously develop colitis, indicating that type II NKT cells play essential roles in maintaining intestinal homeostasis (25). However, LP mononuclear cells (including type II NKT cells) from UC patients express high levels of CD161 and IL-13Rα and produce high amounts of IL-13. Upon stimulation with sulfatide glycolipids, these cells exhibit augmented cytotoxic activity against IECs (26). In addition, a methionine-choline-deficient (MCD) diet lowers the type II NKT population in both the LP and mLNs, ultimately suppressing DSS-induced colitis (27). These findings indicate that type II NKT cells can be either protective or pathogenic in UC. Since IFN-γ produced during intestinal inflammation contributes to the relative distribution of type I and II NKT cells (28, 29), the balance of these subsets plays a significant role in regulating IBD.

3 Effect of glycolipid-stimulated iNKT cells on IBD

Exposure to mucosa-associated microbiota drives pro-inflammatory activation of colonic iNKT cells (mostly $CD161^+$ cells) from IBD patients through TCR-dependent and -independent mechanisms, consequently breaking the epithelial barrier integrity (30). The presentation of commensal-derived glycolipid Ags and exogenous glycolipids by $CD11c^+$ cells, such as DCs, controls the homeostasis and activation of intestinal iNKT cells (31). Furthermore, Wingender et al. demonstrated that intragastric injection of *Sphingomonas yanoikuyae* containing glycolipid Ags activates intestinal iNKT cells with a hyporesponsive phenotype and increases the relative frequency of $V\beta7^+$ iNKT cells in germ-free mice (32). Repeated intraperitoneal (i.p.) injection of either α-GalCer or its more potent analog (7DW8-5) (33, 34) into DSS-treated mice effectively prevents colitis development. Moreover, repeated i.p. injection of α-GalCer inhibits the development of cholangitis complicated by colitis in outbred CD-1 mice by reducing Th1-dominant responses (35). Since repeated i.p. or intravenous (i.v.) injection of α-GalCer increases iNKT10 differentiation (10, 12), it will be worthwhile to investigate whether resolution of colitis by repeated i.p. α-GalCer injection correlates with an increase in colonic iNKT10 cells. Furthermore,

repeated α-GalCer challenge induces memory-like cMAF$^+$ iNKT cells and IL-10-expressing adipose tissue iNKT1 cells, suggesting that iNKT10 cells may differentiate from iNKT1 cells (14). On the other hand, α-GalCer-like glycolipid produced by the commensal bacterium *Bacteroides fragilis* (*B. fragilis*) stimulates splenic iNKT cells to secrete high amounts of IL-10 in Balb/c mice enriched for iNKT2 cells. Upon stimulation with these glycolipids, most iNKT10 cells simultaneously secrete IL-13 but not IFN-γ (36). Fecal microbiota transplantation (enriched for *Lactobacillaceae* and *Bifidobacteriaceae*) suppresses DSS-induced colitis, which is associated with increased iNKT10 cells (37, 38). However, the origin and differentiation of colonic iNKT10 cells during colitis remains unclear. Oral administration of α-GalCer specifically up-regulates expression of TCR engagement markers (e.g., Nur77) in iNKT cells of the mLNs but not the spleen, liver, and thymus and its up-regulated expression selectively occurs in iNKT2 rather than iNKT1 populations in the mLNs (5), supporting the notion that optimal glycolipid administration frequency and route are important factors in treating colitis. Injection of OCH, a derivative of α-GalCer with Th2 selective activity, prevents DSS-induced colitis, which correlates with reduced Th1/Th2 cytokine ratios and increased IL-10 production (39). Treatment with an α-GalCer derivative containing polar functional groups (Bz amide) attenuates DSS-induced colitis, accompanied by expansion and activation of Th2- and Th17-biased iNKT cells rather than Th1-biased iNKT cells (40). Glycolipid Ags derived from the intestinal microbe *B. fragilis* (called GSL-Bf717 or BfaGCs) inhibit iNKT cell activation through competitive binding of their sphinganine branches with CD1d, resulting in protection against oxazolone (Oxa)-induced colitis, an experimental model mimicking UC (41–43). Additional studies have provided evidence that CD1d-dependent pathogenic iNKT cell activation by commensal-derived glycolipid Ags can be inhibited by competitive CD1d binding with globotriaosylceramide (44) and α-lactosylceramide (45) during iNKT cell-mediated Oxa-induced colitis.

4 Cell-intrinsic role of CD1d in IBD

CD1d expression on IECs is significantly decreased in both CD and UC patients compared with healthy controls, suggesting a unique role in controlling intestinal immune responses (46). Exposure of environmental oxazoles derived from either diet or microbes to IECs can play pathogenic roles in colonic inflammation by inhibiting IEC-mediated and CD1d-dependent IL-10 production and promote iNKT cell-derived IL-13 production (47). Lipid-mediated CD1d ligation triggers epithelial cell-derived endogenous IL-10 production, consequently decreasing epithelial permeability induced by IFN-γ (48). Furthermore, one study demonstrated that IECs produce high amounts of IL-10 via CD1d engagement-induced STAT3 activation, suppressing Oxa-induced iNKT cell-mediated colitis (17). These studies suggest that the cross-talk between iNKT cells and CD1d-expressing IECs activated by CD1d-intrinsic signaling controls intestinal homeostasis.

In addition to its effects on IECs, CD1d engagement on various other cell types can induce cell-intrinsic signaling. In mLN B cells, CD1d expression is associated with enhanced IL-10 production under chronic intestinal inflammatory conditions (49). Since IL-10-producing B cells (B10) suppress experimental arthritis in a CD1d-dependent manner (50), it will be interesting to investigate whether iNKT cells cross-talk with B10 cells to regulate colitis. Glycolipid-mediated ligation of CD1d on human monocytes directly triggers marked NFκB activation and excessive IL-12 production, indicating that CD1d-mediated signal transduction is required to induce optimal Th1 responses to glycolipid Ag stimulation (51). Macrophage-specific CD1d deletion alleviates colonic inflammation, highlighting CD1d-intrinsic activating effects on NFκB in macrophages (19). CD1d-deficient macrophages exhibit metabolic reprogramming into an inflammatory phenotype through CD36 internalization and lipid uptake. In addition, mice reconstituted with CD1d-deficient macrophages exhibit increased susceptibility to LPS-induced inflammation (52). Engagement of CD1d on ILC3s by NKT cells drives IL-22 secretion in the mLNs (18). In addition, CD1d-dependent engagement of enterochromaffin cells by iNKT cells triggers the former cells to release peripheral serotonin (5-HT), thereby regulating intestinal hemostasis (16). Finally, a recent study showed that intrinsic hepatocyte-specific CD1d signaling induced by tyrosine phosphorylation of the CD1d cytoplasmic tail protects against hepatocyte apoptosis in mice with non-alcoholic steatohepatitis induced by an HFD or MCD diet (53).

iNKT cells are thought to play protective or pathogenic roles in colitis through their activation via TCR- or cytokine-mediated signaling pathways. Furthermore, various CD1d-expressing cells (e.g., B cells, ILC3s, and macrophages) that can interact with iNKT cells also appear to play protective or pathogenic roles in colitis by producing cytokines (e.g., IL-22 and IL-10) via CD1d-intrinsic signaling. Thus, it will be important to explore how CD1d-intrinsic signaling modulates the function of NKT cells and whether such interactions contribute to colitis development.

5 Immune cells that engage in cross-talk with iNKT cells during IBD

5.1 Myeloid lineage cells: macrophages and neutrophils

CX3CR1^{hi} mononuclear phagocytes guide non-invasive *Salmonellae* to the mLNs where these organisms induce both pathogen-specific T cell responses and IgA antibodies (54). During naive CD4$^+$ T cell transfer-mediated colitis, CD4$^+$ T cells are in close contact with colonic CX3CR1$^+$ phagocytes that present bacterial-derived Ags (55). Interestingly, embryonic CX3CR1$^+$ macrophages are essential for the localization and proliferation of colonic iNKT cells (particularly iNKT17) in their local environment during early life (56). In this regard, it will be worthwhile to further investigate whether CX3CR1$^+$ macrophages contribute to iNKT cell-mediated host defenses during enteropathogen-induced colitis.

Compared with macrophage recruitment, the effect of iNKT cells on neutrophil recruitment remains unclear. For example, the expression of neutrophil-attracting chemokines (i.e., CXCL1, CXCL2, and CXCL3) decreases in the colon of CD1d$^{-/-}$ mice, along with reduced pathogenic neutrophil infiltration (57). In contrast, more neutrophils are recruited to the colon in DSS-treated iNKT cell-deficient Jα18$^{-/-}$ mice and these cells possess a pronounced anti-inflammatory phenotype (58). Since neutrophils are classified into two groups based on their functional differences (59), pro-inflammatory N1 (e.g., TNF-α-secreting) and anti-inflammatory N2 neutrophils (e.g., TGF-β-secreting), further studies are needed to clarify the role of iNKT cells on neutrophil polarization (N1 vs. N2) during colitis development.

5.2 Innate lymphoid cells: ILC2 and ILC3

Blockade of IL-25 signaling is an effective strategy to prevent intestinal inflammation in Oxa-induced colitis. This protection is achieved by inhibiting IL-13 production by ILC2s and NKT cells (60). After helminth infection, tuft cell-derived IL-25 constitutively activates ILC2s to produce IL-13, followed by the differentiation of tuft and goblet cells (61). Moreover, *Tritrichomonas*-generated succinate triggers tuft cell hyperplasia via induction of the tuft cell-IL-25-ILC2-IL-13 axis (62). Lucas et al. demonstrated that IL-25 production from thymic tuft cells skews iNKT cells towards the iNKT2 phenotype in the thymus (63). Moreover, in addition to their interactions within the thymus, the interaction among tuft cells, ILC2s, and iNKT2 cells in the peripheral tissues, particularly the intestine, requires clarification.

iNKT cells (particularly IL-9-producing iNKT cells) also up-regulate IL-22-producing ILC3s in the mLNs, ultimately preventing DSS-induced colitis under IFN-γ-dysregulated conditions (23), indicating that iNKT cells and ILC3s counter-regulate IFN-γ-mediated intestinal inflammation. Moreover, IL-22 increases intestinal barrier function and anti-microbial peptide production (64, 65), and iNKT cells trigger ILC3s to produce IL-22 in the intestine (18), indicating that secretion of IL-22 by ILC3s following their interaction with iNKT cells might be effective in preventing colitis development.

5.3 Adaptive immune cells: CD8$^+$ NKT-like cells and Tregs

A previous study demonstrated that HFD feeding increases susceptibility of mice to DSS-induced colitis via a concurrent increase in CD1d-unrestricted NKT cells (mostly CD8$^+$ and DN T cells) and a decrease in Tregs (66). The severity of intestinal inflammation in transgenic mice expressing human IL-15 in IECs closely correlates with increased numbers of LP CD8$^+$ NKT cells and increased levels of Th1-type cytokines such as IFN-γ and TNF-α (67). We have previously reported that CD1d-independent CD8$^+$ NKT cells are critical effectors that exacerbate DSS-induced colitis in Yeti mice

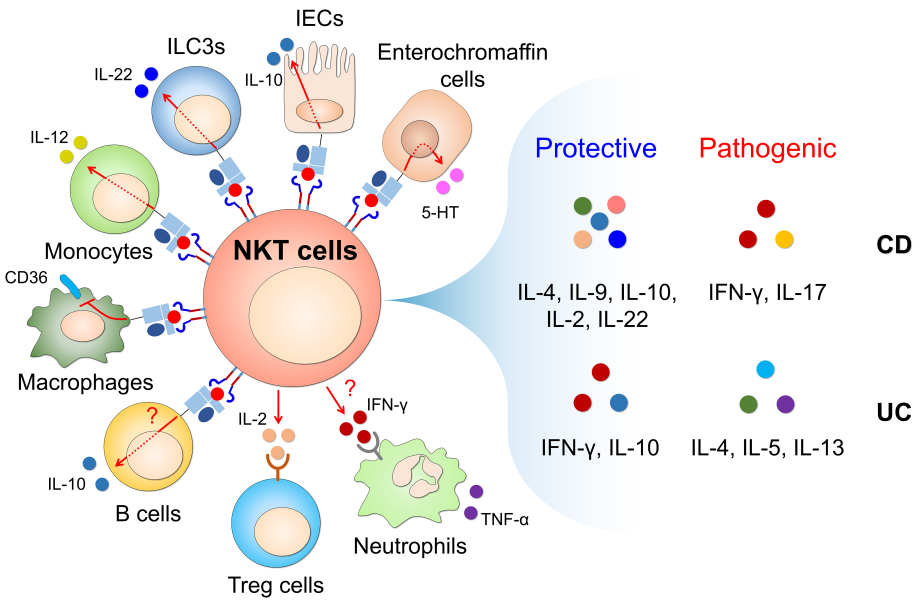

FIGURE 1
CD1d-restricted NKT cells interact with CD1d-expressing cells to produce NKT cell-derived soluble factors that regulate intestinal inflammatory responses. Glycolipid-mediated CD1d engagement triggers the activation of CD1d-expressing non-immune cells (such as IECs and enterochromaffin cells) and immune cells (such as ILC3s, B cells, monocytes and macrophages). This activation occurs through CD1d-intrinsic signaling in non-immune and immune cells and TCR/cytokine-dependent mechanisms in NKT cells. In addition, NKT cells interact with Tregs and neutrophils in a cytokine-dependent manner. Through this cross-talk with diverse cell types, CD1d-restricted NKT cells rapidly produce T helper (Th) 1 cytokines (e.g., IFN-γ), Th2 cytokines (e.g., IL-4, IL-5, IL-9, and IL-13), Th17 cytokines (e.g., IL-17 and IL-22), and regulatory cytokines (e.g., IL-10) upon stimulation with various stimuli (e.g., cytokines, chemokines, toll-like receptor (TLR) ligands, fatty acids, and glycolipids), which can elicit either protective or pathogenic effects in CD and UC. CD, Crohn's disease; UC, ulcerative colitis; IECs, intestinal epithelial cells; ILC3s, type 3 innate lymphoid cells; 5-HT, 5-hydroxytryptamine.

TABLE 1 Roles of gut microbiota and their derivatives in iNKT cell immunity.

Gut microbiota	Glycolipid Ag /TLR ligands/others	CD1d dependency	TLR dependency	Functions	References
Bacteroides fragilis	α-GalCer-like (d17-19:0;βh17:0)	dependent	–	Induce IFN-γ and IL-2 production by iNKT cells	(82)
	polysaccharide A	–	dependent (TLR2)	Induce thymic PLZF$^+$ iNKT cell development	(83)
	GSL-Bf717	dependent	–	Inhibit EC-derived serotonin release induced by iNKT cell-mediated CD1d ligation	(16)
	B. fragilis α-GalCer	dependent	–	Induce activation of and IFN-γ production by iNKT cells	(84)
	GSL-Bf717	dependent	–	Inhibit iNKT cell activation and iNKT cell-mediated colitis	(41)
E. coli LF82	–	dependent	–	Induce IFN-γ production by iNKT cells	(30)
Salmonella typhimurium	–	dependent	–	Induce IFN-γ production by iNKT cells	(30)
Sphingobium yanoikuyae	–	dependent	–	Induce iNKT cell activation	(32)
Helicobacter pylori	Cholesteryl α-Glucosides	dependent	–	Induce iNKT cell activation	(85)
Clostridium scindens	bile acid	–	–	Induce a rapid reduction of hepatic iNKT cells	(86)

–, not evaluated; EC cells, enterochromaffin cells.

characterized by enhanced stability of IFN-γ mRNA transcripts. In contrast, through cooperation with Tregs, iNKT cells are required to control CD1d-independent CD8$^+$ NKT cell-mediated pathogenesis during DSS-induced colitis (68). Pro-inflammatory DCs induce the differentiation of pathogenic Foxp3$^-$CD25$^+$CD4$^+$ T cells with Th1 and Th17 phenotypes and antagonize Treg differentiation in the mLNs of CD1d$^{-/-}$ Yeti mice lacking iNKT cells (69). iNKT cell-primed Tregs produce IL-10 in the presence of bacterial diacylglycerols and show an enhanced suppressive capacity, which provides support for the iNKT-Treg axis in regulating colitis (70). However, the regulatory effects of iNKT cells are restricted to the intestine but not to the spleen during DSS-induced colitis in Yeti mice (68), consistent with the dominance of iNKT2 cells in the intestine.

6 Impact of microbiota, microbiota-derived SCFA, and dietary LCFA on iNKT cells in IBD

Short-chain fatty acid (SCFA)-producing bacteria (e.g., *Faecalibacterium*, *Lachnospiraceae*, *Veillonella Gemmiger*, and *Prevotella*) help to maintain IL-10 production by iNKT cells (13). However, a previous study demonstrated that *Acetatifactor muris* (*A. muris*) belonging to *Firmicutes* is exclusively detected in the fecal samples of NOD2$^{-/-}$CD1d$^{-/-}$ mice lacking iNKT cells, compared to NOD2$^{-/-}$ mice with altered intestinal microbiota. Moreover, oral gavage of *A. muris* promotes colitis in DSS-treated WT mice (71). In addition, increased colonization of pathogenic bacteria (e.g., *Pseudomonas aeruginosa* and *Staphylococcus aureus*) in the intestine of CD1d$^{-/-}$ mice has been implicated in reduced release of Paneth cell-derived lysozyme, a potent anti-microbial protein (72). These anti-bacterial effects in WT mice are enhanced following *in vivo* iNKT cell activation by α-GalCer (72).

In DSS-induced colitis, WT B6 mice that received B6 CD1d KO-derived cecal contents show increased sensitivity to colitis compared with control WT B6 mice injected orally with WT B6-derived cecal contents. Furthermore, an increase in particular segmented filamentous bacteria and a decrease in *Akkermansia muciniphila* (*A. muciniphila*) closely correlates with increased colitis sensitivity in WT B6 mice that received B6 CD1d KO-derived cecal contents (73). Although oral administration of *A. muciniphila* plays a protective role in the Oxa-induced colitis model and *A. muciniphila* stimulates macrophages to release IL-10 (74), it remains unclear whether IL-10 production by *Akkermansia*-stimulated macrophages can induce iNKT10 differentiation in the colon during UC. Thus, it will be important to perform further studies on the protective mechanism of *Akkermansia* in colitis.

The kynurenic acid-GPR35 signaling pathway ameliorates DSS-induced colonic injury and inflammation by suppressing NLRP3-dependent IL-1β production in macrophages (75). Peripheral iNKT cells express high surface levels of GPR35, and the specific activation of GPR35 by its ligand (kynurenic acid) significantly reduces the release of IL-4 but not IFN-γ by α-GalCer-activated human iNKT cells (76). In addition, GPR65 gene expression in intestinal tissues is significantly up-regulated in both CD and UC patients compared with healthy controls (77). Moreover, the progression of DSS-induced colitis is aggravated by GPR65 deficiency, which supports the protective role of GPR65 in this model (77). iNKT cells expressing high levels of GPR65 play a pivotal role in suppressing autoimmune disease. For example, GPR65-deficient mice are more sensitive to experimental autoimmune encephalomyelitis in an iNKT cell-dependent manner (78). Collectively, these studies

suggest that G protein-coupled receptors such as GPR35 and GPR65 play essential roles in IBD protection through iNKT cells.

Butyrate, one of the SCFAs produced by microbiota, suppresses iNKT cell production of both IFN-γ and IL-4 via inhibiting class I histone deacetylases (79). In addition, palmitic acid (C16:0), a saturated fatty acid found in animal and vegetable fats, reduces the levels of both IFN-γ and IL-4 via inositol-requiring enzyme 1α (IRE1α) (80). Moreover, palmitic acid directly triggers NK1.1-negative iNKT cells to produce anti-inflammatory cytokines (e.g., IL-10) via IRE1α-X-box binding protein 1 (81). Thus, combining butyrate and palmitic acid as IFN-γ/IL-4 dual antagonists might represent a promising strategy to induce iNKT cell-mediated suppression in Th1 cell-mediated CD and Th2 cell-mediated UC.

7 Concluding remarks

The studies reviewed here identify iNKT cells as promising targets for designing IBD immune therapies (Figure 1 and Table 1). Although emerging evidence shows the immunological functions of CD1d and iNKT cell subsets (iNKT1, iNKT2, iNKT17, and iNKT10), little is known about their contribution to protection/pathogenesis against CD and UC. Further investigations are needed to explore the precise immunoregulatory mechanisms of intestinal iNKT subsets during IBD. Finally, it will be important to develop new tools to selectively activate or inhibit iNKT cells in the intestine.

Author contributions

SL: Funding acquisition, Writing – original draft, Writing – review & editing. HP: Funding acquisition, Writing – original draft, Writing – review & editing. LVK: Writing – review & editing. SH: Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1I1A1A01054418 to SL; NRF-2021R1I1A1A01051465 to HP; NRF-2022R1A2C1009590 to SH).

Conflict of interest

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

References

1. Roda G, Chien Ng S, Kotze PG, Argollo M, Panaccione R, Spinelli A, et al. Crohn's disease. *Nat Rev Dis Primers* (2020) 6(1):22. doi: 10.1038/s41572-020-0156-2

2. Kobayashi T, Siegmund B, Le Berre C, Wei SC, Ferrante M, Shen B, et al. Ulcerative colitis. *Nat Rev Dis Primers* (2020) 6(1):74. doi: 10.1038/s41572-020-0205-x

3. Van Kaer L, Postoak JL, Wang C, Yang G, Wu L. Innate, innate-like and adaptive lymphocytes in the pathogenesis of MS and EAE. *Cell Mol Immunol* (2019) 16(6):531–9. doi: 10.1038/s41423-019-0221-5

4. Park HJ, Lee SW, Park YH, Kim TC, Van Kaer L, Hong S. CD1d-independent NK1.1(+) Treg cells are IL2-inducible Foxp3(+) T cells co-expressing immunosuppressive and cytotoxic molecules. *Front Immunol* (2022) 13:951592. doi: 10.3389/fimmu.2022.951592

5. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-specific distribution of iNKT cells impacts their cytokine response. *Immunity* (2015) 43 (3):566–78. doi: 10.1016/j.immuni.2015.06.025

6. Nowak M, Schmidt-Wolf IG. Natural killer T cells subsets in cancer, functional defects in prostate cancer and implications for immunotherapy. *Cancers (Basel)* (2011) 3(3):3661–75. doi: 10.3390/cancers3033661

7. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13 (2):101–17. doi: 10.1038/nri3369

8. Lee SW, Park HJ, Van Kaer L, Hong S. Roles and therapeutic potential of CD1d-Restricted NKT cells in inflammatory skin diseases. *Front Immunol* (2022) 13:979370. doi: 10.3389/fimmu.2022.979370

9. Park HJ, Lee SW, Hong S. Regulation of allergic immune responses by microbial metabolites. *Immune Netw* (2018) 18(1):e15. doi: 10.4110/in.2018.18.e15

10. Park HJ, Kim TC, Park YH, Lee SW, Jeon J, Park SH, et al. Repeated alpha-GalCer Administration Induces a Type 2 Cytokine-Biased iNKT Cell Response and Exacerbates Atopic Skin Inflammation in V alpha 14(Tg) NC/Nga Mice. *Biomedicines* (2021) 9(11):1619. doi: 10.3390/biomedicines9111619

11. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol* (2013) 14(11):1146–54. doi: 10.1038/ni.2731

12. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124 (9):3725–40. doi: 10.1172/JCI72308

13. Burrello C, Strati F, Lattanzi G, Diaz-Basabe A, Mileti E, Giuffre MR, et al. IL10 secretion endows intestinal human iNKT cells with regulatory functions towards pathogenic T lymphocytes. *J Crohns Colitis* (2022) 16(9):1461–74. doi: 10.1093/ecco-jcc/jjac049

14. Kane H, LaMarche NM, Ni Scannail A, Garza AE, Koay HF, Azad AI, et al. Longitudinal analysis of invariant natural killer T cell activation reveals a cMAF-associated transcriptional state of NKT10 cells. *Elife* (2022) 11:e76586. doi: 10.7554/eLife.76586

15. Iwabuchi K, Van Kaer L. Editorial: role of CD1- and MR1-restricted T cells in immunity and disease. *Front Immunol* (2019) 10:1837. doi: 10.3389/fimmu.2019.01837

16. Luo J, Chen Z, Castellano D, Bao B, Han W, Li J, et al. Lipids regulate peripheral serotonin release *via* gut CD1d. *Immunity* (2023) 56(7):1533–47.e7. doi: 10.1016/j.immuni.2023.06.001

17. Olszak T, Neves JF, Dowds CM, Baker K, Glickman J, Davidson NO, et al. Protective mucosal immunity mediated by epithelial CD1d and IL-10. *Nature* (2014) 509(7501):497–502. doi: 10.1038/nature13150

18. Saez de Guinoa J, Jimeno R, Farhadi N, Jervis PJ, Cox LR, Besra GS, et al. CD1d-mediated activation of group 3 innate lymphoid cells drives IL-22 production. *EMBO Rep* (2017) 18(1):39–47. doi: 10.15252/embr.201642412

19. Cui S, Wang C, Bai W, Li J, Pan Y, Huang X, et al. CD1d1 intrinsic signaling in macrophages controls NLRP3 inflammasome expression during inflammation. *Sci Adv* (2020) 6(43):eaaz7290. doi: 10.1126/sciadv.aaz7290

20. Grose RH, Thompson FM, Baxter AG, Pellicci DG, Cummins AG. Deficiency of invariant NK T cells in Crohn's disease and ulcerative colitis. *Dig Dis Sci* (2007) 52 (6):1415–22. doi: 10.1007/s10620-006-9261-7

21. Eichele DD, Kharbanda KK. Dextran sodium sulfate colitis murine model: An indispensable tool for advancing our understanding of inflammatory bowel diseases pathogenesis. *World J Gastroenterol* (2017) 23(33):6016–29. doi: 10.3748/wjg.v23.i33.6016

22. Kim HS, Chung DH. IL-9-producing invariant NKT cells protect against DSS-induced colitis in an IL-4-dependent manner. *Mucosal Immunol* (2013) 6(2):347–57. doi: 10.1038/mi.2012.77

23. Park HJ, Lee SW, Van Kaer L, Hong S. CD1d-dependent iNKT cells control DSS-induced colitis in a mouse model of IFNgamma-mediated hyperinflammation by increasing IL22-secreting ILC3 cells. *Int J Mol Sci* (2021) 22(3):1250. doi: 10.3390/ijms22031250

24. Fuss IJ, Heller F, Boirivant M, Leon F, Yoshida M, Fichtner-Feigl S, et al. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest* (2004) 113(10):1490–7. doi: 10.1172/JCI19836

25. Liao CM, Zimmer MI, Shanmuganad S, Yu HT, Cardell SL, Wang CR. dysregulation of CD1d-restricted type ii natural killer T cells leads to spontaneous development of colitis in mice. *Gastroenterology* (2012) 142(2):326–34.e1-2. doi: 10.1053/j.gastro.2011.10.030

26. Fuss IJ, Joshi B, Yang Z, Degheidy H, Fichtner-Feigl S, de Souza H, et al. IL-13Ralpha2-bearing, type II NKT cells reactive to sulfatide self-antigen populate the mucosa of ulcerative colitis. *Gut* (2014) 63(11):1728–36. doi: 10.1136/gutjnl-2013-305671

27. Sagami S, Ueno Y, Tanaka S, Fujita A, Niitsu H, Hayashi R, et al. Choline deficiency causes colonic type II natural killer T (NKT) cell loss and alleviates murine colitis under type I NKT cell deficiency. *PLoS One* (2017) 12(1):e0169681. doi: 10.1371/journal.pone.0169681

28. Liao CM, Zimmer MI, Wang CR. The functions of type I and type II natural killer T cells in inflammatory bowel diseases. *Inflammation Bowel Dis* (2013) 19 (6):1330–8. doi: 10.1097/MIB.0b013e318280b1e3

29. Hashimoto M, Hiwatashi K, Ichiyama K, Morita R, Sekiya T, Kimura A, et al. SOCS1 regulates type I/type II NKT cell balance by regulating IFNgamma signaling. *Int Immunol* (2011) 23(3):165–76. doi: 10.1093/intimm/dxq469

30. Burrello C, Pellegrino G, Giuffre MR, Lovati G, Magagna I, Bertocchi A, et al. Mucosa-associated microbiota drives pathogenic functions in IBD-derived intestinal iNKT cells. *Life Sci Alliance* (2019) 2(1):e201800229. doi: 10.26508/lsa.201800229

31. Saez de Guinoa J, Jimeno R, Gaya M, Kipling D, Garzon MJ, Dunn-Walters D, et al. CD1d-mediated lipid presentation by CD11c(+) cells regulates intestinal homeostasis. *EMBO J* (2018) 37(5):e97537. doi: 10.15252/embj.201797537

32. Wingender G, Stepniak D, Krebs P, Lin L, McBride S, Wei B, et al. Intestinal microbes affect phenotypes and functions of invariant natural killer T cells in mice. *Gastroenterology* (2012) 143(2):418–28. doi: 10.1053/j.gastro.2012.04.017

33. Saubermann LJ, Beck P, De Jong YP, Pitman RS, Ryan MS, Kim HS, et al. Activation of natural killer T cells by alpha-galactosylceramide in the presence of CD1d provides protection against colitis in mice. *Gastroenterology* (2000) 119(1):119–28. doi: 10.1053/gast.2000.9114

34. Lee C, Hong SN, Kim YH. A glycolipid adjuvant, 7DW8-5, provides a protective effect against colonic inflammation in mice by the recruitment of CD1d-restricted natural killer T cells. *Intest Res* (2020) 18(4):402–11. doi: 10.5217/ir.2019.00132

35. Numata Y, Tazuma S, Ueno Y, Nishioka T, Hyogo H, Chayama K. Therapeutic effect of repeated natural killer T cell stimulation in mouse cholangitis complicated by colitis. *Dig Dis Sci* (2005) 50(10):1844–51. doi: 10.1007/s10620-005-2949-2

36. Cameron G, Nguyen T, Ciula M, Williams SJ, Godfrey DI. Glycolipids from the gut symbiont Bacteroides fragilis are agonists for natural killer T cells and induce their regulatory differentiation. *Chem Sci* (2023) 14(29):7887–96. doi: 10.1039/d3sc02124f

37. Strati F, Pujolassos M, Burrello C, Giuffre MR, Lattanzi G, Caprioli F, et al. Antibiotic-associated dysbiosis affects the ability of the gut microbiota to control intestinal inflammation upon fecal microbiota transplantation in experimental colitis models. *Microbiome* (2021) 9(1):39. doi: 10.1186/s40168-020-00991-x

38. Burrello C, Garavaglia F, Cribiu FM, Ercoli G, Lopez G, Troisi J, et al. Therapeutic faecal microbiota transplantation controls intestinal inflammation through IL10 secretion by immune cells. *Nat Commun* (2018) 9(1):5184. doi: 10.1038/s41467-018-07359-8

39. Ueno Y, Tanaka S, Sumii M, Miyake S, Tazuma S, Taniguchi M, et al. Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of valpha14 natural killer T cells in mice. *Inflammation Bowel Dis* (2005) 11(1):35–41. doi: 10.1097/00054725-200501000-00005

40. Inuki S, Hirata N, Kashiwabara E, Kishi J, Aiba T, Teratani T, et al. Polar functional group-containing glycolipid CD1d ligands modulate cytokine-biasing responses and prevent experimental colitis. *Sci Rep* (2020) 10(1):15766. doi: 10.1038/s41598-020-72280-4

41. An D, Oh SF, Olszak T, Neves JF, Avci FY, Erturk-Hasdemir D, et al. Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. *Cell* (2014) 156(1-2):123–33. doi: 10.1016/j.cell.2013.11.042

42. Oh SF, Praveena T, Song H, Yoo JS, Jung DJ, Erturk-Hasdemir D, et al. Host immunomodulatory lipids created by symbionts from dietary amino acids. *Nature* (2021) 600(7888):302–7. doi: 10.1038/s41586-021-04083-0

43. Song X, Zhang H, Zhang Y, Goh B, Bao B, Mello SS, et al. Gut microbial fatty acid isomerization modulates intraepithelial T cells. *Nature* (2023) 619(7971):837–43. doi: 10.1038/s41586-023-06265-4

44. Pereira CS, Sa-Miranda C, De Libero G, Mori L, Macedo MF. Globotriaosylceramide inhibits iNKT-cell activation in a CD1d-dependent manner. *Eur J Immunol* (2016) 46(1):147–53. doi: 10.1002/eji.201545725

45. Lai AC, Chi PY, Thio CL, Han YC, Kao HN, Hsieh HW, et al. alpha-lactosylceramide protects against iNKT-mediated murine airway hyperreactivity and liver injury through competitive inhibition of cd1d binding. *Front Chem* (2019) 7:811. doi: 10.3389/fchem.2019.00811

46. Perera L, Shao L, Patel A, Evans K, Meresse B, Blumberg R, et al. Expression of nonclassical class I molecules by intestinal epithelial cells. *Inflammation Bowel Dis* (2007) 13(3):298–307. doi: 10.1002/ibd.20026

47. Iyer SS, Gensollen T, Gandhi A, Oh SF, Neves JF, Collin F, et al. Dietary and microbial oxazoles induce intestinal inflammation by modulating aryl hydrocarbon receptor responses. *Cell* (2018) 173(5):1123–34.e11. doi: 10.1016/j.cell.2018.04.037

48. Colgan SP, Hershberg RM, Furuta GT, Blumberg RS. Ligation of intestinal epithelial CD1d induces bioactive IL-10: critical role of the cytoplasmic tail in autocrine signaling. *Proc Natl Acad Sci U S A* (1999) 96(24):13938–43. doi: 10.1073/pnas.96.24.13938

49. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity* (2002) 16(2):219–30. doi: 10.1016/s1074-7613(02)00274-1

50. Oleinika K, Rosser EC, Matei DE, Nistala K, Bosma A, Drozdov I, et al. CD1d-dependent immune suppression mediated by regulatory B cells through modulations of iNKT cells. *Nat Commun* (2018) 9(1):684. doi: 10.1038/s41467-018-02911-y

51. Yue SC, Shaulov A, Wang R, Balk SP, Exley MA. CD1d ligation on human monocytes directly signals rapid NF-kappaB activation and production of bioactive IL-12. *Proc Natl Acad Sci U S A* (2005) 102(33):11811–6. doi: 10.1073/pnas.0503366102

52. Brailey PM, Evans L, Lopez-Rodriguez JC, Sinadinos A, Tyrrel V, Kelly G, et al. CD1d-dependent rewiring of lipid metabolism in macrophages regulates innate immune responses. *Nat Commun* (2022) 13(1):6723. doi: 10.1038/s41467-022-34532-x

53. Zhigang Lei JY, Wu Yu, Shen J, Lin S, Xue W, Mao C, et al. CD1d protects against hepatocyte apoptosis in non-alcoholic steatohepatitis. *J Hepatol* (2023) 23: S0168–8278. doi: 10.1016/j.jhep.2023.10.025

54. Diehl GE, Longman RS, Zhang JX, Breart B, Galan C, Cuesta A, et al. Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX(3)CR1(hi) cells. *Nature* (2013) 494(7435):116–20. doi: 10.1038/nature11809

55. Rossini V, Zhurina D, Radulovic K, Manta C, Walther P, Riedel CU, et al. CX3CR1(+) cells facilitate the activation of CD4 T cells in the colonic lamina propria during antigen-driven colitis. *Mucosal Immunol* (2014) 7(3):533–48. doi: 10.1038/mi.2013.70

56. Gensollen T, Lin X, Zhang T, Pyzik M, See P, Glickman JN, et al. Embryonic macrophages function during early life to determine invariant natural killer T cell levels at barrier surfaces. *Nat Immunol* (2021) 22(6):699–710. doi: 10.1038/s41590-021-00934-0

57. Huang E, Liu R, Lu Z, Liu J, Liu X, Zhang D, et al. NKT cells mediate the recruitment of neutrophils by stimulating epithelial chemokine secretion during colitis. *Biochem Biophys Res Commun* (2016) 474(2):252–8. doi: 10.1016/j.bbrc.2016.04.024

58. Shen S, Prame Kumar K, Stanley D, Moore RJ, Van TTH, Wen SW, et al. Invariant natural killer T cells shape the gut microbiota and regulate neutrophil recruitment and function during intestinal inflammation. *Front Immunol* (2018) 9:999. doi: 10.3389/fimmu.2018.00999

59. Ohms M, Moller S, Laskay T. An attempt to polarize human neutrophils toward N1 and N2 phenotypes in vitro. *Front Immunol* (2020) 11:532. doi: 10.3389/fimmu.2020.00532

60. Camelo A, Barlow JL, Drynan LF, Neill DR, Ballantyne SJ, Wong SH, et al. Blocking IL-25 signalling protects against gut inflammation in a type-2 model of colitis by suppressing nuocyte and NKT derived IL-13. *J Gastroenterol* (2012) 47(11):1198–211. doi: 10.1007/s00535-012-0591-2

61. von Moltke J, Ji M, Liang HE, Locksley RM. Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit. *Nature* (2016) 529(7585):221–5. doi: 10.1038/nature16161

62. Schneider C, O'Leary CE, von Moltke J, Liang HE, Ang QY, Turnbaugh PJ, et al. A metabolite-triggered tuft cell-ILC2 circuit drives small intestinal remodeling. *Cell* (2018) 174(2):271–84.e14. doi: 10.1016/j.cell.2018.05.014

63. Lucas B, White AJ, Cosway EJ, Parnell SM, James KD, Jones ND, et al. Diversity in medullary thymic epithelial cells controls the activity and availability of iNKT cells. *Nat Commun* (2020) 11(1):2198. doi: 10.1038/s41467-020-16041-x

64. Gulhane M, Murray L, Lourie R, Tong H, Sheng YH, Wang R, et al. High fat diets induce colonic epithelial cell stress and inflammation that is reversed by IL-22. *Sci Rep* (2016) 6:28990. doi: 10.1038/srep28990

65. Coorens M, Rao A, Grafe SK, Unelius D, Lindforss U, Agerberth B, et al. Innate lymphoid cell type 3-derived interleukin-22 boosts lipocalin-2 production in intestinal epithelial cells *via* synergy between STAT3 and NF-kappaB. *J Biol Chem* (2019) 294 (15):6027–41. doi: 10.1074/jbc.RA118.007290

66. Ma X, Torbenson M, Hamad AR, Soloski MJ, Li Z. High-fat diet modulates non-CD1d-restricted natural killer T cells and regulatory T cells in mouse colon and exacerbates experimental colitis. *Clin Exp Immunol* (2008) 151(1):130–8. doi: 10.1111/j.1365-2249.2007.03530.x

67. Yoshihara K, Yajima T, Kubo C, Yoshikai Y. Role of interleukin 15 in colitis induced by dextran sulphate sodium in mice. *Gut* (2006) 55(3):334–41. doi: 10.1136/gut.2005.076000

68. Lee SW, Park HJ, Cheon JH, Wu L, Van Kaer L, Hong S. iNKT cells suppress pathogenic NK1.1(+)CD8(+) T cells in DSS-induced colitis. *Front Immunol* (2018) 9:2168. doi: 10.3389/fimmu.2018.02168

69. Lee SW, Park HJ, Van Kaer L, Hong S. Opposing Roles of DCs and iNKT Cells in the Induction of Foxp3 Expression by MLN CD25(+)CD4(+) T Cells during IFNgamma-Driven Colitis. *Int J Mol Sci* (2022) 23(23):15316. doi: 10.3390/ijms232315316

70. Venken K, Decruy T, Aspeslagh S, Van Calenbergh S, Lambrecht BN, Elewaut D. Bacterial CD1d-restricted glycolipids induce IL-10 production by human regulatory T cells upon cross-talk with invariant NKT cells. *J Immunol* (2013) 191(5):2174–83. doi: 10.4049/jimmunol.1300562

71. Lee C, Hong SN, Paik NY, Kim TJ, Kim ER, Chang DK, et al. CD1d modulates colonic inflammation in NOD2-/- mice by altering the intestinal microbial composition comprising acetatifactor muris. *J Crohns Colitis* (2019) 13(8):1081–91. doi: 10.1093/ecco-jcc/jjz025

72. Nieuwenhuis EE, Matsumoto T, Lindenbergh D, Willemsen R, Kaser A, Simons-Oosterhuis Y, et al. Cd1d-dependent regulation of bacterial colonization in the intestine of mice. *J Clin Invest* (2009) 119(5):1241–50. doi: 10.1172/JCI36509

73. Selvanantham T, Lin Q, Guo CX, Surendra A, Fieve S, Escalante NK, et al. NKT cell-deficient mice harbor an altered microbiota that fuels intestinal inflammation during chemically induced colitis. *J Immunol* (2016) 197(11):4464–72. doi: 10.4049/jimmunol.1601410

74. Qu S, Fan L, Qi Y, Xu C, Hu Y, Chen S, et al. Akkermansia muciniphila alleviates dextran sulfate sodium (DSS)-induced acute colitis by NLRP3 activation. *Microbiol Spectr* (2021) 9(2):e0073021. doi: 10.1128/Spectrum.00730-21

75. Zheng X, Hu M, Zang X, Fan Q, Liu Y, Che Y, et al. Kynurenic acid/GPR35 axis restricts NLRP3 inflammasome activation and exacerbates colitis in mice with social stress. *Brain Behav Immun* (2019) 79:244–55. doi: 10.1016/j.bbi.2019.02.009

76. Fallarini S, Magliulo L, Paoletti T, de Lalla C, Lombardi G. Expression of functional GPR35 in human iNKT cells. *Biochem Biophys Res Commun* (2010) 398 (3):420–5. doi: 10.1016/j.bbrc.2010.06.091

77. Marie MA, Sanderlin EJ, Satturwar S, Hong H, Lertpiriyapong K, Donthi D, et al. GPR65 (TDAG8) inhibits intestinal inflammation and colitis-associated colorectal cancer development in experimental mouse models. *Biochim Biophys Acta Mol Basis Dis* (2022) 1868(1):166288. doi: 10.1016/j.bbadis.2021.166288

78. Wirasinha RC, Vijayan D, Smith NJ, Parnell GP, Swarbrick A, Brink R, et al. GPR65 inhibits experimental autoimmune encephalomyelitis through CD4(+) T cell independent mechanisms that include effects on iNKT cells. *Immunol Cell Biol* (2018) 96(2):128–36. doi: 10.1111/imcb.1031

79. Lee S, Koh J, Chang Y, Kim HY, Chung DH. Invariant NKT cells functionally link microbiota-induced butyrate production and joint inflammation. *J Immunol* (2019) 203(12):3199–208. doi: 10.4049/jimmunol.1801314

80. Ko JS, Koh JM, So JS, Jeon YK, Kim HY, Chung DH. Palmitate inhibits arthritis by inducing t-bet and gata-3 mRNA degradation in iNKT cells *via* IRE1alpha-dependent decay. *Sci Rep* (2017) 7(1):14940. doi: 10.1038/s41598-017-14780-4

81. LaMarche NM, Kane H, Kohlgruber AC, Dong H, Lynch L, Brenner MB. Distinct iNKT cell populations use IFNgamma or ER stress-induced IL-10 to control adipose tissue homeostasis. *Cell Metab* (2020) 32(2):243–58.e6. doi: 10.1016/j.cmet.2020.05.017

82. von Gerichten J, Lamprecht D, Opalka L, Soulard D, Marsching C, Pilz R, et al. Bacterial immunogenic alpha-galactosylceramide identified in the murine large intestine: dependency on diet and inflammation. *J Lipid Res* (2019) 60(11):1892–904. doi: 10.1194/jlr.RA119000236

83. Ennamorati M, Vasudevan C, Clerkin K, Halvorsen S, Verma S, Ibrahim S, et al. Intestinal microbes influence development of thymic lymphocytes in early life. *Proc Natl Acad Sci U S A* (2020) 117(5):2570–8. doi: 10.1073/pnas.1915047117

84. Wieland Brown LC, Penaranda C, Kashyap PC, Williams BB, Clardy J, Kronenberg M, et al. Production of alpha-galactosylceramide by a prominent member of the human gut microbiota. *PLoS Biol* (2013) 11(7):e1001610. doi: 10.1371/journal.pbio.1001610

85. Ito Y, Vela JL, Matsumura F, Hoshino H, Tyznik A, Lee H, et al. Helicobacter pylori cholesteryl alpha-glucosides contribute to its pathogenicity and immune response by natural killer T cells. *PLoS One* (2013) 8(12):e78191. doi: 10.1371/journal.pone.0078191

86. Ma C, Han M, Heinrich B, Fu Q, Zhang Q, Sandhu M, et al. Gut microbiome-mediated bile acid metabolism regulates liver cancer *via* NKT cells. *Science* (2018) 360 (6391):eaan5931. doi: 10.1126/science.aan5931

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 01 February 2024
DOI 10.3389/fimmu.2024.1345037

OPEN ACCESS

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RECEIVED 27 November 2023
ACCEPTED 15 January 2024
PUBLISHED 01 February 2024

CITATION
Yamasaki S, Shimizu K and Fujii S (2024) Tumor epitope spreading by a novel multivalent therapeutic cellular vaccine targeting cancer antigens to invariant NKT-triggered dendritic cells *in situ*. *Front. Immunol.* 15:1345037. doi: 10.3389/fimmu.2024.1345037

Tumor epitope spreading by a novel multivalent therapeutic cellular vaccine targeting cancer antigens to invariant NKT-triggered dendritic cells *in situ*

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Introduction: Cancer is categorized into two types based on the microenvironment: cold and hot tumors. The former is challenging to stimulate through immunity. The immunogenicity of cancer relies on the quality and quantity of cancer antigens, whether recognized by T cells or not. Successful cancer immunotherapy hinges on the cancer cell type, antigenicity and subsequent immune reactions. The T cell response is particularly crucial for secondary epitope spreading, although the factors affecting these mechanisms remain unknown. Prostate cancer often becomes resistant to standard therapy despite identifying several antigens, placing it among immunologically cold tumors. We aim to leverage prostate cancer antigens to investigate the potential induction of epitope spreading in cold tumors. This study specifically focuses on identifying factors involved in secondary epitope spreading based on artificial adjuvant vector cell (aAVC) therapy, a method established as invariant natural killer T (iNKT) -licensed DC therapy.

Methods: We concentrated on three prostate cancer antigens (prostate-specific membrane antigen (PSMA), prostate-specific antigen (PSA), and prostatic acid phosphatase (PAP)). By introducing allogeneic cells with the antigen and murine CD1d mRNA, followed by α-galactosylceramide (α-GalCer) loading, we generated five types of aAVCs, i.e, monovalent, divalent and trivalent antigen-expressing aAVCs and four types of prostate antigen-expressing cold tumors. We evaluated iNKT activation and antigen-specific CD8+ T cell responses against tumor cells prompted by the aAVCs.

Results: Our study revealed that monovalent aAVCs, expressing a single prostate antigen, primed T cells for primary tumor antigens and also induced T cells targeting additional tumor antigens by triggering a tumor antigen-spreading response. When we investigated the immune response by trivalent aAVC (aAVC-PROS), aAVC-PROS therapy elicited multiple antigen-specific CD8+ T cells simultaneously. These CD8+ T cells exhibited both preventive and therapeutic effects against tumor progression.

Conclusions: The findings from this study highlight the promising role of tumor antigen-expressing aAVCs, in inducing efficient epitope spreading and generating robust immune responses against cancer. Our results also propose that multivalent antigen-expressing aAVCs present a promising therapeutic option and could be a more comprehensive therapy for treating cold tumors like prostate cancer.

KEYWORDS

cancer, cytotoxic T cell, dendritic cell, immunotherapy, iNKT cell

1 Introduction

Prostate cancer (PCa) is considered a "cold tumor," characterized by low immune cell infiltration, an immunosuppressive tumor immune microenvironment (TIME), and infiltrating effector lymphocytes with a dysfunctional phenotype (1). However, recent single-cell analyses have allowed stratification, and immunotherapy is effective in some cases. Clinically, PCa is the second most prevalent cancer in men and the fifth most common cause of cancer-related deaths worldwide (2). Prostatectomy, radiation therapy, chemotherapy, and hormone deprivation therapy have been used as traditional treatments for prostate cancer (3). However, these treatments have limited efficacy; thus, new approaches for combating prostate cancer are warranted (4). One of the reasons for the limited treatment options relies on the host's immune dysfunction in innate and adaptive immunity. By investigating prostate cancer as an example, the clinical concerns of cold tumors can be analogized.

PCa stands as a significant example among cancers recognized for their diminished innate immunity. Patients with advanced PCa exhibit a decreased number of peripheral blood iNKT cells. Patients with aggressive stages of the disease had fewer iNKT cells (5). In considering the functional restoration of innate immunity, the advantage of the antitumor effect mediated by iNKT cells extends beyond the direct antitumor effect. It also includes the adjuvant effect on dendritic cells and the adjunct effect on natural killer (NK) cells. The adjunct effect on NK cells, wherein iNKT cells activate NK cells even in the presence of tumors, has been well-known because the validation of NK activation is an important indicator. Indeed, NK cells are major effector cells in cancer immunotherapy (6, 7). NK cell function is often inhibited at multiple levels in the PCa TME (8). Decreased or impaired NK activity has been reported as cancer progresses (9), and the frequency of circulating NK cells is reduced in metastatic PCa (10). Further, higher intratumoral NK cell numbers have been shown to confer longer distant metastasis-free survival (11). Thus, iNKT and NK cells, as innate lymphocytes, play a protective role against the aggressive state or metastasis of prostate cancer. PCa is a cancer known to have reduced T-cell immunity. Important evidence has emerged from clinical studies regarding the role of T cells in prostate cancer. Patients with PCa show limited responses to immune checkpoint inhibitors, which have revolutionized the treatment of several cancer types (12–15). This poor response to immune checkpoint blockade (ICB) treatment is attributed to the low infiltration of $CD8^+$ T cells into prostate tumors compared to other solid tumors that are more responsive to checkpoint blockade (16, 17). Despite this, prostate cancer cells typically express several prostate-specific antigens, such as prostatic acid phosphatase (PAP), prostate-specific antigen (PSA), and prostate-specific membrane antigen (PSMA), which have been used as vaccine targets (18). Therefore, developing a strategy for immunotherapy against prostate cancer requires the expansion of prostate tumor antigen-specific $CD8^+$ T cells and their trafficking into tumor tissues.

There are several cancers, such as PCa, pancreatic, ovarian and breast cancers, in which both NK and T cells are dysfunctional from a clinical standpoint (19). Immunological strategies that boost both should be considered for such cold tumors. Simultaneously generating NK cell and $CD8^+$ T cell responses is ideal for preventing tumor cells from evading immune surveillance. Previous animal and clinical studies have reported on α-galactosylceramide (α-GalCer)-pulsed dendritic cell (DC) therapy. iNKT cells produce systemic IFN-γ responses, leading to NK cell-mediated antitumor effects (20–25). Recently, we reported that the number of granzyme B-expressing NK cells increases in the early stages of lung cancer (26). However, therapies focused solely on NK cell activation exhibit limited efficacy. We developed a cellular cancer vaccine termed artificial adjuvant vector cells (aAVCs) (27–29), based on the relationship between innate immunity and the induction of adaptive immunity via *in vivo* DC targeting (30, 31). These cells are allogeneic cells (NIH3T3 for murine and HEK293 for human) co-transfected with an antigen-derived mRNA and CD1d mRNA and then loaded with iNKT cell ligand. aAVC can activate iNKT and NK

Abbreviations: α-GalCer, α-galactosylceramide; aAVC, artificial adjuvant vector cell; DC, dendritic cell; aAVC-PROS, PROS-expressing aAVCs; NK cell, natural killer cell; iNKT cell, invariant natural killer T cell; ELISPOT, enzyme-linked immune absorbent spot; TIME, tumor immune microenvironment.

cells as an adjunct activity by iNKT cells. In a series of immune responses, after activation by aAVC, innate iNKT/NK cells reject the aAVCs, and then, the killed aAVCs are taken up by DCs *in situ*, thereby promoting several immunogenic features of DCs. The *in vivo* DCs in the lungs, liver, and spleen that captured aAVCs undergo maturation via interaction with iNKT cells, which is brought about by CD40L/CD40 interactions and inflammatory cytokines (IFN-γ and TNF-α) (27–29, 32, 33). aAVC therapy is a cellular therapy platform, and we demonstrated that many types of antigens were used, such as OVA, MART-1, NY-ESO-1, and WT1 antigens in murine models (27–29, 34–36) and also against human relapsed and refractory acute myelogenous leukemia patients (37). Such *in vivo* DC-targeting therapies promote $CD8^+$ T cell responses more effectively than ex vivo DC therapies (28, 32, 33, 38).

This study has two primary aims. First, we attempted to establish an aAVC containing the cancer antigens and demonstrate an antitumor effect against the relevant antigen-expressing, cold tumor cells. We investigated whether aAVCs expressing a single antigen can induce tumor antigen-specific responses and demonstrate antigen spreading. Furthermore, we developed aAVCs containing two or three cancer antigens (PSMA, PSA, and PAP) aiming to induce multiple types of $CD8^+$ T cells, thereby preventing tumor cells from evading immune detection. We examined how aAVCs, which express several antigens, activate multiple antigen-specific $CD8^+$ T cells *in vivo*. We then compared the condition because the strength of the $CD8^+$ T cell response depends on the antigenicity of the tumor antigen. Our findings demonstrate that multiple prostate-cancer antigen-expressing aAVCs can promote tumor epitope spreading as well as activate multiple antigen-specific $CD8^+$ T cells, representing a promising therapeutic option for conventional therapy-resistant prostate cancer. Hence, restoring and activating innate immunity would lead to useful immunotherapy.

2 Materials and methods

2.1 Animals and cell lines

Pathogen-free C57BL/6 (B6) mice were purchased from Charles River at 6 to 8 weeks of age. Rag1 $^{-/-}$ (B6.129S7-*Rag1*^{tm1Mom}/J) mice were purchased from the Jackson Laboratory. $CD1d^{-/-}$ mice with B6 genetic background were kindly provided from L. Van Kaer (Vanderbilt University, Nashville, TN). Ly5.1 congenic OT-1 mice were generated by cross/backcross breeding of OT-1 with B6. Ly5.1 mice. All mice were maintained under specific pathogen-free conditions at the RIKEN animal facility, and all procedures were performed in compliance with the protocols approved by the Institutional Animal Care Committee at RIKEN (AEY2022-020). NIH3T3 cells were obtained from the RIKEN Cell Bank. NKT hybridoma (1.B2) and B16 cell line were kindly provided by Dr. M. Kronenberg (La Jolla Institute) and Dr. RM Steinman (Rockefeller University) respectively. All cell lines were maintained and treated according to the supplier's recommendations. B16-PSMA, B16-PSA, and B16-PAP cells were generated by retroviral transduction of human PSMA, PSA, or PAP genes into B16 cells.

2.2 Reagents

2.2.1 Antibodies, peptides and proteins

The following monoclonal antibody (mAbs) were purchased from BD Biosciences, or BioLegend: anti-CD1d (1B1), anti-CD3 (145-2C11), anti-CD8 (53-6.7), CD19(6D5), CD45.1 (A20), TCRβ (H57-597), Vα2/TCRVα2 (B20.1), TNF-α (MP6-XT22) and IFN-γ (XMG1.2) and CD1d-DimerX. The OVA-tetramer and CD1d-tetramer were purchased from MBL. Anti-PSMA (clone: UMAB27), anti-PSA (clone: EP1588Y), and anti-PAP (clone: EPR4067) antibodies were obtained from OriGene. OVA257–264 peptide (SIINFEKL) and α-GalCer were purchased from Toray Research Center, Inc., and Funakoshi, respectively. PepTivator PSMA (#130-099-795), PepTivator PSA (#130-099-800), and PepTivator PAP (#130-096-767) were purchased from Miltenyi Biotech Inc. The recombinant human PSMA (FOLH1), human PSA (KLK3), and human PAP (ACPP) proteins were purchased from Origene (Rockville, MD, USA).

2.2.2 Cell preparation

Mononuclear cells (MNCs) from the spleen and liver were isolated as previously described. Briefly, spleen cells were obtained by passing the spleen through a 70 μm cell strainer. Subsequently, erythrocytes were lysed with ACK lysing buffer (Life Technologies). For the isolation of liver MNCs, the minced liver tissues were digested with collagenase D (Roche) and layered on Percoll gradients (40/70%) (Amersham Pharmacia Biotec), followed by centrifugation.

2.3 Flow cytometry

The spleen cells were preincubated with anti-CD16/CD32 (BioLegend) in PBS with 2% heat-inactivated FBS and then stained with surface antibodies and viability dye for 30 min on ice. A Fixable Aqua or Violet-Dead Cell Stain Kit (Invitrogen) was used to remove the dead cells. The cytokine production was analyzed by intracellular staining using a BD Intracellular Cytokine Staining Kit (BD Biosciences). For these, LSRFortessa X-20 or Canto II instrument and CELLQuest, FACSDiva (BD Biosciences), or the FlowJo software (v10.3B2) were used.

2.4 Preparation of aAVC-OVA, PSMA, aAVC-PSA, and aAVC-PAP

Murine CD1d and OVA plasmids used in this study was previously described (29). Coding sequences for PSMA (GenBank Accession Number: NM_004476), PSA (GenBank Accession Number: NM_001648), and PAP (GenBank Accession Number: NM_001099) were generated via gene synthesis (Takara) and cloned into the HindIII and BamHI, HindIII and EcoRI, and HindIII and BamHI sites of the pGEM-4Z vector (Promega), respectively. The resultant plasmid was then linearized with BamHI and EcoRI and purified using the QIAquick PCR Purification Kit (Qiagen). *In vitro* transcription was conducted

using the mMessage mMachine T7 Ultra Kit (Ambion), and then RNA was purified using the RNeasy Mini/Midi Kit (Qiagen). aAVCs were prepared as previously described (27). Briefly, NIH3T3 cells resuspended in OptiMEM and RNA were transferred simultaneously to a cuvette. This cell suspension was pulsed in an ECM 830 Square Wave Electroporation System (Harvard Apparatus). After electroporation, the cells were transferred to a culture medium and cultured in 500 ng/mL of α-GalCer. Protein expression in the transfected cells was assessed by flow cytometry for CD1d, ELISA (ITEA) for OVA and western blot analysis for antigen proteins, such as PSMA, PSA and PAP.

2.5 Western blotting

To quantify the protein levels of prostate antigens (PSMA, PSA, and PAP), western blotting was used as previously described (35). Briefly, the relevant antigen-expressing aAVC was lysed in Laemmli sample buffer (Bio-Rad) and boiled at 95°C. Samples were loaded onto a polyacrylamide gel and electrotransferred to a PVDF membrane. Membranes were blocked and incubated with primary anti-PSMA, anti-PSA, or anti-PAP antibodies overnight at 4°C with gentle shaking. The membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (R&D Systems) at room temperature. Chemiluminescence images were obtained using a luminescence image analyzer (LAS 1000) and Image Gauge software (Fujifilm Co.). Protein levels were evaluated using the Image Gauge software, and image processing (resizing, cropping, and merging) was conducted using Adobe Photoshop (Adobe Systems).

2.6 ELISA

Ten thousand mouse aAVCs were co-cultured with 1×10^5 NKT hybridoma (1.B2) for 24 h in 96-well round-bottom plates. Culture supernatants were analyzed for mouse IL-2 production using ELISA for cytokines with a mouse IL-2 Duo set (R&D Systems).

2.7 Enzyme-linked immune absorbent spot assay

Murine IFN-γ-secreting $CD8^+$ T cells were performed by ELISPOT assays as previously described (27). Ninety six well filtration plates were coated with rat anti-mouse IFN-γ (R4-6A2; BD) overnight at 10 μg/mL. $CD8^+$ T cells were isolated from the spleens of naïve or aAVC-treated mice by using CD8 MACS beads (Miltenyi Biotec). To prepare for antigen-presenting cells (APCs), splenic DCs from naïve mice were isolated using CD11c MACS beads. Additionally, $CD8^+$ T cells (5×10^5/well) were co-cultured with DCs (1×10^5/well) pulsed with or without PepTivator (Miltenyi Biotec) for 24 h. After the culture, the plates were incubated with biotinylated anti-mouse IFN-γ (XMG1.2; BD) (2 μg/mL) for 2 h. Finally, IFN-γ-secreting spots were developed with streptavidin-HRP (BD) and stable DAB substrate (Research Genetics). IFN-γ SFCs were quantified using microscopy.

2.8 Prophylactic and therapeutic antitumor effect of aAVCs in mice

For the prophylactic model for each antigen, female C57BL/6 mice were immunized intravenously with 5×10^5 aAVC expressing the relevant antigen and inoculated subcutaneously with 1×10^5 B16 cells expressing the relevant antigen after two weeks. For the therapeutic model, C57BL/6 mice were injected subcutaneously with 5×10^5 tumor cells and treated with 5×10^5 aAVC when tumor size reached around $50mm^3$. Tumor diameters were measured using a Vernier caliper every 2–3 days, and the tumor volume was calculated using the following equation: $V = L \times W2 \times 0.52$, where V is the volume, L is the length, and W is the width. Mice with high tumor burden (volume > 2000 mm^3) were sacrificed under anesthesia.

2.9 Statistical analysis

StatMate V software (Nihon 3 B Scientific Inc., Japan) was used for the statistical analyses. A student's *t*-test (two-tailed) was used for the analysis of differences between the two groups. One-way analysis of variance (ANOVA) and *post-hoc* Dunnett's tests were used for multiple comparisons. *P* values less than 0.05 were considered significant (*P<0.05; **P<0.01; ***P<0.001).

3 Results

3.1 Establishment of prostate antigen-expressing aAVCs

Mouse aAVCs were created by co-transfecting α-GalCer-loaded, mCD1d mRNA NIH3T3 cells with three human prostate antigen mRNAs (PSMA-mRNA, PSA-mRNA, or PAP-mRNA) and denoted as aAVC-PSMA (Figures 1A, B), aAVC-PSA (Figures 1C, D), and aAVC-PAP (Figures 1E, F), respectively. After transfection, we verified the expression of each protein using western blotting (Figures 1A, C, E). aAVC-PSMA, aAVC-PSA, and aAVC-PAP expressed the corresponding proteins at 1546 ± 202, 564 ± 97, and 172 ± 34 ng/10^6 cells, respectively. We verified that the expression rate of mCD1d in each aAVC was > 90% (Figures 1B, D, F).

3.2 iNKT cell activation by prostate antigen-expressing aAVCs

After verifying the establishment of each aAVC type, we evaluated whether aAVC-PSMA, aAVC-PSA, and aAVC-PAP induced iNKT cell activation. We examined the iNKT response by culturing these aAVCs with iNKT cell hybridomas *in vitro*.

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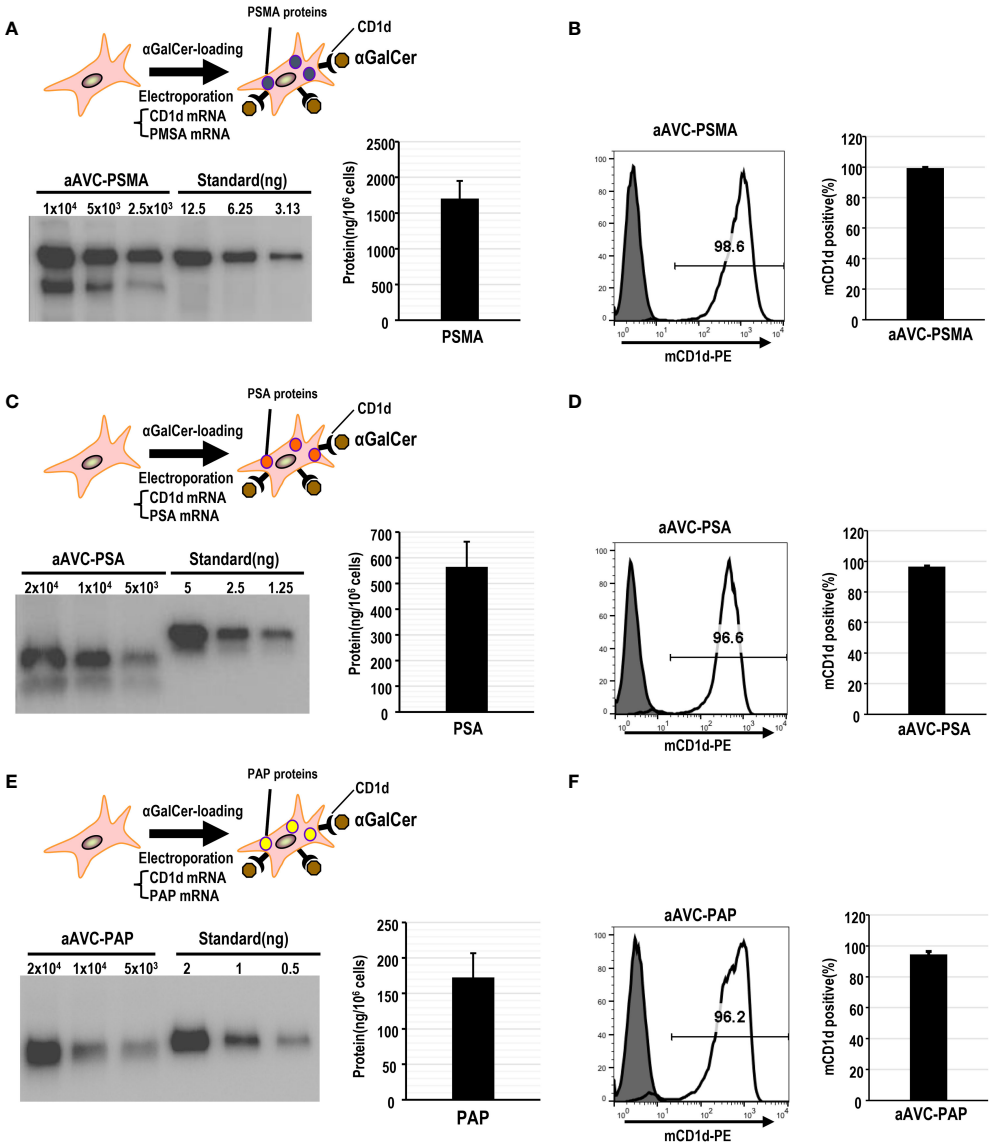

FIGURE 1
Establishment of monovalent aAVCs co-expressing CD1d and prostate tumor antigens for cancer therapy. **(A)** aAVCs expressing PSMA were created by electroporating NIH3T3 cells with PSMA and murine CD1d mRNA, followed by loading with α-galactosylceramide (α-GalCer). PSMA protein expression level was assessed using western blotting. **(B)** CD1d surface expression on aAVC-PSMA was assessed by flow cytometry (open, aAVC-PSMA; shaded, isotype). **(C)** aAVC-PSA was prepared as described in **(A)**, with PSA being transfected instead. PSA protein expression in aAVC-PSA was measured using western blot analysis. **(D)** CD1d surface expression on aAVC-PSA was assessed using flow cytometry (open, aAVC-PSA; shaded, isotype). **(E)** aAVC-PAP was prepared as described in **(A)**, with PAP transfected instead. The PAP protein in aAVC-PAP was measured using western blot analysis. **(F)** as similar to **(A)**, but CD1d surface expression on aAVC-PAP was assessed by flow cytometry (open, aAVC-PAP; shaded, isotype).

After culturing aAVC-PSMA, aAVC-PSA, and aAVC-PAP with iNKT cells, IL-2 was produced by iNKT in the presence of aAVC, but not parental NIH cells in all groups (Figures 2A, C, E). Next, we investigated the capacity of each aAVC to expand iNKT cells *in vivo*. The percentage of iNKT cells in the spleen was examined three days after administering each aAVC. The iNKT cell frequency in all aAVC-PSMA-, aAVC-PSA-, and aAVC-PAP-injected mice was five times higher than that in the naïve mice (Figures 2B, D, F). Therefore, aAVC-PSMA, aAVC-PSA, and aAVC-PAP similarly stimulated iNKT cells *in vivo*, with aAVC-PSMA demonstrating the highest potency as an activator of iNKT cells in this context.

3.3 Prophylactic effects of aAVC-PSMA, aAVC-PSA, or aAVC-PAP administration on prostate antigens

Owing to its resistance to ICB, B16 melanoma is widely recognized as a cold tumor. To evaluate whether the three types of prostate antigen-expressing aAVCs exhibit antitumor effects, we established three permanent B16 cell lines: B16 expressing PSMA, PSA, or PAP (Figures 3A, C, E). C57BL/6 mice were intravenously administered aAVC-PSMA, aAVC-PSA, or aAVC-PAP individually and then inoculated with PSMA-, PSA-, or PAP-expressing melanoma cell lines (B16-PSMA, B16-PSA, and B16-

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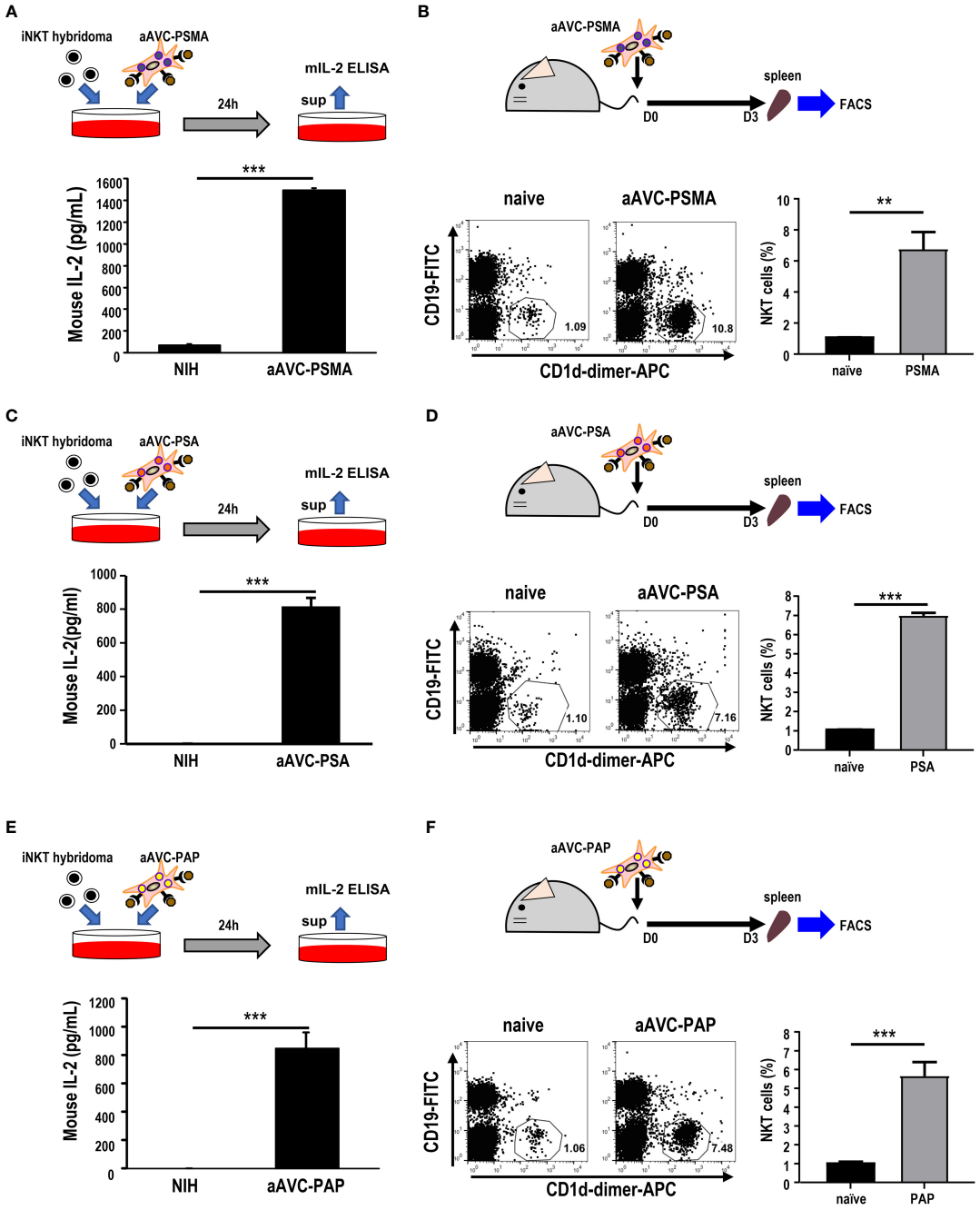

FIGURE 2
iNKT cell activation by aAVCs. **(A, C, E)** α-GalCer presentation on aAVC-PSMA **(A)**, aAVC-PSA **(B)**, and aAVC-PAP **(C)**. aAVCs were co-cultured with the Vα14 iNKT cell hybridoma 1.B2 for 24 h (upper), and IL-2 production in the culture supernatant was evaluated using IL-2 ELISA (n=4, mean ± SEM) (lower); ****P<0.001* (Mann-Whiteny). **(B, D, F)** C57BL/6 mice were injected intravenously with 5×10^5 aAVC-PSMA **(B)**, aAVC-PSA **(D)**, and aAVC-PAP **(F)**. Spleens were removed after 3 days, and splenic iNKT was analyzed after staining with CD19-FITC, CD1d-dimer$^+$ APC, and 7-AAD (upper). Representative dot plots showing the frequency of splenic iNKT cells (lower left) and summary(n=3, mean ± SEM) (lower right) were shown. **P<0.01, ***P<0.001(Mann-Whiteny).

PAP) respectively after 14 days. Compared with the mice in the untreated control group, the aAVC-PSMA-, aAVC-PSA-, or aAVC-PAP-vaccinated mice were protected from the growth of B16-PSMA, B16-PSA, and B16-PAP (Figures 3B, D, F). Vaccination with monovalent prostate antigen-expressing aAVC exhibited substantial antitumor effects.

3.4 Relationship between iNKT frequency and antigen-specific T cell immunity in aAVC therapy

Next, we examined the relationship between iNKT cell frequency and antigen-specific T cell immunity in aAVC therapy.

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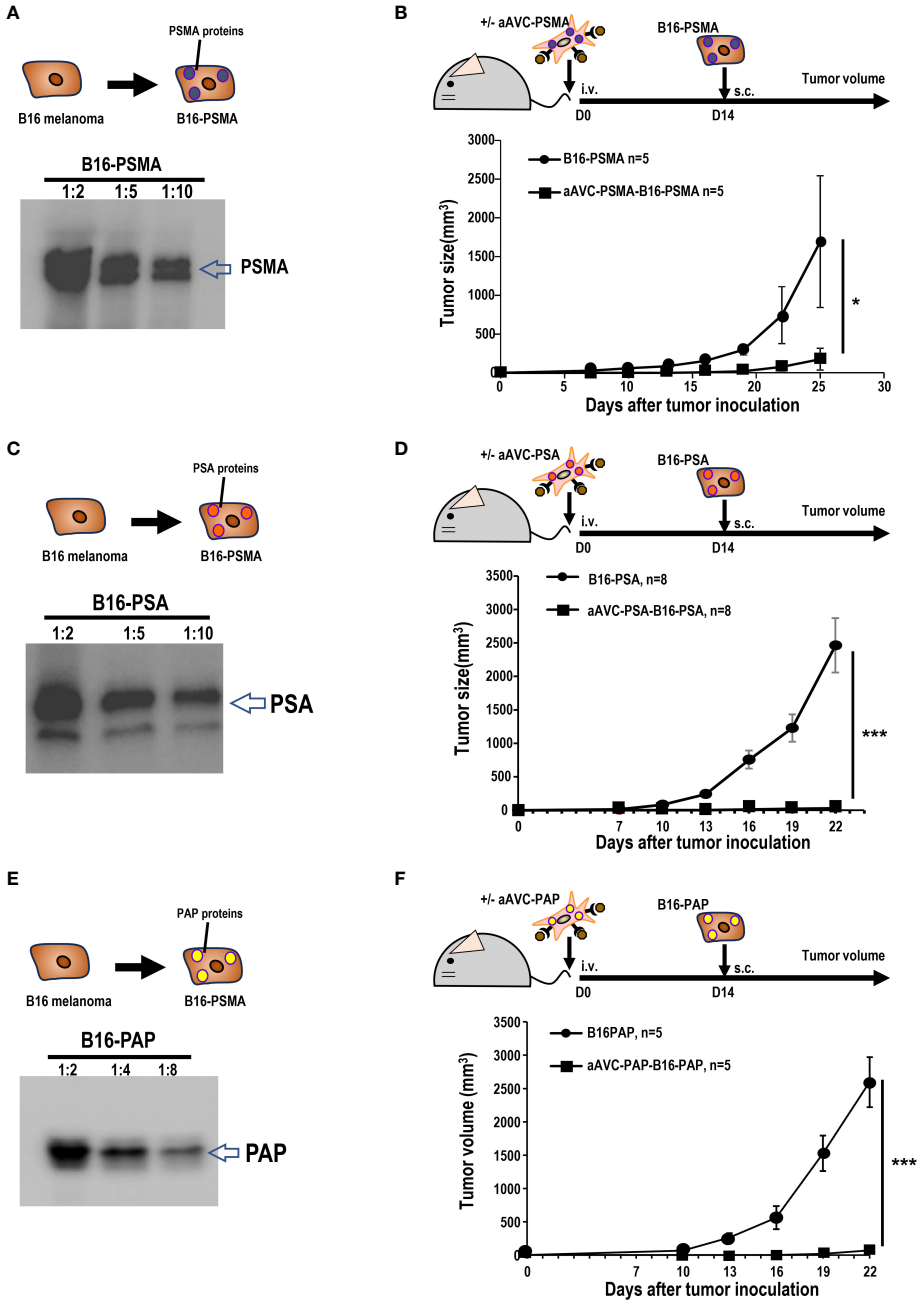

FIGURE 3
Antitumor response by aAVCs in a prophylactic model. **(A)** Establishment of B16-PSMA **(A)**, B16-PSA **(C)**, and B16-PAP **(E)** cells. After introducing the PSMA, PSA, or PAP gene into B16, stable PSMA, PSA, or PAP protein expression was verified using western blot analysis. **(B, D, F)** Mice were immunized with or without 5×10^5 aAVC-PSMA, aAVC-PSA, or aAVC-PAP on day 0. Following this, mice were challenged with 5×10^5 B16-PSMA **(B)**, B16-PSA **(D)**, or B16-PAP **(F)**, respectively on day 14 (n=5 per group, mean ± SEM); *$P<0.05$. ***$P<0.001$.

For this, we transferred different number of iNKT cells to Rag1$^{-/-}$ mice. We harvested B cell-depleted MNCs from spleen and liver of WT mice and CD1d$^{-/-}$ mice that are deficient in iNKT cells at the ratio of 100:0(%) or 50:50 (%) to Rag1$^{-/-}$ mice (Figures 4A, B). We verified that the number of iNKT cells (1st; 0.74x 10^6 cells/mouse, 2nd 0.53 x 10^6 cells/mouse) included in MNCs from WT mice and CD1d$^{-/-}$ mice 50:50 (%) was half the number of iNKT cells included n MNCs from WT mice (1st; 1.41x 10^6 cells/mouse, 2nd 1.05x 10^6 cells/mouse). Then, to trace and measure the antigen-specific T cell response, we administered low number of OT-1 cells (1x 10^5 cells/mouse) and subsequently aAVC-OVA cells the following day. Despite the reduced frequency of iNKT cells, the OVA-specific CD8$^+$ T cell response in mice given iNKT cells from WT mice and CD1d$^{-/-}$ mice wassimilar to those from WT mice-derived iNKT cells transferred Rag1$^{-/-}$ mice (Figures 4C, D). Thus, although the activation of iNKT cells is important factor, even if low frequent

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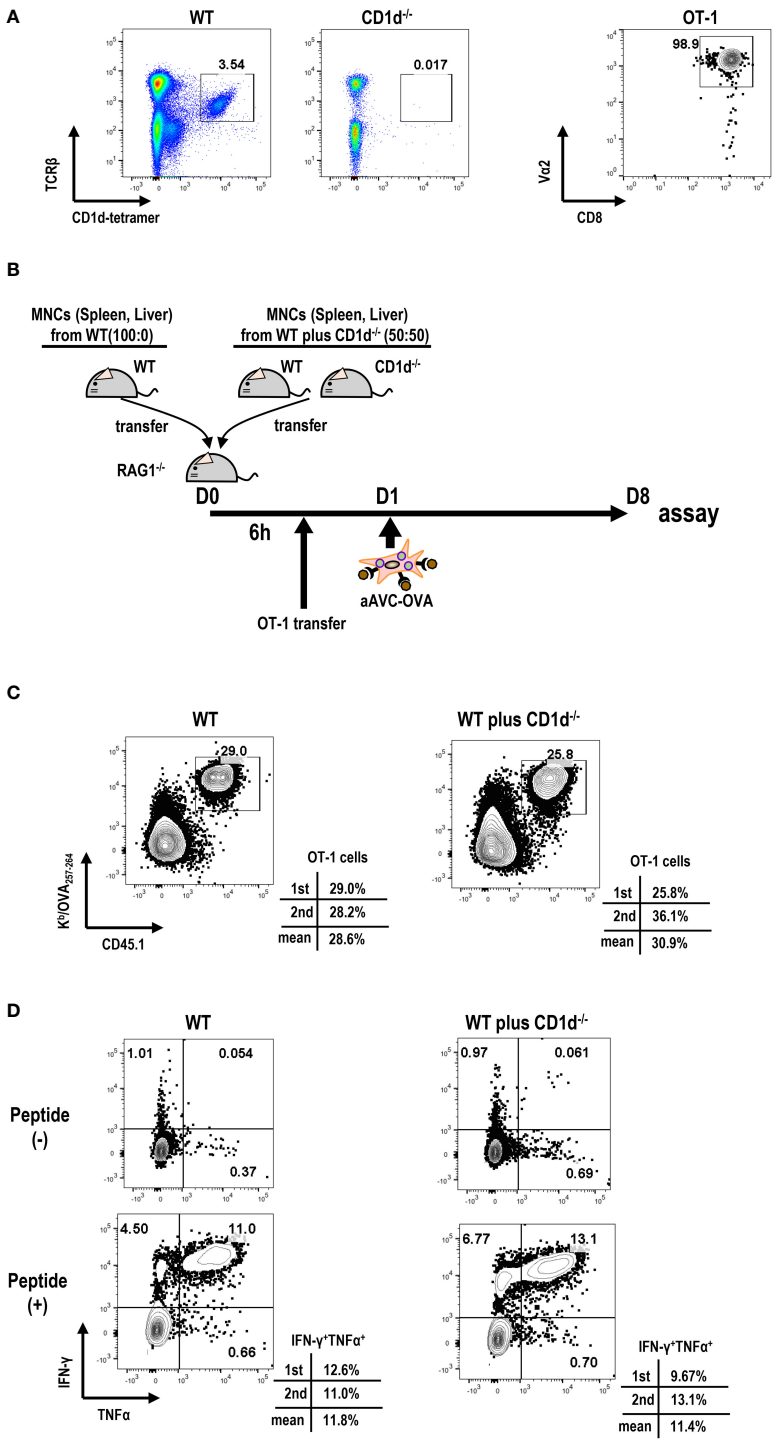

FIGURE 4
Relation of iNKT cells in host to antigen-specific T cell immunity by aAVC therapy. **(A)** Preparation of MNCs and OT-1 cells. Frequency of iNKT cells in B cell-depleted MNCs from WT and CD1d$^{-/-}$ mice (right) and purity of OT-1 CD8^{+} T cells (left) were analyzed by flow cytometry. OT-1 CD8^{+} T cells were isolated from spleen and lymph nodes of Ly5.1 OT-1Tg mice using CD8 MACS beads. The purity of OT -1 cells was analyzed using CD8a-FITC and Va2-PE. **(B)** Experimental protocol. The MNCs from WT mice and CD1d$^{-/-}$ mice (40x10^{6}/mouse) at the ratio of 50:50 (%) or 100:0(%) were transferred to Rag1$^{-/-}$ mice. OT-1 cells (1x10^{5}/mouse) were transferred 6 h later. aAVC-OVA cells (5x10^{5}/mouse) were administered the following day. **(C, D)** Frequency and cytokine production of OVA-specific CD8^{+} T cells. A week later, the Kb/SIINFEKL-tetramer^{+} CD8^{+}T cells **(C)** and OVA257–264 peptide specific cytokine production (IFN-γ and TNF-α) **(D)** were analyzed. The data are representative data from two experiments independently and each data are also provided.

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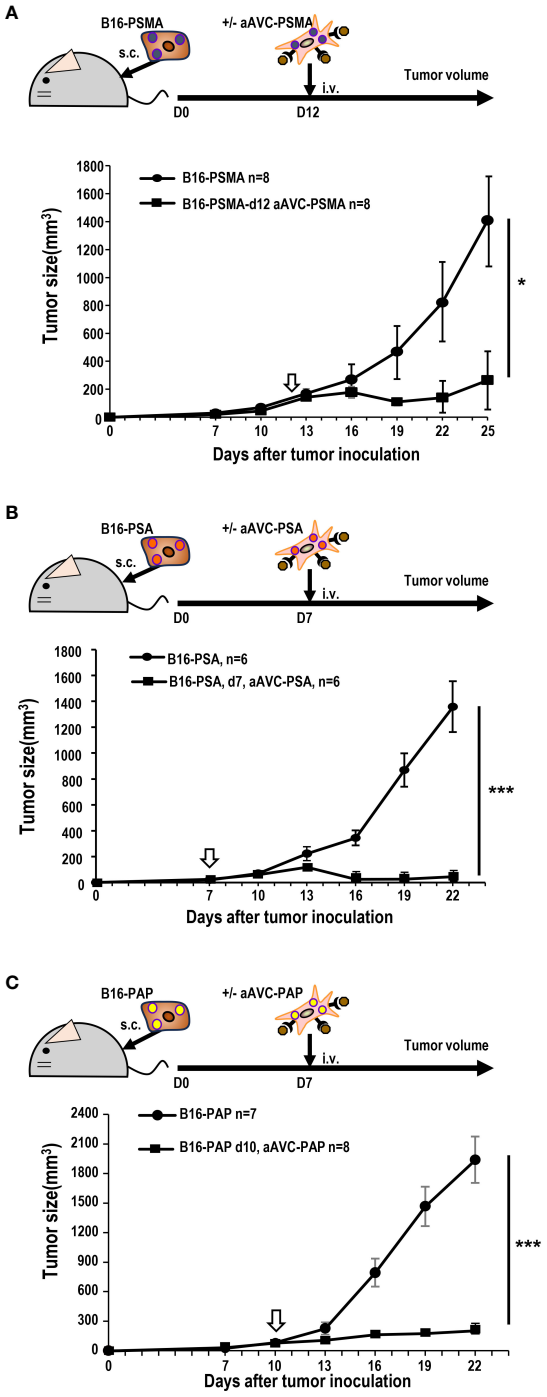

FIGURE 5
Antitumor response by aAVCs in a therapeutic model. The antitumor response was evaluated in a therapeutic model in which the therapy was administered at a tumor volume of around 50 mm^3. **(A)** Mice were inoculated with 5×10^5 B16-PSMA cells and treated with or without 5×10^5 aAVC-PSMA on day 12 (n=8 per group, mean ± SEM); *P<0.05. **(B)** Mice were inoculated with 5×10^5 B16-PSA cells and treated with or without 5×10^5 aAVC-PSA on day 7 (n=6 per group, mean ± SEM); ***P<0.001. **(C)** Mice were inoculated with 5×10^5 B16-PAP cells and treated with or without 5×10^5 aAVC-PAP on day 10 (n=7–8 per group, mean ± SEM); ***P<0.001.

iNKT cells *in vivo* can show the adjuvant activity for T cell priming sufficiently in aAVC therapy.

3.5 Therapeutic effect of aAVC-PSMA, aAVC-PSA, or aAVC-PAP on prostate antigen-expressing tumor

Next, we investigated whether aAVC therapy had a therapeutic effect on prostate antigen-expressing tumor-bearing mice. B16-PSMA, B16-PSA, and B16-PAP cells were inoculated subcutaneously into mice individually. When we observed tumors measuring around 50 mm^3 in all tumor-bearing mice, they were treated with aAVC-PSMA (Figure 5A), aAVC-PSA (Figure 5B), or aAVC-PAP (Figure 5C), respectively. Each aAVC exhibited tumor abrogation individually (Figures 5A–C). Among these, aAVC-PSA appears to have the most potent therapeutic effect.

3.6 Demonstration of tumor epitope spreading by aAVC therapy

Next, we investigated whether aAVC therapy induces antigen spreading in a therapeutic model. We established three antigen-expressing tumor cell lines by introducing DNA of PSMA, PSA, and PAP into B16 cells (hereafter, B16-PSMA/PSA/PAP) (Figure 6A). We verified that these cells expressed each antigen using RT-PCR and western blot analyses (Figure 6B). B16-PSMA/PSA/PAP-bearing mice were treated with monovalent prostate cancer antigen expressing aAVC, that is, either aAVC-PSMA, aAVC-PSA, or aAVC-PAP on day 7. We found that immunization with aAVC-PSMA, aAVC-PSA, or aAVC-PAP abrogated the tumors (Figures 6C, E, G). After verifying the antitumor effect on day 16, we sacrificed the mice. We analyzed the antigen-specific $CD8^+$ T cell response: PSMA-specific T cell response in aAVC-PSMA-treated mice (Figure 6D), PSA-specific T cell response in aAVC-PSA-treated mice (Figure 6F), and PAP-specific T cell response in aAVC-PAP-treated mice (Figure 6H). We detected a higher antigen-specific $CD8^+$ T cell response following treatment with the relevant antigen-expressing aAVC. We found the antitumor effects through the therapy with aAVC-PSMA, aAVC-PSA or aAVC-PAP respectively (Figures 6C, E, G). In addition, intriguingly, we found that monovalent-aAVC induced not only $CD8^+$ T cell responses against the target antigen, but also elicited antigen-specific $CD8^+$ T cell responses against other tumor antigens simultaneously. This discrepancy might be explained by the capacity of aAVC therapy to induce significant epitope spreading from the tumor to $CD8^+$ T cells.

3.7 Multiple T cell responses induced by treatment with a multivalent antigen-expressing aAVC

Subsequently, we generated a multivalent aAVC expressing the three prostate cancer antigens (Figure 7A). After simultaneous transfection with PSMA, PSA, and PAP mRNA in addition to murine CD1d mRNA, we verified the expression of PSMA, PSA, and PAP protein by western blot analysis (Figure 7B). The trivalent

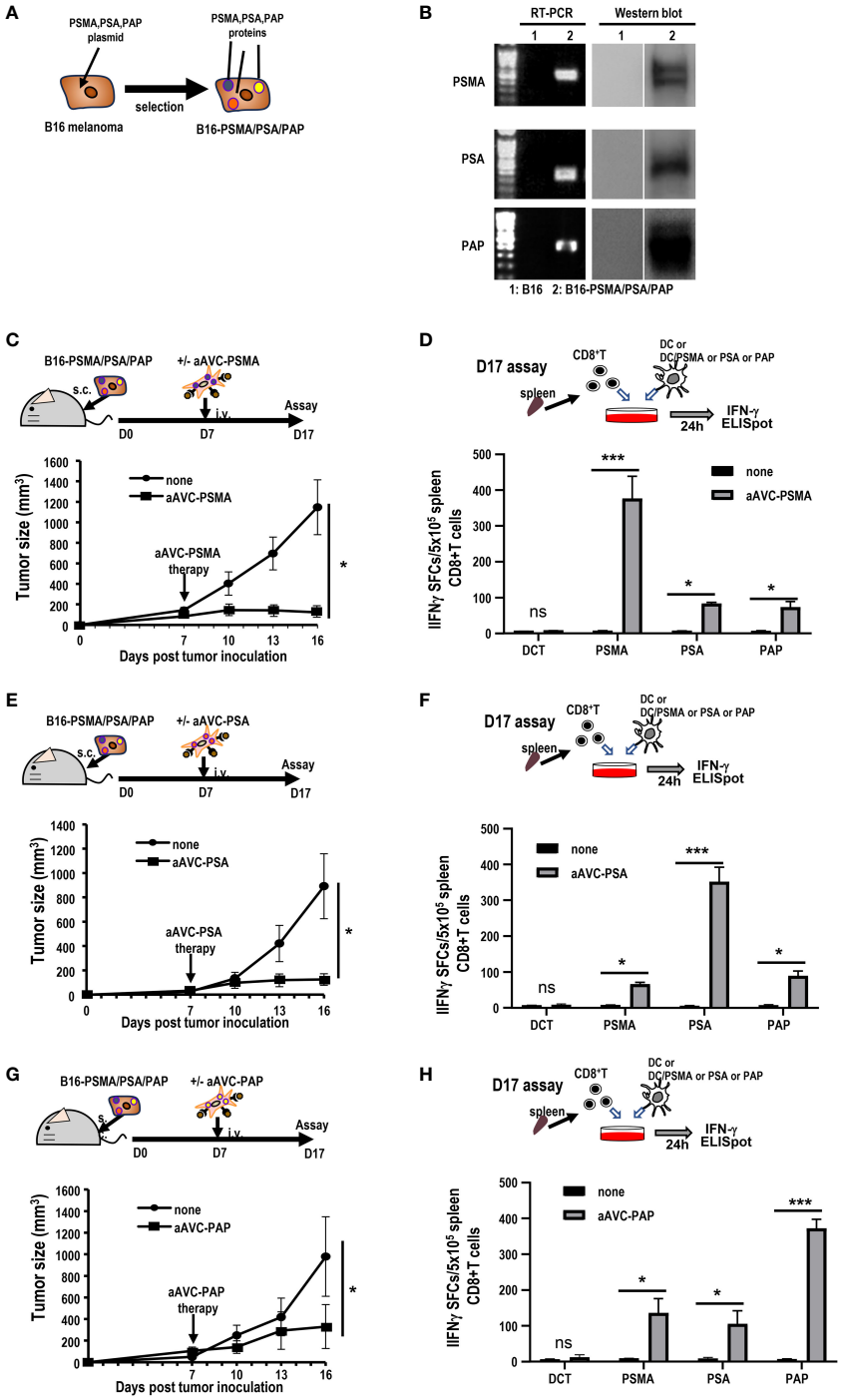

FIGURE 6
Induction of tumor epitope spreading by aAVC vaccination. **(A, B)** B16-PSMA/PSA/PAP cells were established using a retroviral vector expressing PSMA, PSA, or PAP, respectively. Tumor expression was verified using RT-PCR and western blotting. **(C, E, G)** Antitumor effect of various prostate antigen-expressing aAVC therapies. Mice were inoculated with 5×10^5 B16-PSMA/PSA/PAP cells and treated with or without 5×10^5 aAVC-PSMA **(C)**, aAVC-PSA **(E)**, or aAVC-PAP **(G)** on day 7 (n=5 per group, mean ± SEM); **P*<0.05. **(D, F, H)** Epitope spread of prostate antigens in $CD8^+$ T cells after aAVC therapy. Ten days after vaccination with aAVC-PSMA **(B)**, aAVC-PSA **(D)**, or aAVC-PAP **(F)**, PSMA-, PSA-, or PAP-specific $CD8^+$ T cells were analyzed using an IFN-γ ELISPOT assay. For this, splenic $CD8^+$ T cells isolated from the immunized mice were cultured with splenic $CD11c^+$ dendritic cells (DCs) from naïve mice that had been cultured in the presence or absence of PSMA, PSA, or PAP-PepTivator for 24 h. (n=4 per group, mean ± SEM); **P*<0.05; ****P*<0.001; *ns*: not significant, according to Tukey's test.

cancer antigen aAVC, denoted as aAVC-PROS, effectively expressed PSMA (483 ± 31ng/10^6 cells), PSA (59.2 ± 5.0 ng/10^6 cells), and PAP (17.9 ± 1.2 ng/10^6 cells). $CD8^+$ T cells were isolated from the spleen cells of vaccinated mice that had been administered aAVC-triPROS to analyze the antigen-specific T cell response. At day 7, they were cocultured with splenic DCs from naïve mice in the

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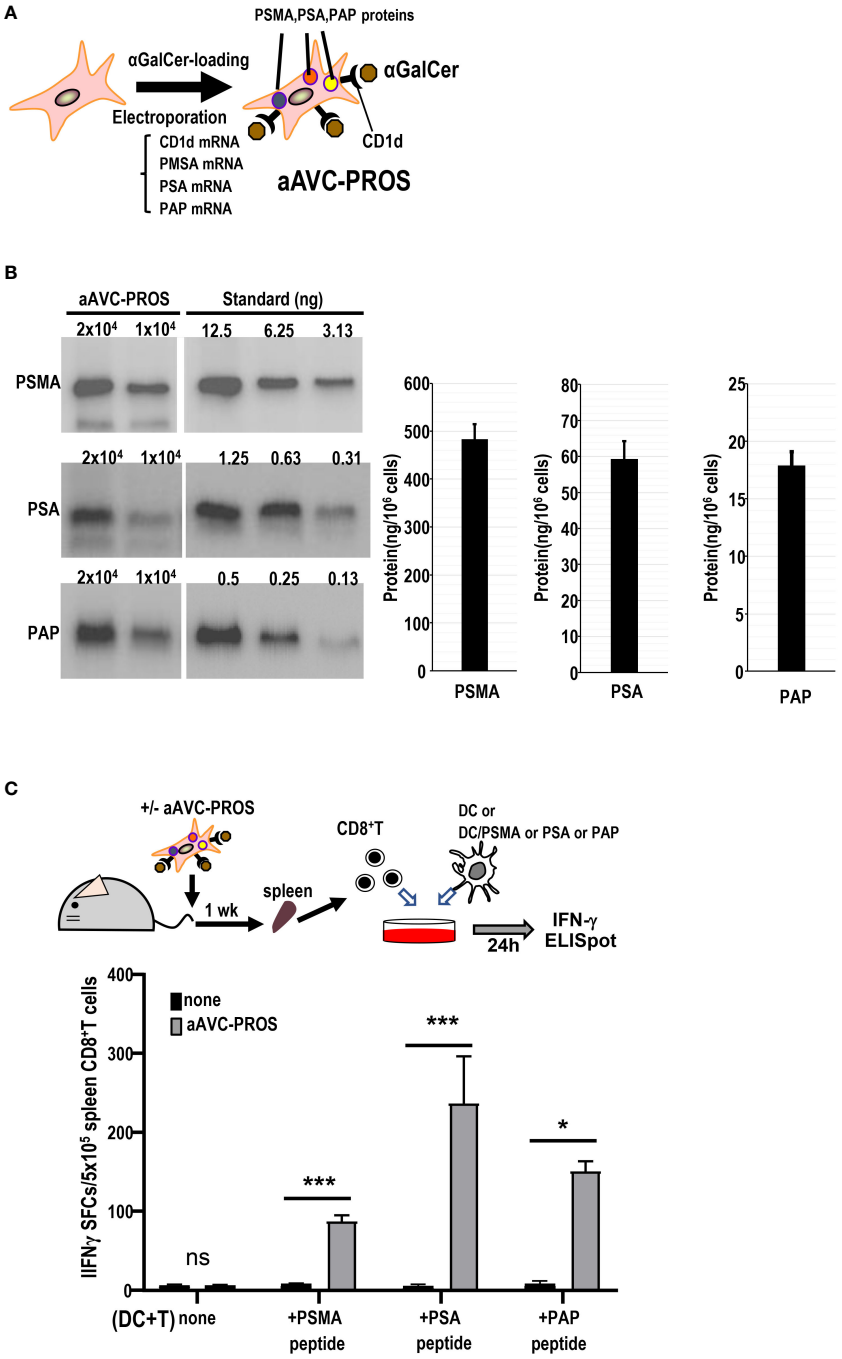

FIGURE 7
Establishment of a multivalent aAVC and prostate cancer antigen-specific CD8$^+$ T cell response. **(A)** Establishment of multivalent artificial adjuvant vector cells for the prostate (aAVC-PROS). Three prostate cancer antigen-expressing artificial adjuvant cells (aAVC-PSMA/PSA/PAP and aAVC-PROS) were established by co-transfecting NIH3T3 cells with PSMA, PSA, PAP, and murine CD1d mRNA and then loaded with α-GalCer. **(B)** Tumor protein antigen in aAVC-PROS. The amount of PSMA, PSA, and PAP was determined using western blot analysis. **(C)** The prostate antigen-specific CD8$^+$ T cell response induced by aAVC. Mice were intravenously immunized with 5 × 10^5 aAVC-PROS. After one week, PSMA-, PSA-, or PAP-specific CD8$^+$ T cells were analyzed using an IFN-γ ELISPOT assay. Splenic CD8$^+$ T cells isolated from the immunized mice were cultured with splenic CD11c$^+$ DCs from naïve mice that had been cultured in the presence or absence of PSMA-, PSA-, or PAP-PepTivator for 24 h. (n=4 per group, mean ± SEM) **P<0.05*; ****P<0.001*; *ns*: not significant, according to Tukey's test.

presence or absence of PSMA, PSA or PAP peptivators. The antigen-specific CD8$^+$ T cells produced in response to the peptides were determined using the ELISpot assay (Figure 7C). Although the amounts of each protein expressed in aAVC-PROS were different, the T cell response induced by aAVC-PROS was higher in PSA-specific T cells, followed in order by PAP-specific T cells and PSMA-specific T cells. The amount of protein expressed in the aAVC did not always correlate with the T cell immune response. T cell immune response depends on the amount of antigen present and antigenicity of the specific expressed protein. Our results

revealed that the response depends on the antigen quantity when the antigens are identical. However, when dealing with different antigens, the response relies more on antigenicity than the amount of antigen present in the aAVCs. This suggests that the T cell response may depend on the immunogenicity of the antigen involved in aAVC when we use multifunctional epitope-expressing aAVC.

3.8 Therapeutic effect of divalent and trivalent antigen-expressing aAVCs

Having determined that monovalent antigen-expressing aAVC therapy is effective against multiple antigen-expressing tumors and that this effect was caused by epitope spreading, we subsequently investigated whether divalent or trivalent antigen-expressing aAVC generated antitumor effects more effectively. Because PSMA and PSA were more effective than PAP, suggesting that combining these two antigens would be more potent (Figure 6), we established a divalent aAVC expressing PSMA and PSA. We compared the antitumor effects of divalent aAVC-PSMA/PSA and trivalent aAVC-PROS against B16-PSMA/PSA/PAP (Figure 8A). Both vaccinations showed an abrogation of tumor cells similarly. Further analysis indicates that divalent aAVC is better than monovalent aAVC; however, there was no difference in efficacy between divalent and trivalent aAVCs. Finally, we compared the antitumor effects of the three vaccines during the early and late tumor phases. When we assessed the tumor size on days 10 (early) and 19 (late), we did not observe any difference between divalent and trivalent aAVC vaccination until day 10, but found that the antitumor effect of trivalent aAVC was superior to that of monovalent or divalent aAVC on day 19 (Figure 8B). These findings imply that even monovalent aAVC show the antitumor effect sufficiently during the early phase. Notably, only trivalent antigen-expressing aAVC exhibits increased potency in the late phase. Thus, multivalent antigen-expressing aAVC can elicit multiple T cell responses more effectively than monovalent antigen-expressing aAVC for the long period.

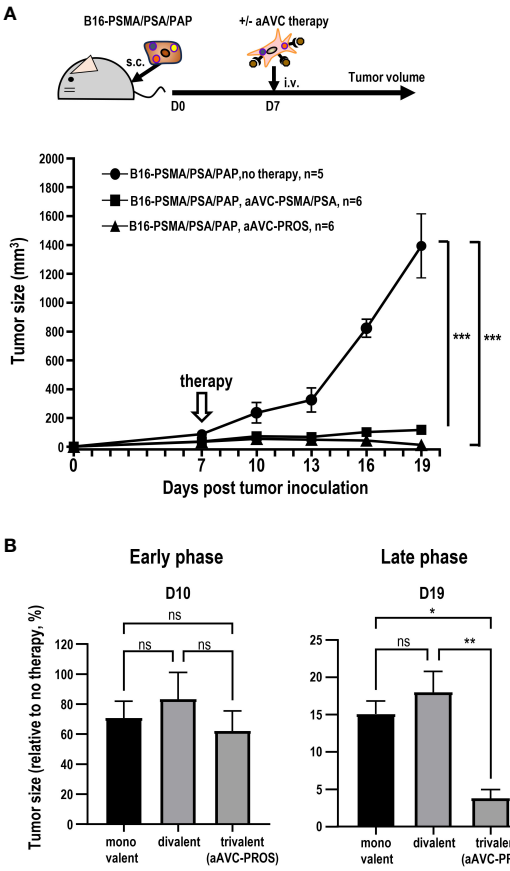

FIGURE 8
Comparison of the antitumor effect of divalent and trivalent prostate antigen-expressing aAVCs. Divalent artificial adjuvant cells (aAVC-PSMA/PSA) were established by co-transfecting NIH3T3 cells with PSMA, PSA, and murine CD1d mRNA. **(A)** Mice were inoculated with 5×10^5 B16-PSMA/PSA/PAP cells and treated with or without 5×10^5 divalent aAVC-PSMA/PSA or trivalent aAVC-PROS on day 7. **(B)** Comparison of the antitumor effects of monovalent (aAVC-PSMA, aAVC-PSA, or aAVC-PAP), divalent (aAVC-PSMA/PSA), or trivalent aAVCs (aAVC-PROS). The tumor size was evaluated by comparing untreated mice with treated mice on days 10 (Early phase) and 19 (Late phase). *Plt;0.05, **P<0.01, ***P<0.001; ns: Tukey's test.

4 Discussion

In this study, we established an aAVC approach using three prostate cancer antigens as aAVC-PROS and verified the advantages of this vaccine in a cold tumor melanoma model. Differences in the immune response and antitumor effects were evaluated for each antigen. T-cell responsiveness differs depending on the tumor antigen characteristics. Essentially, the strength of the $CD8^+$ T cell response depends on the antigenicity and quantity of tumor antigen in the aAVC. Therefore, we first compared the capacity of aAVCs expressing three different antigens to activate innate and adaptive immunity. The results showed that iNKT responses, antigen-specific $CD8^+$ T cells, and antitumor activity were successfully induced by all aAVCs. We also found that if the antigen in each aAVC were the same one, ensuing T cell response would depend on the amount of antigen, however, if the antigen in aAVC was different, it would depend on the antigenicity. Multivalent vaccines are regarded as an effective strategy to prevent immune escape in cases where one type of tumor antigens is lost. In the current study, we verified that aAVC-PROS carrying three antigens exhibited sufficient antitumor effects and induced three antigen-specific $CD8^+$ T cells. Interestingly, when tumors expressing multiple antigens were treated with monovalent aAVCs, $CD8^+$ T cells were elicited against antigens that differed from those with which the mice were immunized. Thus, we demonstrate that aAVC induces epitope spreading. Finally, we evaluated the direct activity of multivalent antigen-expressing aAVC in conjunction with the indirect activity of tumor epitope spreading by aAVC by comparing monovalent, divalent, or trivalent antigen-expressing aAVC. In the early phase, there was a minimal difference among the vaccines; however, multivalent antigen-expressing aAVC exhibited more potent antitumor effects in the late phase. Our results suggest

that antigen-spreading ability and multivalent antigen vaccines are beneficial for long term-antitumor effects. aAVCs targeting multiple prostate cancer antigens would be promising therapeutic options for conventional therapy-resistant prostate cancer.

Epitope spreading was first described in experimental autoimmune encephalomyelitis (39). These antigens often segregate into different epitopes derived from defined proteins (intramolecular spreading) and other antigens (intermolecular spreading). Antigen spread following treatment with sipuleucel-T has been demonstrated most clearly through studies of antibody responses in patients with prostate cancer in a phase III trial (40). Evidence of antigen spread in response to immunotherapy has also been reported in several other tumor types, including metastatic breast cancer (41) and melanomas (42–44). Moreover, emerging data have revealed that patients who acquire broad immunological responses have improved clinical outcomes (41, 45, 46). Although it is theoretically understood that epitope spreading is induced, it cannot be simply and easily induced using radiation, chemotherapy drugs, or ICB. Therefore, it is essential to determine whether the immune response can adapt to amplify and diversify the endogenous T cell response against diverse tumor antigens. This clarification is crucial for understanding the potential of immune responses against multiple tumor antigens. Epitope spreading and endogenous T cell activation within the tumor can be promoted by cDC1 in the tumor (47). Since DCs *in vivo* seem to play a critical role in the success of epitope spreading in the antitumor effect, they could serve as a surrogate molecular indicator of the clinical impact of the aAVC platform.

According to concerns for patients who have the low frequency of iNKT cells, several groups demonstrated that NK cell response was shown after α-GalCer-pulsed DC therapy (24, 26, 37, 48). Particularly, an initial study showed that NK cell response depends on the function of iNKT cells, but not the frequency. We observed a notable granzyme B-expressing NK cell response in most lung cancer patients undergoing autologous DC/Gal therapy (26). However, we did not observe a similar response in T cells. Recently, we conducted a phase I trial involving aAVC-expressing WT1 therapy targeted at patients with relapsed and refractory AML (37). Our results revealed that either iNKT or NK cells or both were activated in all the patients, even in patients with lung cancer and relapse and refractory AML, including those with low counts of iNKT cells. We demonstrated that a small number of iNKT cells exert an adjuvant effect in murine model (Figure 4). Our future research will explore whether an increased number of transferred iNKT cells can enhance T cell functionality more effectively.

Several strategies have been explored to improve the efficacy of immunotherapy, including monotherapy, multivalent therapy, and combination therapies. In the present study, we compared monovalent and multivalent (divalent or trivalent) antigen-expressing aAVCs. We demonstrated that multivalent antigen-expressing aAVC was more effective than monovalent-aAVC. When we subsequently compared divalent or trivalent aAVC, we revealed that trivalent antigen-expressing aAVCs enhance antigen-specific T cells under tumor-bearing, immunosuppressive conditions. When considering combination therapy for prostate cancer, it is crucial to consider amplifying antigen-specific $CD8^+$ T cells, efficiently trafficking them by TIME reprogramming, and reinvigorating and sustaining their survival via ICB and cytokines. A recent study investigating ICB resistance in prostate cancer showed that androgens worsen the response to ICB (49). Androgen receptor (AR) blockade directly enhances the PSA antigen-specific $CD8^+$ T cell response and restores the activation of pathways in $CD8^+$ T cells, including TCR, PD-1, and IFN-γ signaling (49). The enhancement of these pathways corresponds to the deactivation of the NR4A1 pathway, which limits the efficacy of anti-PD-1 antibody-targeted immunotherapy (50). We previously demonstrated that the two-fold T cell response in the TIME was elevated by the synergistic effect of aAVC and anti-PD1Ab on T cells in the TIME (29), indicating its potential application in prostate cancer. Thus, analysis of the TIME may reveal various mechanisms for effective checkpoint blockade in tumor-bearing hosts, leading to the development of new therapeutic strategies.

Data availability statement

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

Ethics statement

The animal study was approved by Institutional Animal Care Committee of RIKEN. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SY: Formal Analysis, Investigation, Methodology, Visualization, Writing – original draft. KS: Formal Analysis, Investigation, Methodology, Validation, Writing – original draft. SF: Conceptualization, Formal Analysis, Funding acquisition, Methodology, Supervision, Validation, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The authors declare that this study received funding from Astellas Pharma, Inc. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

Conflict of interest

SF received research funding and honoraria from Astellas Pharma, Inc. KS received honoraria from Astellas Pharma Inc.

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

References

1. Boettcher AN, Usman A, Morgans A, VanderWeele DJ, Sosman J, Wu JD. Past, current, and future of immunotherapies for prostate cancer. *Front Oncol* (2019) 9:884. doi: 10.3389/fonc.2019.00884

2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* (2021) 71(3):209–49. doi: 10.3322/caac.21660

3. Jones CU, Hunt D, McGowan DG, Amin MB, Chetner MP, Bruner DW, et al. Radiotherapy and short-term androgen deprivation for localized prostate cancer. *N Engl J Med* (2011) 365(2):107–18. doi: 10.1056/NEJMoa1012348

4. Arndt C, Feldmann A, Koristka S, Cartellieri M, Dimmel M, Ehninger A, et al. Simultaneous targeting of prostate stem cell antigen and prostate-specific membrane antigen improves the killing of prostate cancer cells using a novel modular T cell-retargeting system. *Prostate* (2014) 74(13):1335–46. doi: 10.1002/pros.22850

5. Wang J, Cheng X, Jin Y, Xia B, Qin R, Zhang W, et al. Safety and clinical response to combined immunotherapy with autologous iNKT cells and PD-1(+)CD8(+) T cells in patients failing first-line chemotherapy in stage IV pancreatic cancer. *Cancer Res Commun* (2023) 3(6):991–1003. doi: 10.1158/2767-9764.CRC-23-0137

6. Lopez-Soto A, Gonzalez S, Smyth MJ, Galluzzi L. Control of metastasis by NK cells. *Cancer Cell* (2017) 32(2):135–54. doi: 10.1016/j.ccell.2017.06.009

7. Bald T, Krummel MF, Smyth MJ, Barry KC. The NK cell-cancer cycle: advances and new challenges in NK cell-based immunotherapies. *Nat Immunol* (2020) 21 (8):835–47. doi: 10.1038/s41590-020-0728-z

8. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* (1986) 319:675–8. doi: 10.1038/319675a0

9. Pasero C, Gravis G, Guerin M, Granjeaud S, Thomassin-Piana J, Rocchi P, et al. Inherent and tumor-driven immune tolerance in the prostate microenvironment impairs natural killer cell antitumor activity. *Cancer Res* (2016) 76(8):2153–65. doi: 10.1158/0008-5472.CAN-15-1965

10. Liu G, Lu S, Wang X, Page ST, Higano CS, Plymate SR, et al. Perturbation of NK cell peripheral homeostasis accelerates prostate carcinoma metastasis. *J Clin Invest* (2013) 123(10):4410–22. doi: 10.1172/JCI69369

11. Zhao SG, Lehrer J, Chang SL, Das R, Erho N, Liu Y, et al. The immune landscape of prostate cancer and nomination of PD-L2 as a potential therapeutic target. *J Natl Cancer Inst* (2019) 111(3):301–10. doi: 10.1093/jnci/djy141

12. Beer TM, Kwon ED, Drake CG, Fizazi K, Logothetis C, Gravis G, et al. Randomized, double-blind, phase III trial of ipilimumab versus placebo in asymptomatic or minimally symptomatic patients with metastatic chemotherapy-naive castration-resistant prostate cancer. *J Clin Oncol* (2017) 35(1):40–7. doi: 10.1200/JCO.2016.69.1584

13. Kwon ED, Drake CG, Scher HI, Fizazi K, Bossi A, van den Eertwegh AJ, et al. Ipilimumab versus placebo after radiotherapy in patients with metastatic castration-resistant prostate cancer that had progressed after docetaxel chemotherapy (CA184-043): a multicentre, randomised, double-blind, phase 3 trial. *Lancet Oncol* (2014) 15 (7):700–12. doi: 10.1016/S1470-2045(14)70189-5

14. Sharma P, Pachynski RK, Narayan V, Flechon A, Gravis G, Galsky MD, et al. Nivolumab plus ipilimumab for metastatic castration-resistant prostate cancer: preliminary analysis of patients in the checkMate 650 trial. *Cancer Cell* (2020) 38 (4):489–99.e3. doi: 10.1016/j.ccell.2020.08.007

15. Antonarakis ES, Piulats JM, Gross-Goupil M, Goh J, Ojamaa K, Hoimes CJ, et al. Pembrolizumab for treatment-refractory metastatic castration-resistant prostate cancer: multicohort, open-label phase II KEYNOTE-199 study. *J Clin Oncol* (2020) 38(5):395–405. doi: 10.1200/JCO.19.01638

16. Steele KE, Tan TH, Korn R, Dacosta K, Brown C, Kuziora M, et al. Measuring multiple parameters of CD8+ tumor-infiltrating lymphocytes in human cancers by image analysis. *J Immunother Cancer* (2018) 6(1):20. doi: 10.1186/s40425-018-0326-x

17. Topalian SL, Taube JM, Anders RA, Pardoll DM. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. *Nat Rev Cancer* (2016) 16 (5):275–87. doi: 10.1038/nrc.2016.36

18. Westdorp H, Skold AE, Snijer BA, Franik S, Mulder SF, Major PP, et al. Immunotherapy for prostate cancer: lessons from responses to tumor-associated antigens. *Front Immunol* (2014) 5:191. doi: 10.3389/fimmu.2014.00191

19. Zhang J, Huang D, Saw PE, Song E. Turning cold tumors hot: from molecular mechanisms to clinical applications. *Trends Immunol* (2022) 43(7):523–45. doi: 10.1016/j.it.2022.04.010

20. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-specific distribution of iNKT cells impacts their cytokine response. *Immunity* (2015) 43 (3):566–78. doi: 10.1016/j.immuni.2015.06.025

21. Slauenwhite D, Johnston B. Regulation of NKT cell localization in homeostasis and infection. *Front Immunol* (2015) 6:255. doi: 10.3389/fimmu.2015.00255

22. Fujii S, Shimizu K, Hemmi H, Fukui M, Bonito AJ, Chen G, et al. Glycolipid alpha-C-galactosylceramide is a distinct inducer of dendritic cell function during innate and adaptive immune responses of mice. *Proc Natl Acad Sci U S A* (2006) 103 (30):11252–7. doi: 10.1073/pnas.0604812103

23. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged interferon-γ producing NKT response induced with α-galactosylceramide-loaded dendritic cells. *Nat Immunol* (2002) 3:867–74. doi: 10.1038/ni827

24. Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, et al. A phase I-II study of α-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* (2009) 182(4):2492–501. doi: 10.4049/jimmunol.0800126

25. Iyoda T, Yamasaki S, Ueda S, Shimizu K, Fujii S. Natural killer T and natural killer cell-based immunotherapy strategies targeting cancer. *Biomolecules* (2023) 13(2). doi: 10.3390/biom13020348

26. Iyoda T, Shimizu K, Kawamura M, Shinga J, Watanabe T, Fukunaga K, et al. Augmenting granzyme B-expressing NK cells by invariant NKT ligand-loaded APCs in patients with postoperative early stage non-small cell lung cancer: results of a randomized phase II study. *Immunohorizons* (2023) 7(1):1–16. doi: 10.4049/immunohorizons.2200091

27. Fujii S, Goto A, Shimizu K. Antigen mRNA-transfected, allogeneic fibroblasts loaded with NKT-cell ligand confer antitumor immunity. *Blood* (2009) 113(18):4262–72. doi: 10.1182/blood-2008-08-176446

28. Shimizu K, Mizuno T, Shinga J, Asakura M, Kakimi K, Ishii Y, et al. Vaccination with antigen-transfected, NKT cell ligand-loaded, human cells elicits robust in *situ* immune responses by dendritic cells. *Cancer Res* (2013) 73(1):62–73. doi: 10.1158/0008-5472.CAN-12-07590008-5472.CAN-12-0759

29. Shimizu K, Yamasaki S, Shinga J, Sato Y, Watanabe T, Ohara O, et al. Systemic DC activation modulates the tumor microenvironment and shapes the long-lived tumor-specific memory mediated by CD8+ T cells. *Cancer Res* (2016) 76(13):3756–66. doi: 10.1158/0008-5472.CAN-15-3219

30. Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii S, Soares H, et al. *In vivo* targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination. *J Exp Med* (2004) 199(6):815–24. doi: 10.1084/jem.20032220

31. Dhodapkar MV, Sznol M, Zhao B, Wang D, Carvajal RD, Keohan ML, et al. Induction of antigen-specific immunity with a vaccine targeting NY-ESO-1 to the dendritic cell receptor DEC-205. *Sci Transl Med* (2014) 6(232):232ra51. doi: 10.1126/scitranslmed.3008068

32. Fujii S, Shimizu K. Exploiting antitumor immunotherapeutic novel strategies by deciphering the cross talk between invariant NKT cells and dendritic cells. *Front Immunol* (2017) 8:886. doi: 10.3389/fimmu.2017.00886

33. Fujii S, Shimizu K. Immune networks and therapeutic targeting of iNKT cells in cancer. *Trends Immunol* (2019) 40(11):984–97. doi: 10.1016/j.it.2019.09.008

34. Fujii S, Shimizu K, Hemmi H, Steinman RM. Innate Valpha14(+) natural killer T cells mature dendritic cells, leading to strong adaptive immunity. *Immunol Rev* (2007) 220:183–98. doi: 10.1111/j.1600-065X.2007.00561.x

35. Fujii S, Yamasaki S, Hanada K, Ueda S, Kawamura M, Shimizu K. Cancer immunotherapy using artificial adjuvant vector cells to deliver NY-ESO-1 antigen to dendritic cells in situ. *Cancer Sci* (2022) 113(3):864–74. doi: 10.1111/cas.15259

36. Shimizu K, Ueda S, Kawamura M, Satoh M, Fujii S. A single immunization with cellular vaccine confers dual protection against SARS-CoV-2 and cancer. *Cancer Sci* (2022) 113(8):2536–47. doi: 10.1111/cas.15434

37. Fujii S, Kawamata T, Shimizu K, Nakabayashi J, Yamasaki S, Iyoda T, et al. Reinvigoration of innate and adaptive immunity via therapeutic cellular vaccine for patients with AML. *Mol Therapy-Oncolytics* (2022) 27:315–32. doi: 10.1016/j.omto.2022.09.001

38. Fujii S, Yamasaki S, Sato Y, Shimizu K. Vaccine designs utilizing invariant NKT-licensed antigen-presenting cells provide NKT or T cell help for B cell responses. *Front Immunol* (2018) 9:1267. doi: 10.3389/fimmu.2018.01267

39. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. *Nature* (1992) 358 (6382):155–7. doi: 10.1038/358155a0

40. GuhaThakurta D, Sheikh NA, Fan LQ, Kandadi H, Meagher TC, Hall SJ, et al. Humoral Immune Response against Nontargeted Tumor Antigens after Treatment with Sipuleucel-T and Its Association with Improved Clinical Outcome. *Clin Cancer Res* (2015) 21(16):3619–30. doi: 10.1158/1078-0432.CCR-14-2334

41. Disis ML, Wallace DR, Gooley TA, Dang Y, Slota M, Lu H, et al. Concurrent trastuzumab and HER2/neu-specific vaccination in patients with metastatic breast cancer. *J Clin Oncol* (2009) 27(28):4685–92. doi: 10.1200/JCO.2008.20.6789

42. Hardwick N, Chain B. Epitope spreading contributes to effective immunotherapy in metastatic melanoma patients. *Immunotherapy* (2011) 3(6):731–3. doi: 10.2217/imt.11.62

43. Corbiere V, Chapiro J, Stroobant V, Ma W, Lurquin C, Lethe B, et al. Antigen spreading contributes to MAGE vaccination-induced regression of melanoma metastases. *Cancer Res* (2011) 71(4):1253–62. doi: 10.1158/0008-5472.CAN-10-2693

44. Chapuis AG, Roberts IM, Thompson JA, Margolin KA, Bhatia S, Lee SM, et al. T-cell therapy using interleukin-21-primed cytotoxic T-cell lymphocytes combined with cytotoxic T-cell lymphocyte antigen-4 blockade results in long-term cell persistence and durable tumor regression. *J Clin Oncol* (2016) 34(31):3787–95. doi: 10.1200/JCO.2015.65.5142

45. Walter S, Weinschenk T, Stenzl A, Zdrojowy R, Pluzanska A, Szczylik C, et al. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nat Med* (2012) 18 (8):1254–61. doi: 10.1038/nm.2883

46. Hu Z, Leet DE, Allesoe RL, Oliveira G, Li S, Luoma AM, et al. Personal neoantigen vaccines induce persistent memory T cell responses and epitope spreading in patients with melanoma. *Nat Med* (2021) 27(3):515–25. doi: 10.1038/s41591-020-01206-4

47. Etxeberria I, Bolanos E, Quetglas JI, Gros A, Villanueva A, Palomero J, et al. Intratumor adoptive transfer of IL-12 mRNA transiently engineered antitumor CD8(+) T cells. *Cancer Cell* (2019) 36(6):613–29.e7. doi: 10.1016/j.ccell.2019.10.006

48. Richter J, Neparidze N, Zhang L, Nair S, Monesmith T, Sundaram R, et al. Clinical regressions and broad immune activation following combination therapy targeting human NKT cells in myeloma. *Blood* (2013) 121(3):423–30. doi: 10.1182/blood-2012-06-435503

49. Guan X, Polesso F, Wang C, Sehrawat A, Hawkins RM, Murray SE, et al. Androgen receptor activity in T cells limits checkpoint blockade efficacy. *Nature* (2022) 606(7915):791–6. doi: 10.1038/s41586-022-04522-6

50. Chen J, Lopez-Moyado IF, Seo H, Lio CJ, Hempleman LJ, Sekiya T, et al. NR4A transcription factors limit CAR T cell function in solid tumours. *Nature* (2019) 567 (7749):530–4. doi: 10.1038/s41586-019-0985-x

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 08 February 2024
DOI 10.3389/fimmu.2024.1357483

OPEN ACCESS

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RECEIVED 18 December 2023
ACCEPTED 16 January 2024
PUBLISHED 08 February 2024

CITATION
Liu J, Joseph S, Manohar K, Lee J, Brokaw JP, Shelley WC and Markel TA (2024) Role of innate T cells in necrotizing enterocolitis. *Front. Immunol.* 15:1357483. doi: 10.3389/fimmu.2024.1357483

Role of innate T cells in necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) is a destructive gastrointestinal disease primarily affecting preterm babies. Despite advancements in neonatal care, NEC remains a significant cause of morbidity and mortality in neonatal intensive care units worldwide and the etiology of NEC is still unclear. Risk factors for NEC include prematurity, very low birth weight, feeding with formula, intestinal dysbiosis and bacterial infection. A review of the literature would suggest that supplementation of prebiotics and probiotics prevents NEC by altering the immune responses. Innate T cells, a highly conserved subpopulation of T cells that responds quickly to stimulation, develops differently from conventional T cells in neonates. This review aims to provide a succinct overview of innate T cells in neonates, encompassing their phenotypic characteristics, functional roles, likely involvement in the pathogenesis of NEC, and potential therapeutic implications.

KEYWORDS

immunity, innate T cells, NKT, MAIT, γδ T, neonates, preterm, necrotizing enterocolitis

Introduction

Necrotizing enterocolitis (NEC) is a devastating disease that affects neonates born prematurely. Approximately 10% of infants are born pre-term, and about 7% of them develop NEC (1). While etiology of NEC is unknown, several risk factors have been previously reported: prematurity, very low birth weight, formula feeding, microbial dysbiosis and bacterial infection (2–7). The current treatment of NEC includes cessation of enteral feeding, use of broad-spectrum antibiotics, and parenteral administration of nutrition. About 50% of infants with NEC progress to requiring surgical intervention due to intestinal ischemia and necrosis, which is often associated with high mortality and long-term complications including intestinal stricture, short-gut syndrome, and neurodevelopmental delays (1, 8). Discovering new approaches to treat NEC medically is imperative to avoid disease progression and surgical interventions.

The diagnosis, classification, management, outcome, and complications of NEC have been summarized in many great review articles (2–6). This review focuses on exploring the possibility therapeutic role of innate T cells for NEC.

Unique characteristics of neonatal immune response

Tolerogenic nature

The neonatal immune system can be characterized as tolerogenic, immature, and naïve. During pregnancy, microchimerism occurs, leading to the presence of maternal cells in the fetuses and vice versa. As a result, fetal immune cells are tolerant towards maternal antigens, while maternal immune cells and antibodies are vertically transferred to offspring (9, 10). Due to this tolerogenic nature and inherent bias toward Th2-cell polarization, the developing immune system in neonates can be more susceptible to infections (11, 12).

Immaturity and naivety

Neonatal immune cells are quantitatively fewer and qualitatively different from their adult immune cells (12, 13). As a result, maternal immune cells and antibodies circulate in offspring long after birth and impact neonatal immune responses (13–15). While maternal IgG is critical in preventing bacterial infection in neonates (16), the presence of maternal antibodies worsen the intrinsic defect of the infant's primary antibody response but does not appear to affect their T cell response (13). Though neonatal T cells are mostly recent thymic emigrants, less antigen-experienced, and produce less IL-2 and IFN-γ (17), these cells may be more sensitive to cytokines than to antigen stimulations due to higher expression of cytokine receptors on their cell surface (18).

Impact of microchimerism

The adaptive immune system in neonates is naïve and defective due to limited exposure to antigens and the tolerogenic environment *in utero* (19), therefore innate immunity is important in providing protection from infections (12, 20) despite its immaturity and hyporesponsiveness to stimulations (11, 21). A recent study showed that maternal microchimeric cells are enriched in fetal bone marrow and favor fetal monocyte differentiation (22), suggesting maternal/offspring microchimerism also affects neonatal innate immunity.

Neonatal T cells have full potential

Many investigations suggest that fetuses and neonates are capable of mounting robust T cell responses (13, 19). Early in life, neonates experience rapid growth and may allocate energy towards growth rather than mounting an immune response, leading to environmental enteric dysfunction (EED). Neonates need to maintain a balance between host defense against pathogens and other essential physiological processes (17).

While significant knowledge in neonatal immunity has been acquired, little is known about neonatal innate T cells and their role in the immune responses in neonatal diseases.

Innate T cells in neonates

Characteristics of innate T cells

Conventional T cells express highly diverse T-cell receptors (TCRs) and respond to peptide antigens presented by polymorphic MHC class I or II molecules. In contrast, innate T cells often have limited TCR diversity and predominantly recognize non-peptide antigens presented by monomorphic non-MHC molecules (23). Many of the non-peptide antigens that activate innate T cells are microbially derived (24–31). Therefore, their development and function are changed by the microbiome (32–34).

There are three subsets of innate T cells: Natural Killer T (NKT) cells, Mucosal-Associated Invariant T (MAIT) cells and Gamma Delta (γδ) T cells. NKT and MAIT cells are mostly semi-invariant αβ T cells. All three types of innate T cells develop in the thymus and localize in non-lymphoid tissues such as the liver, lung, and intestine (24, 35).

As shown in Table 1, innate T cells share some common characteristics, such as their ability to bridge the innate and adaptive immune systems by quickly responding to antigens and producing large amounts of pro- and anti-inflammatory cytokines (23). The development of all three types of innate T cells is regulated by the same transcription factor, PLZF (promyelocytic leukemia zinc finger; ZBTB16) (35, 53–55). Transcriptomic analyses have demonstrated that both NKT and MAIT cells are more similar with one another compared to conventional T cells (56, 57). While all innate T cells express IL-12 receptor (IL-12R), IL-18R and other surface markers (24, 51), both NKT and MAIT cells also express NK and T cell markers (51). The similar transcriptomic profiles of NKT and MAIT cells are likely acquired by their residence in the thymus (56).

Early in life, all three types of innate T cells seem to be more responsive and mature than conventional T cells in responding to stimulation (24, 42–44). These innate T cells are speculated to play important roles in tissue homeostasis and fighting against infection during early life when conventional T cells are still naïve and immature (58).

Neonatal NKT cells

NKT cells are innate T cells that can be activated by lipid antigens, with CD1d, an MHC class I-like molecule, acting as the antigen presenting molecule (59–62). The most potent lipid antigen for NKT cells is alpha-Galactosylceramide (a-GalCer, KRN7000), which is a synthetic glycolipid derived from the marine sponge *Agelas mauritianus* (63, 64). NKT cells can also be activated by various endogenous and microbial lipid antigens such as iGb3, sulfatide, and α-glucuronosylceramide (GSL-1) (28, 40, 65, 66). Activated NKT cells may be utilized in vaccine development and the treatment of conditions like autoimmune diseases, graft-versus-host disease, infections, neurological diseases, and cancer (67–77).

NKT cells are either $CD4^+$ or $CD4^-CD8^-$ T cells. Based on TCR usage, NKT cells can be further categorized as Type I (invariant

TABLE 1 Comparison of fetal/neonatal innate T cells.

	NKT cells	MAIT cells	γδ T cells	References
Maturation marker	CD45RO+ CD161+CD25+CD122+ CD127+	CD45RO+CD161+CD25+	CD27+CD28+	(36, 37)
Frequency in infants	<0.1% of CD3 T cells in cord blood	~0.1% of CD3 T cells in cord blood	~2% of CD3 T cells in cord blood	(36, 37)
Frequency in neonatal mice	Little is known	undetectable	3-4 times of αβ T cells in small intestine	(38, 39)
Microbial antigens/ligand	Microbial lipids such as α-glucuronosylceramide (GSL-1)	Microbial vitamin B metabolites	Phosphoantigens, Butyrophilins	(24, 40, 41)
Cytokine secretion	IFN-γ, IL-4	IFN-γ, IL-22	IFN-γ, TNF-α, IL-10	(42–47)
NEC impact	unknown	More MAIT cells accumulated in NEC intestines	Reduced in NEC	(48–50)
Common Characteristics	a) Limited TCR diversity; b) non-peptide antigens; c) enriched in non-lymphoid organs; d) developed in thymus; e) expressing IL-12 receptor and IL-18 receptor; f) PLZF as transcription factor; g) more cytokine production upon stimulation comparing to conventional T cells; h) proportion in T cells negatively correlates with gestational age; i) hyperproliferative potential			(23, 24, 35, 36 42–45, 51, 52)

TCR) and the much less studied Type II (variable CD1d-restricted TCR) NKT cells. In this review only type I NKT cells related work is discussed.

Studies have shown that a low number of neonatal NKT cells are present in cord blood (45, 78) and they are less responsive to stimulation compared to adult NKT cells (45). However, neonatal NKT cells were more responsive to stimulation compared to neonatal conventional T cells (45). The population of neonatal NKT cells was higher in the blood of day 14 preterm infants compared to those from age-matched full-term infants. In the subsequent 2-3 weeks, however, that higher proportion of NKT cells decreases to a level similar to that of full-term infants. This is likely due to gestational development because the proportion of $CD3^+$ T cells expands and positively correlates with gestational age (52). The proportion of NKT cells in the blood should expand as infants grow older since the proportion of NKT cells in adult blood is much higher than that in cord blood (45).

Neonates have a Th-2 biased immunity with neonatal NKT cells producing more IL-4 than IFN-γ (46, 47). Interestingly, NKT cells are enriched in the fetal small intestine. These small intestinal NKT cells, different from NKT cells from other fetal organs, express mature markers and IFN-γ upon stimulation, resembling adult NKT cells (44).

As shown in Table 1, not much is known about the frequency of neonatal NKT cells in mice.

Neonatal MAIT cells

MAIT cells predominantly recognize non-peptide microbial antigens presented by monomorphic MHC class I-like molecule (MR1) (23, 30, 79). MAIT cells express limited TCR diversity (Vα19 in mice, Vα7.2 in humans with limited variation of TCR-β chains). The research of MAIT cell antigens experienced a breakthrough when 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) was identified and remains the most potent MAIT cell agonist to date (31, 41, 80). MAIT cells are mostly $CD8^+$ or $CD4^-$ $CD8^-$ T cells. Emerging research demonstrates that MAIT cells are involved in many conditions, such as infection, cancer, tissue repair, autoimmunity, inflammation, and metabolic diseases (81–89).

Data on how gestational age impacts MAIT cell levels is conflicting. One study suggests that the proportion of neonatal MAIT cells is low and does not seem to be affected by gestational age ranged 23 to 28 weeks (52). However, another study using cord blood from broader range of gestational ages (24 weeks to full term) showed that the proportion of MAIT cells in $CD3^+$ T cells negatively correlated with gestational age (36). Currently, there are two ways to identify human MAIT cells: $CD3^+Vα7.2^+$ $CD161^{high}$ T and $CD3^+$MR1:5-OP-RU $tetramer^+$ cells. In adult blood, these two populations almost fully overlap. However, in cord blood, only a small portion of $CD3^+Vα7.2^+$ $CD161^{high}$ T cells are also MR1:5-OP-RU $tetramer^+$ (36, 53). This is likely due to specific expansion after encountering microbial antigens. Cord blood-derived MAIT cells consistently are more capable to proliferate upon stimulation compared to adult MAIT cells (36). Allogeneic hematopoietic cell transplantation study showed expansion of MAIT cells in recipients after cord blood transplantation but not in adult bone-marrow or peripheral blood stem cell transplantations, supporting the high proliferative capacity of neonatal MAIT cells (90).

Mouse MAIT cell studies have been lagging due to the scarcity of mouse MAIT cells. To solve this problem, a wild-derived inbred CAST/EiJ mouse model was discovered with frequencies of MAIT cells 20 times more than those in C57BL/6J mice (38). MAIT cells also increase significantly in the transgenic mice expressing the TCR Vα19, but its application is limited due to high non-specific binding of MR1:5-OP-RU tetramer in other T cells (91–93). Like human MAIT cells, mouse MAIT cells are almost undetectable at birth but expand significantly after encountering the developing microbiome (38).

Neonatal γδ T cells

Most mammalian T cells express αβ TCR. A small population of T cells express gamma and delta (γδ) TCR and these cells are called γδ T cells. The antigen presenting molecule for γδ T cells is not known. γδ TCR may interact with antigens in an antibody/antigen binding fashion (24, 94, 95). The functions of γδ T cells include immune surveillance, thermogenesis, and tissue homeostasis (96). γδ T cells are known to be important for maintaining mucosal tolerance (97, 98). Although similar numbers of γδ T cells can be found in the intestine of germ-free and specific pathogen-free mice (99), the crosstalk between microbiome and γδ T cells is important for the effector function of γδ T cells (32). Removal of gut microbiome by antibiotic treatment in drinking water impairs oral tolerance and also transiently removes intestinal γδ T cells.

Neonatal γδ T cells are Th2-prone and more naïve than adult γδ T cells, but more Th1-prone compared to neonatal αβ T cells. Thus, it seems reasonable to hypothesize that neonatal γδ T cells may be key providers of immunoprotection and immunomodulation in the perinatal period (42). γδ intraepithelial lymphocytes (IEL) are the first T-cell subset present in the intestine during embryogenesis (39, 100). Neonatal mouse γδ IELs were found to produce higher levels of cytokines, such as IFN-γ and IL-10, as compared to neonatal αβ IELs and adult γδ IELs, indicating enhanced activity of γδ IELs during early life (39).

Neonatal γδ T cells are more diverse compared to adult γδ T cells. The dominant Vγ9Vδ2 subset in human adult blood is due to the post-natal expansion of cells expressing unique CDR3 formed in response to encountering phosphor-antigens derived from the microbe-specific isoprenoid synthesis pathway. During mouse embryonic development, there are waves of γδ T cell development that start as early as day 15 of gestation so most peripheral tissues are colonized by long-lived γδ T cells early in life (96). The first wave of mouse γδ T cells are dendritic epidermal T cells (DETCs). These DETCs migrate to mouse skin and proliferate there during fetal development (24, 96). While some γδ T cells can be restored in 2 weeks in adult mice, fetal γδ T cells cannot be regenerated in the adult thymus (24).

Similar to neonatal NKT cells, the proportion of neonatal γδ T cells are larger in the blood from preterm infants than that from full-term infants, and the proportion of γδ T cells decreases to a similar level as that from full-term infants in the next 2-4 weeks (52). This is likely due to the expansion of $CD3^+$ T cells in late gestational stages.

The role of innate T cells in NEC

Gut microbial community perturbations are the most consequential risk factor for NEC (101). The intestinal microbiome of preterm infants is distinct and less diverse than that of term-born infants. Interestingly, the gut microbiome in preterm infants seems to have an orderly progression where the bacterial classes switch from Bacilli to Gammaproteobacteria to Clostridia, and is minimally influenced by mode of delivery, antibiotics, or feeds (101, 102).

It is not clear how the bacterial class switch in preterm infants increases their risk of developing NEC but analysis of gene expression analysis in NEC tissues does reveal an altered immune response (48, 103–106). The microbial dysbiosis in NEC likely alters the development of innate T cells given the microbiome's known influence on innate T cell maturation, activation, and expansion via changes in microbial antigens and modulation of the mucosal microenvironment (32, 81, 107, 108). Immune cell development needs microbial exposure, but there seems to be a "window of opportunity" (17, 58, 109). Using mouse models, several groups have demonstrated that exposure to certain microbiome early in life defines hosts' T cell functions in adulthood (17, 58, 109). It is reasonable to speculate that the microbial dysbiosis in NEC impacts not only neonatal immunity but also long-term immunity beyond when the disease is resolved.

Studies about the relationship between innate T cells and NEC are sparse. A recent report has shown that more MAIT cells accumulate in the intestine of NEC patients compared to control infants. However, these MAIT cells within NEC intestine are mostly $CD4^-CD8^-$, while MAIT cells from healthy intestine are mainly $CD8\alpha\alpha^+$ MAIT cells (49). $CD8\alpha\alpha^+$ MAIT cells are known to be more mature than $CD4^-CD8^-$ or $CD8\alpha\beta^+$ MAIT cells (43, 110). These results suggest that there are more immature MAIT cells residing in NEC intestines. Weitkamp et al. discovered significantly lower $CD8^+$ γδ IEL in preterm infants with NEC compared to control infants, suggesting that γδ IELs depletion occurs during the development of NEC (39).

It is worth noting that a unique population of IELs, called innate CD8alpha (*i*CD8α) cells, that expresses the CD8αα homodimer and may be involved with NEC pathogenesis. Though neither T cells nor dendritic cells, they are IL-12R positive and responsive to stimulation by IL-12 and PMA/Ionomycin. *i*CD8α cells show capacity in antigen processing/presentation and protection from bacterial infection (111). *i*CD8α cells are also reduced in NEC patients compared to control infants, consistent with reduced $CD8\alpha\alpha^+$ MAIT and $CD8^+$ γδ T cells in NEC (39, 49, 111). These observations indicate mucosal $CD8^+$ lymphocytes, either TCR^+ (MAIT and γδ T cells) or TCR^- (innate lymphoid cells) may be important in preventing NEC.

Deficiency of MR1 in neonatal mice renders protection from NEC pathogenesis (112) while TCRδ-deficient neonatal mice develop worse NEC disease compared to WT controls (39). These data suggest that innate T cells, probably altered by microbial dysbiosis, play a role in NEC pathogenesis (Figure 1A). Little is known how innate T cells may contribute to the pathogenesis of NEC. Innate T cells are known to bridge the innate and adaptive immune system and can mediate immune tolerance (113–116). It is speculated that the function of innate T cells may be altered with reduced immune tolerance due to microbial dysbiosis and immaturity in preterm infants. Another possible factor is IL-17 production that plays a critical role in pathogenesis of NEC (117). Innate T cells are known to produce IL-17 (118–120). Innate T cells from preterm infants may produce more IL-17 due to immaturity and microbial dysbiosis, contributing to NEC pathogenesis.

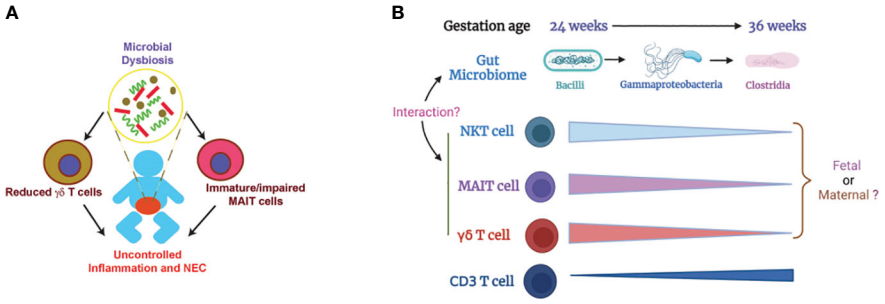

FIGURE 1
Illustration of how microbial dysbiosis and immaturity of innate T cells may affect NEC pathogenesis. **(A)** Immature/impaired MAIT cells and/or reduced γδ T cells, possibly caused by microbial dysbiosis, contribute to NEC pathogenesis (39, 48, 49, 112). **(B)** Change of gut microbiome and innate T cells in preterm infants based on gestation age. The proportions of innate T cells negatively correlate with gestational age. Little is known about whether the innate T cells in preterm infants are of fetal or maternal origin. It is also unclear how the transition of gut microbiome affects the development and function of innate T cells (36, 52, 101, 102). .

Conclusions and future direction

The current standard treatment regimen of NEC includes cessation of enteral feeding, institution of parenteral nutrition, initiating broad-spectrum antibiotics, respiratory support, and surgical intervention as needed (1). Human breastmilk has long been utilized as a way to reduce NEC (1). The expansion of Bifidobacteriaceae in gut microbiome after birth also decreases the risk of NEC (3, 100, 121). Human milk oligosaccharide (HMO) is important to bifidobacterial colonization, consistent with the observation that breast-feeding lowers the incidence of NEC (100). Prebiotics (*e.g.* human milk oligosaccharide) and probiotics (*e.g. Bifidobacteria*) are being investigated as potential preventative and therapeutics approaches for NEC (3, 7, 121, 122). A few acting mechanisms of probiotics in preventing NEC have been proposed (123), but little is known about how prebiotics and probiotics therapies may change innate T cells in NEC.

Because of their limited TCR diversity and monomorphism, innate T cells would be an off-the-shelf cell-based therapy with minimal graft-versus-host disease (124). Innate T cells can quickly respond to antigens and produce large amounts of pro- and anti-inflammatory cytokines to bridge the innate and adaptive immune systems (23, 51, 125–127). Innate T cells can also acquire effector T cell characteristics and accumulate in mucosal tissues early in life (42–44). IL-22 has been shown to alleviate NEC (128) and innate T cells are capable of producing IL-22 (43, 118). Genetic engineering technologies, such as CRISPR/Cre and CAR-T cells (76, 129–131), may facilitate the production of IL-22-producing γδ T and/or MAIT cells, even *i*CD8α IELs, for cell-based immunotherapy for NEC.

Contrarily, the accumulation of immature innate T cells in preterm infants may lead to the development of NEC. Blocking the activation of immature innate T cells may reduce the incidence of NEC. Antibodies for CD1d and MR1, the antigen presenting molecules for NKT and MAIT cells respectively, are effective in suppressing NKT and MAIT cell activation (132–134). These MR1 and CD1d specific antibodies may also be a potential avenue for further investigations in NEC immunotherapy.

Preterm infants are not developmentally primed to be colonized by microorganisms and the normal neonatal microbial adaptation may be hazardous in preterm infants (135). It is well-established that the development and maturation of innate T cells are influenced by the microbiome. The levels of innate T cells in preterm infants negatively correlates with gestational age (Figure 1B, Table 1) but the mechanism is unknown. Because the mother and offspring form a microchimer, innate T cells in fetus and preterm infants can be from fetal or maternal origin. The gut microbiome in preterm infants is transitional and different from term infants, but it is unclear exactly how the innate T cells in preterm infants are influenced by the altered gut microbiome. Future work should focus on identifying the origin of fetal and neonatal innate T cells and how their development and function are impacted by beneficial or pathogenic microbiome. The knowledge gained from this work will help facilitate the development of a novel innate T cell-based therapy for NEC.

Author contributions

JL: Writing – original draft, Writing – review & editing. KM: Writing – review & editing. SJ: Writing – review & editing. JL: Writing – review & editing. JB: Writing – review & editing. CS: Writing – review & editing. TM: Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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

References

1. Rich BS, Dolgin SE. Necrotizing enterocolitis. *Pediatr Rev* (2017) 38(12):552–9. doi: 10.1542/pir.2017-0002

2. Markel TA, Engelstad H, Poindexter BB. Predicting disease severity of necrotizing enterocolitis: how to identify infants for future novel therapies. *J Clin Neonatol.* (2014) 3(1):1–9. doi: 10.4103/2249-4847.128717

3. Kim CS, Claud EC. Necrotizing enterocolitis pathophysiology: how microbiome data alter our understanding. *Clin Perinatol.* (2019) 46(1):29–38. doi: 10.1016/j.clp.2018.10.003

4. Fundora JB, Guha P, Shores DR, Pammi M, Maheshwari A. Intestinal dysbiosis and necrotizing enterocolitis: assessment for causality using Bradford Hill criteria. *Pediatr Res* (2020) 87(2):235–48. doi: 10.1038/s41390-019-0482-9

5. Neu J, Walker WA. Necrotizing enterocolitis. *N Engl J Med* (2011) 364(3):255–64. doi: 10.1056/NEJMra1005408

6. Alganabi M, Lee C, Bindi E, Li B, Pierro A. Recent advances in understanding necrotizing enterocolitis. *F1000Res.* (2019) 8:107. doi: 10.12688/f1000research.17228.1

7. Campos-Martinez AM, Exposito-Herrera J, Gonzalez-Bolivar M, Fernandez-Marin E, Uberos J. Evaluation of risk and preventive factors for necrotizing enterocolitis in premature newborns. *A Systematic Rev Literature. Front Pediatr* (2022) 10:874976. doi: 10.3389/fped.2022.874976

8. Drucker NA, McCulloh CJ, Li B, Pierro A, Besner GE, Markel TA. Stem cell therapy in necrotizing enterocolitis: Current state and future directions. *Semin Pediatr Surg* (2018) 27(1):57–64. doi: 10.1053/j.sempedsurg.2017.11.011

9. Kinder JM, Stelzer IA, Arck PC, Way SS. Immunological implications of pregnancy-induced microchimerism. *Nat Rev Immunol* (2017) 17(8):483–94. doi: 10.1038/nri.2017.38

10. Adams KM, Nelson JL. Microchimerism: an investigative frontier in autoimmunity and transplantation. *Jama.* (2004) 291(9):1127–31. doi: 10.1001/jama.291.9.1127

11. Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat Rev Immunol* (2007) 7(5):379–90. doi: 10.1038/nri2075

12. Basha S, Surendran N, Pichichero M. Immune responses in neonates. *Expert Rev Clin Immunol* (2014) 10(9):1171–84. doi: 10.1586/1744666X.2014.942288

13. Adkins B, Leclerc C, Marshall-Clarke S. Neonatal adaptive immunity comes of age. *Nat Rev Immunol* (2004) 4(7):553–64. doi: 10.1038/nri1394

14. Touzot F, Dal-Cortivo L, Verkarre V, Lim A, Crucis-Armengaud A, Moshous D, et al. Massive expansion of maternal T cells in response to EBV infection in a patient with SCID-Xl. *Blood.* (2012) 120(9):1957–9. doi: 10.1182/blood-2012-04-426833

15. Maloney S, Smith A, Furst DE, Myerson D, Rupert K, Evans PC, et al. Microchimerism of maternal origin persists into adult life. *J Clin Invest.* (1999) 104 (1):41–7. doi: 10.1172/JCI6611

16. Sanidad KZ, Amir M, Ananthanarayanan A, Singaraju A, Shiland NB, Hong HS, et al. Maternal gut microbiome-induced IgG regulates neonatal gut microbiome and immunity. *Sci Immunol* (2022) 7(72):eabh3816. doi: 10.1126/sciimmunol.abh3816

17. Torow N, Hand TW, Hornef MW. Programmed and environmental determinants driving neonatal mucosal immune development. *Immunity.* (2023) 56 (3):485–99. doi: 10.1016/j.immuni.2023.02.013

18. Rudd BD. Neonatal T cells: A reinterpretation. *Annu Rev Immunol* (2020) 38:229–47. doi: 10.1146/annurev-immunol-091319-083608

19. Rackaityte E, Halkias J. Mechanisms of fetal T cell tolerance and immune regulation. *Front Immunol* (2020) 11:588. doi: 10.3389/fimmu.2020.00588

20. Kumar SK, Bhat BV. Distinct mechanisms of the newborn innate immunity. *Immunol Lett* (2016) 173:42–54. doi: 10.1016/j.imlet.2016.03.009

21. Yu JC, Khodadadi H, Malik A, Davidson B, Salles É DSL, Bhatia J, et al. Innate immunity of neonates and infants. *Front Immunol* (2018) 9:1759. doi: 10.3389/fimmu.2018.01759

22. Stelzer IA, Urbschat C, Schepanski S, Thiele K, Triviai I, Wieczorek A, et al. Vertically transferred maternal immune cells promote neonatal immunity against early life infections. *Nat Commun* (2021) 12(1):4706. doi: 10.1038/s41467-021-24719-z

23. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nat Immunol* (2015) 16(11):1114–23. doi: 10.1038/ni.3298

24. Vermijlen D, Prinz I. Ontogeny of innate T lymphocytes - some innate lymphocytes are more innate than others. *Front Immunol* (2014) 5:486. doi: 10.3389/fimmu.2014.00486

25. Wu D, Xing GW, Poles MA, Horowitz A, Kinjo Y, Sullivan B, et al. Bacterial glycolipids and analogs as antigens for CD1d-restricted NKT cells. *Proc Natl Acad Sci U S A.* (2005) 102(5):1351–6. doi: 10.1073/pnas.0408696102

26. Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD, et al. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature.* (2005) 434(7032):520–5. doi: 10.1038/nature03407

27. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C3rd, Zhou D, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature.* (2005) 434(7032):525–9. doi: 10.1038/nature03408

28. Sriram V, Du W, Gervay-Hague J, Brutkiewicz RR. Cell wall glycosphingolipids of *Sphingomonas paucimobilis* are CD1d-specific ligands for NKT cells. *Eur J Immunol* (2005) 35(6):1692–701. doi: 10.1002/eji.200526157

29. Altincicek B, Moll J, Campos N, Foerster G, Beck E, Hoeffler JF, et al. Cutting edge: human gamma delta T cells are activated by intermediates of the 2-C-methyl-D-erythritol 4-phosphate pathway of isoprenoid biosynthesis. *J Immunol* (2001) 166 (6):3655–8. doi: 10.4049/jimmunol.166.6.3655

30. Le Bourhis L, Martin E, Peguillet I, Guihot A, Froux N, Core M, et al. Antimicrobial activity of mucosal-associated invariant T cells. *Nat Immunol* (2010) 11(8):701–8. doi: 10.1038/ni.1890

31. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature.* (2012) 491(7426):717–23. doi: 10.1038/nature11605

32. Papotto PH, Yilmaz B, Silva-Santos B. Crosstalk between gammadelta T cells and the microbiota. *Nat Microbiol* (2021) 6(9):1110–7. doi: 10.1038/s41564-021-00948-2

33. Su X, Gao Y, Yang R. Gut microbiota derived bile acid metabolites maintain the homeostasis of gut and systemic immunity. *Front Immunol* (2023) 14:1127743. doi: 10.3389/fimmu.2023.1127743

34. Jabeen MF, Hinks TSC. MAIT cells and the microbiome. *Front Immunol* (2023) 14:1127588. doi: 10.3389/fimmu.2023.1127588

35. Pellicci DG, Koay HF, Berzins SP. Thymic development of unconventional T cells: how NKT cells, MAIT cells and gammadelta T cells emerge. *Nat Rev Immunol* (2020) 20(12):756–70. doi: 10.1038/s41577-020-0345-y

36. Ben Youssef G, Tourret M, Salou M, Ghazarian L, Houdouin V, Mondot S, et al. Ontogeny of human mucosal-associated invariant T cells and related T cell subsets. *J Exp Med* (2018) 215(2):459–79. doi: 10.1084/jem.20171739

37. van der Heiden M, Bjorkander S, Rahman Qazi K, Bittmann J, Hell L, Jenmalm MC, et al. Characterization of the gammadelta T-cell compartment during infancy reveals clear differences between the early neonatal period and 2 years of age. *Immunol Cell Biol* (2020) 98(1):79–87. doi: 10.1111/imcb.12303

38. Cui Y, Franciszkiewicz K, Mburu YK, Mondot S, Le Bourhis L, Premel V, et al. Mucosal-associated invariant T cell-rich congenic mouse strain allows functional evaluation. *J Clin Invest.* (2015) 125(11):4171–85. doi: 10.1172/JCI82424

39. Weitkamp JH, Rosen MJ, Zhao Z, Koyama T, Geem D, Denning TL, et al. Small intestinal intraepithelial TCRgammadelta+ T lymphocytes are present in the premature intestine but selectively reduced in surgical necrotizing enterocolitis. *PloS One* (2014) 9 (6):e99042. doi: 10.1371/journal.pone.0099042

40. Brutkiewicz RR. CD1d ligands: the good, the bad, and the ugly. *J Immunol* (2006) 177(2):769–75. doi: 10.4049/jimmunol.177.2.769

41. Corbett AJ, Awad W, Wang H, Chen Z. Antigen recognition by MR1-reactive T cells; MAIT cells, metabolites, and remaining mysteries. *Front Immunol* (2020) 11:1961. doi: 10.3389/fimmu.2020.01961

42. Gibbons DL, Haque SF, Silberzahn T, Hamilton K, Langford C, Ellis P, et al. Neonates harbour highly active gammadelta T cells with selective impairments in preterm infants. *Eur J Immunol* (2009) 39(7):1794–806. doi: 10.1002/eji.200939222

43. Leeansyah E, Loh L, Nixon DF, Sandberg JK. Acquisition of innate-like microbial reactivity in mucosal tissues during human fetal MAIT-cell development. *Nat Commun* (2014) 5:3143. doi: 10.1038/ncomms4143

44. Loh L, Ivarsson MA, Michaëlsson J, Sandberg JK, Nixon DF. Invariant natural killer T cells developing in the human fetus accumulate and mature in the small intestine. *Mucosal Immunol* (2014) 7(5):1233–43. doi: 10.1038/mi.2014.13

45. Swieboda D, Rice TF, Guo Y, Nadel S, Thwaites RS, Openshaw PJM, et al. Natural killer cells and innate lymphoid cells but not NKT cells are mature in their cytokine production at birth. *Clin Exp Immunol* ((2024)) 215(1):1–14. doi: 10.1093/cei/uxad094

46. Harner S, Noessner E, Nadas K, Leumann-Runge A, Schiemann M, Faber FL, et al. Cord blood Valpha24-Vbeta11 natural killer T cells display a Th2-chemokine receptor profile and cytokine responses. *PloS One* (2011) 6(1):e15714. doi: 10.1371/journal.pone.0015714

47. Kadowaki N, Antonenko S, Ho S, Rissoan MC, Soumelis V, Porcelli SA, et al. Distinct cytokine profiles of neonatal natural killer T cells after expansion with subsets of dendritic cells. *J Exp Med* (2001) 193(10):1221–6. doi: 10.1084/jem.193.10.1221

48. Weitkamp JH, Koyama T, Rock MT, Correa H, Goettel JA, Matta P, et al. Necrotising enterocolitis is characterised by disrupted immune regulation and diminished mucosal regulatory (FOXP3)/effector (CD4, CD8) T cell ratios. *Gut.* (2013) 62(1):73–82. doi: 10.1136/gutjnl-2011-301551

49. Tian J, Yan C, Jiang Y, Zhou H, Li L, Shen J, et al. Peripheral and intestinal mucosal-associated invariant T cells in premature infants with necrotizing enterocolitis. *Front Pharmacol* (2022) 13:1008080. doi: 10.3389/fphar.2022.1008080

50. Sproat T, Payne RP, Embleton ND, Berrington J, Hambleton S. T cells in preterm infants and the influence of milk diet. *Front Immunol* (2020) 11:1035. doi: 10.3389/fimmu.2020.01035

51. Treiner E, Lantz O. CD1d- and MR1-restricted invariant T cells: of mice and men. *Curr Opin Immunol* (2006) 18(5):519–26. doi: 10.1016/j.coi.2006.07.001

52. Rahman Qazi K, Jensen GB, van der Heiden M, Bjorkander S, Marchini G, Jenmalm MC, et al. Extreme prematurity and sepsis strongly influence frequencies and functional characteristics of circulating gammadelta T and natural killer cells. *Clin Transl Immunol* (2021) 10(6):e1294. doi: 10.1002/cti2.1294

53. Koay HF, Gherardin NA, Enders A, Loh L, Mackay LK, Almeida CF, et al. A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage. *Nat Immunol* (2016) 17(11):1300–11. doi: 10.1038/ni.3565

54. Kovalovsky D, Uche OU, Eladad S, Hobbs RM, Yi W, Alonzo E, et al. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nat Immunol* (2008) 9(9):1055–64. doi: 10.1038/ni.1641

55. Lu Y, Cao X, Zhang X, Kovalovsky D. PLZF controls the development of fetal-derived IL-17+Vgamma6+ gammadelta T cells. *J Immunol* (2015) 195(9):4273–81. doi: 10.4049/jimmunol.1500939

56. Salou M, Legoux F, Gilet J, Darbois A, du Halgouet A, Alonso R, et al. A common transcriptomic program acquired in the thymus defines tissue residency of MAIT and NKT subsets. *J Exp Med* (2019) 216(1):133–51. doi: 10.1084/jem.20181483

57. Gutierrez-Arcelus M, Teslovich N, Mola AR, Polidoro RB, Nathan A, Kim H, et al. Lymphocyte innateness defined by transcriptional states reflects a balance between proliferation and effector functions. *Nat Commun* (2019) 10(1):687. doi: 10.1038/s41467-019-08604-4

58. Constantinides MG, Belkaid Y. Early-life imprinting of unconventional T cells and tissue homeostasis. *Science* (2021) 374(6573):eabf0095. doi: 10.1126/science.abf0095

59. Cardell S, Tangri S, Chan S, Kronenberg M, Benoist C, Mathis D. CD1-restricted CD4+ T cells in major histocompatibility complex class II-deficient mice. *J Exp Med* (1995) 182(4):993–1004. doi: 10.1084/jem.182.4.993

60. Chen YH, Chiu NM, Mandal M, Wang N, Wang CR. Impaired NK1$^+$ T cell development and early IL-4 production in CD1-deficient mice. *Immunity.* (1997) 6 (4):459–67. doi: 10.1016/S1074-7613(00)80289-7

61. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1$^+$ T lymphocytes. *Science.* (1995) 268:863–5. doi: 10.1126/science.7538697

62. Mendiratta SK, Martin WD, Hong S, Boesteanu A, Joyce S, Van Kaer L. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity.* (1997) 6(4):469–77. doi: 10.1016/S1074-7613(00)80290-3

63. Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, et al. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem* (1995) 38(12):2176–87. doi: 10.1021/jm00012a018

64. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of V_a14 NKT cells by glycosylceramides. *Science.* (1997) 278(5343):1626–9. doi: 10.1126/science.278.5343.1626

65. Zhou D, Mattner J, Cantu C 3rd, Schrantz N, Yin N, Gao Y, et al. Lysosomal glycosphingolipid recognition by NKT cells. *Science.* (2004) 306(5702):1786–9. doi: 10.1126/science.1103440

66. Blomqvist M, Rhost S, Teneberg S, Lofbom L, Osterbye T, Brigl M, et al. Multiple tissue-specific isoforms of sulfatide activate CD1d-restricted type II NKT cells. *Eur J Immunol* (2009) 39(7):1726–35. doi: 10.1002/eji.200839001

67. Wu L, Van Kaer L. Natural killer T cells in health and disease. *Front Biosci (Schol Ed).* (2011) 3(1):236–51. doi: 10.2741/s148

68. Wu X, Liu J, Li W, Khan MF, Dai H, Tian J, et al. CD1d-dependent neuroinflammation impairs tissue repair and functional recovery following a spinal cord injury. *bioRxiv* (2023). doi: 10.1101/2023.10.13.562047

69. Liu J, Renukaradhya GJ, Brutkiewicz RR. The Regulation of CD1d+ and CD1d- Tumors by NKT Cells: The roles of NKT cells in regulating CD1d+ and CD1d- tumor immunity. In: Terabe M, Berzofsky JA, editors. *Natural killer T cells: balancing the regulation of tumor immunity*. New York: Springer Science+Business Media, LLC (2011). p. 71–94.

70. Liu J, Gallo RM, Khan MA, Renukaradhya GJ, Brutkiewicz RR. Neurofibromin 1 impairs natural killer T-cell-dependent antitumor immunity against a T-cell lymphoma. *Front Immunol* (1901) 2018:8. doi: 10.3389/fimmu.2017.01901

71. Cui Y, Wan Q. NKT cells in neurological diseases. *Front Cell Neurosci* (2019) 13:245. doi: 10.3389/fncel.2019.00245

72. Jeong D, Woo YD, Chung DH. Invariant natural killer T cells in lung diseases. *Exp Mol Med* (2023) 55(9):1885–94. doi: 10.1038/s12276-023-01024-x

73. Miyake S, Yamamura T. NKT cells and autoimmune diseases: unraveling the complexity. In: Moody DB, editor. *T cell activation by CD1 and lipid antigens, current topics in microbiology and immunology*. Berlin, Heidelberg: Springer (2007).

74. Hadiloo K, Tahmasebi S, Esmaeilzadeh A. CAR-NKT cell therapy: a new promising paradigm of cancer immunotherapy. *Cancer Cell Int* (2023) 23(1):86. doi: 10.1186/s12935-023-02923-9

75. Ahmadi A, Fallah Vastani Z, Abounoori M, Azizi M, Labani-Motlagh A, Mami S, et al. The role of NK and NKT cells in the pathogenesis and improvement of multiple sclerosis following disease-modifying therapies. *Health Sci Rep* (2022) 5(1):e489. doi: 10.1002/hsr2.489

76. Heczey A, Courtney AN, Montalbano A, Robinson S, Liu K, Li M, et al. Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: an interim analysis. *Nat Med* (2020) 26(11):1686–90. doi: 10.1038/s41591-020-1074-2

77. Coman T, Rossignol J, D'Aveni M, Fabiani B, Dussiot M, Rignault R, et al. Human CD4- invariant NKT lymphocytes regulate graft versus host disease. *Oncoimmunology.* (2018) 7(11):e1470735. doi: 10.1080/2162402X.2018.1470735

78. Prabhu SB, Rathore DK, Nair D, Chaudhary A, Raza S, Kanodia P, et al. Comparison of human neonatal and adult blood leukocyte subset composition phenotypes. *PloS One* (2016) 11(9):e0162242. doi: 10.1371/journal.pone.0162242

79. Huang S, Martin E, Kim S, Yu L, Soudais C, Fremont DH, et al. MR1 antigen presentation to mucosal-associated invariant T cells was highly conserved in evolution. *Proc Natl Acad Sci U S A.* (2009) 106(20):8290–5. doi: 10.1073/pnas.0903196106

80. Corbett AJ, Eckle SB, Birkinshaw RW, Liu L, Patel O, Mahony J, et al. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature.* (2014) 509(7500):361–5. doi: 10.1038/nature13160

81. Godfrey DI, Koay HF, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. *Nat Immunol* (2019) 20(9):1110–28. doi: 10.1038/s41590-019-0444-8

82. Nel I, Bertrand L, Toubal A, Lehuen A. MAIT cells, guardians of skin and mucosa? *Mucosal Immunol* (2021) 14(4):803–14. doi: 10.1038/s41385-021-00391-w

83. Toubal A, Lehuen A. Role of MAIT cells in metabolic diseases. *Mol Immunol* (2021) 130:142–7. doi: 10.1016/j.molimm.2020.12.014

84. Kubica P, Lara-Velazquez M, Bam M, Siraj S, Ong I, Liu P, et al. MR1 overexpression correlates with poor clinical prognosis in glioma patients. *Neurooncol Adv* (2021) 3(1):vdab034. doi: 10.1093/noajnl/vdab034

85. Serriari NE, Eoche M, Lamotte L, Lion J, Fumery M, Marcelo P, et al. Innate mucosal-associated invariant T (MAIT) cells are activated in inflammatory bowel diseases. *Clin Exp Immunol* (2014) 176(2):266–74. doi: 10.1111/cei.12277

86. Constantinides MG, Link VM, Tamoutounour S, Wong AC, Perez-Chaparro PJ, Han SJ, et al. MAIT cells are imprinted by the microbiota in early life and promote tissue repair. *Science* (2019) 366(6464):eaax6624. doi: 10.1126/science.aax6624

87. Magalhaes I, Pingris K, Poitou C, Bessoles S, Venteclef N, Kiaf B, et al. Mucosal-associated invariant T cell alterations in obese and type 2 diabetic patients. *J Clin Invest.* (2015) 125(4):1752–62. doi: 10.1172/JCI78941

88. Liu J, Nan H, Brutkiewicz RR, Casasnovas J, Kua KL. Sex discrepancy in the reduction of mucosal-associated invariant T cells caused by obesity. *Immun Inflammation Dis* (2021) 9(1):299–309. doi: 10.1002/iid3.393

89. Eberhard JM, Hartjen P, Kummer S, Schmidt RE, Bockhorn M, Lehmann C, et al. CD161+ MAIT cells are severely reduced in peripheral blood and lymph nodes of HIV-infected individuals independently of disease progression. *PloS One* (2014) 9(11): e111323. doi: 10.1371/journal.pone.0111323

90. Konuma T, Kohara C, Watanabe E, Takahashi S, Ozawa G, Suzuki K, et al. Reconstitution of circulating mucosal-associated invariant T cells after allogeneic hematopoietic cell transplantation: its association with the riboflavin synthetic pathway of gut microbiota in cord blood transplant recipients. *J Immunol* (2020) 204 (6):1462–73. doi: 10.4049/jimmunol.1900681

91. Reantragoon R, Corbett AJ, Sakala IG, Gherardin NA, Furness JB, Chen Z, et al. Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. *J Exp Med* (2013) 210(11):2305–20. doi: 10.1084/jem.20130958

92. Rahimpour A, Koay HF, Enders A, Clanchy R, Eckle SB, Meehan B, et al. Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. *J Exp Med* (2015) 212(7):1095–108. doi: 10.1084/jem.20142110

93. Sakala IG, Kjer-Nielsen L, Eickhoff CS, Wang X, Blazevic A, Liu L, et al. Functional heterogeneity and antimycobacterial effects of mouse mucosal-associated invariant T cells specific for riboflavin metabolites. *J Immunol* (2015) 195(2):587–601. doi: 10.4049/jimmunol.1402545

94. Willcox CR, Pitard V, Netzer S, Couzi L, Salim M, Silberzahn T, et al. Cytomegalovirus and tumor stress surveillance by binding of a human gammadelta T cell antigen receptor to endothelial protein C receptor. *Nat Immunol* (2012) 13 (9):872–9. doi: 10.1038/ni.2394

95. Zeng X, Wei YL, Huang J, Newell EW, Yu H, Kidd BA, et al. gammadelta T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific

interleukin-17 response. *Immunity*. (2012) 37(3):524–34. doi: 10.1016/j.immuni.2012.06.011

96. Ribot JC, Lopes N, Silva-Santos B. gammadelta T cells in tissue physiology and surveillance. *Nat Rev Immunol* (2021) 21(4):221–32. doi: 10.1038/s41577-020-00452-4

97. Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R. Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A*. (2002) 99 (22):14338–43. doi: 10.1073/pnas.212290499

98. Rezende RM, Cox LM, Moreira TG, Liu S, Boulenouar S, Dhang F, et al. Gamma-delta T cells modulate the microbiota and fecal micro-RNAs to maintain mucosal tolerance. *Microbiome*. (2023) 11(1):32. doi: 10.1186/s40168-023-01478-1

99. Bandeira A, Mota-Santos T, Itohara S, Degermann S, Heusser C, Tonegawa S, et al. Localization of gamma/delta T cells to the intestinal epithelium is independent of normal microbial colonization. *J Exp Med* (1990) 172(1):239–44. doi: 10.1084/jem.172.1.239

100. Henrick BM, Rodriguez L, Lakshmikanth T, Pou C, Henckel E, Arzoomand A, et al. Bifidobacteria-mediated immune system imprinting early in life. *Cell*. (2021) 184 (15):3884–98e11. doi: 10.1016/j.cell.2021.05.030

101. La Rosa PS, Warner BB, Zhou Y, Weinstock GM, Sodergren E, Hall-Moore CM, et al. Patterned progression of bacterial populations in the premature infant gut. *Proc Natl Acad Sci U S A*. (2014) 111(34):12522–7. doi: 10.1073/pnas.1409497111

102. Thanert R, Keen EC, Dantas G, Warner BB, Tarr PI. Necrotizing enterocolitis and the microbiome: current status and future directions. *J Infect Dis* (2021) 223(12 Suppl 2):S257–S63. doi: 10.1093/infdis/jiaa604

103. Tremblay É, Thibault MP, Ferretti E, Babakissa C, Bertelle V, Bettolli M, et al. Gene expression profiling in necrotizing enterocolitis reveals pathways common to those reported in Crohn's disease. *BMC Med Genomics* (2016) 9:6. doi: 10.1186/s12920-016-0166-9

104. Egozi A, Olaloye O, Werner L, Silva T, McCourt B, Pierce RW, et al. Single cell atlas of the neonatal small intestine with necrotizing enterocolitis. *bioRxiv*. (2022) 2022:03.01.482508. doi: 10.2139/ssrn.4032068

105. Olaloye OO, Liu P, Toothaker JM, McCourt BT, McCourt CC, Xiao J, et al. CD16+CD163+ monocytes traffic to sites of inflammation during necrotizing enterocolitis in premature infants. *J Exp Med* (2021) 218(9):e20200344. doi: 10.1084/jem.20200344

106. Egozi A, Olaloye O, Werner L, Silva T, McCourt B, Pierce RW, et al. Single-cell atlas of the human neonatal small intestine affected by necrotizing enterocolitis. *PloS Biol* (2023) 21(5):e3002124. doi: 10.1371/journal.pbio.3002124

107. Wingender G, Stepniak D, Krebs P, Lin L, McBride S, Wei B, et al. Intestinal microbes affect phenotypes and functions of invariant natural killer T cells in mice. *Gastroenterology*. (2012) 143(2):418–28. doi: 10.1053/j.gastro.2012.04.017

108. Olszak T, An D, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science*. (2012) 336(6080):489–93. doi: 10.1126/science.1219328

109. Tabilas C, Iu DS, Daly CWP, Yee Mon KJ, Reynaldi A, Wesnak SP, et al. Early microbial exposure shapes adult immunity by altering CD8+ T cell development. *Proc Natl Acad Sci U S A*. (2022) 119(49):e2212548119. doi: 10.1073/pnas.2212548119

110. Dias J, Boulouis C, Gorin JB, van den Biggelaar R, Lal KG, Gibbs A, et al. The CD4(-)CD8(-) MAIT cell subpopulation is a functionally distinct subset developmentally related to the main CD8(+) MAIT cell pool. *Proc Natl Acad Sci U S A*. (2018) 115(49):E11513–E22. doi: 10.1073/pnas.1812273115

111. Van Kaer L, Algood HMS, Singh K, Parekh VV, Greer MJ, Piazuelo MB, et al. CD8alphaalpha(+) innate-type lymphocytes in the intestinal epithelium mediate mucosal immunity. *Immunity*. (2014) 41(3):451–64. doi: 10.1016/j.immuni.2014.08.010

112. Liu J, Hunter C, Manohar K, Mesfin F, Shelley WC, Brokaw J, et al. MR1-deficiency in neonatal mice renders protection from necrotizing enterocolitis. *J Immunol* (2023) 210 (1_Supplement):66.16–6. doi: 10.4049/jimmunol.210.Supp.66.16

113. Bharadwaj NS, Gumperz JE. Harnessing invariant natural killer T cells to control pathological inflammation. *Front Immunol* (2022) 13:998378. doi: 10.3389/fimmu.2022.998378

114. Kapp JA, Kapp LM, McKenna KC. Gammadelta T cells play an essential role in several forms of tolerance. *Immunol Res* (2004) 29(1-3):93–102. doi: 10.1385/IR:29:1-3:093

115. Giri S, Lal G. Differentiation and functional plasticity of gamma-delta (γδ) T cells under homeostatic and disease conditions. *Mol Immunol* (2021) 136:138–49. doi: 10.1016/j.molimm.2021.06.006

116. Giri S, Meitei HT, Mishra A, Lal G. Vγ2(+) γδ T cells in the presence of anti-CD40L control surgical inflammation and promote skin allograft survival. *J Invest Dermatol* (2022) 142(10):2706–14.e3. doi: 10.1016/j.jid.2022.03.016

117. Egan CE, Sodhi CP, Good M, Lin J, Jia H, Yamaguchi Y, et al. Toll-like receptor 4-mediated lymphocyte influx induces neonatal necrotizing enterocolitis. *J Clin Invest*. (2016) 126(2):495–508. doi: 10.1172/JCI83356

118. Ness-Schwickerath KJ, Morita CT. Regulation and function of IL-17A- and IL-22-producing gammadelta T cells. *Cell Mol Life Sci* (2011) 68(14):2371–90. doi: 10.1007/s00018-011-0700-z

119. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol* (2013) 14(11):1146–54. doi: 10.1038/ni.2731

120. Park KJ, Jin HM, Cho YN, Yoon JH, Kee SJ, Kim HS, et al. Altered frequency, activation, and clinical relevance of circulating innate and innate-like lymphocytes in patients with alcoholic liver cirrhosis. *Immune Netw* (2023) 23(3):e22. doi: 10.4110/in.2023.23.e22

121. Deshpande G, Rao S, Patole S, Bulsara M. Updated meta-analysis of probiotics for preventing necrotizing enterocolitis in preterm neonates. *Pediatrics*. (2010) 125 (5):921–30. doi: 10.1542/peds.2009-1301

122. AlFaleh K, Anabrees J. Probiotics for prevention of necrotizing enterocolitis in preterm infants. *Cochrane Database Syst Rev* (2022) PUB4:CD005496. doi: 10.1002/14651858.CD005496.pub4

123. Patel RM, Underwood MA. Probiotics and necrotizing enterocolitis. *Semin Pediatr Surg* (2018) 27(1):39–46. doi: 10.1053/j.sempedsurg.2017.11.008

124. Godfrey DI, Le Nours J, Andrews DM, Uldrich AP, Rossjohn J. Unconventional T cell targets for cancer immunotherapy. *Immunity*. (2018) 48(3):453–73. doi: 10.1016/j.immuni.2018.03.009

125. Huang S. Targeting innate-like T cells in tuberculosis. *Front Immunol* (2016) 7:594. doi: 10.3389/fimmu.2016.00594

126. Huang W, He W, Shi X, He X, Dou L, Gao Y. The role of CD1d and MR1 restricted T cells in the liver. *Front Immunol* (2018) 9:2424. doi: 10.3389/fimmu.2018.02424

127. Stolk D, van der Vliet HJ, de Gruijl TD, van Kooyk Y, Exley MA. Positive & Negative roles of innate effector cells in controlling cancer progression. *Front Immunol* (2018) 9:1990. doi: 10.3389/fimmu.2018.01990

128. Mihi B, Gong Q, Nolan LS, Gale SE, Goree M, Hu E, et al. Interleukin-22 signaling attenuates necrotizing enterocolitis by promoting epithelial cell regeneration. *Cell Rep Med* (2021) 2(6):100320. doi: 10.1016/j.xcrm.2021.100320

129. Makkouk A, Yang XC, Barca T, Lucas A, Turkoz M, Wong JTS, et al. Off-the-shelf Vδ1 gamma delta T cells engineered with glypican-3 (GPC-3)-specific chimeric antigen receptor (CAR) and soluble IL-15 display robust antitumor efficacy against hepatocellular carcinoma. *J Immunother Cancer* (2021) 9(12):e003441. doi: 10.1136/jitc-2021-003441

130. Bohineust A, Tourret M, Derivry L, Caillat-Zucman S. Mucosal-associated invariant T (MAIT) cells, a new source of universal immune cells for chimeric antigen receptor (CAR)-cell therapy. *Bull Cancer* (2021) 108(10s):S92–s5. doi: 10.1016/j.bulcan.2021.07.003

131. Stadtmauer EA, Fraietta JA, Davis MM, Cohen AD, Weber KL, Lancaster E, et al. CRISPR-engineered T cells in patients with refractory cancer. *Science* (2020) 367 (6481):eaba7365. doi: 10.1126/science.aba7365

132. Liu J, Brutkiewicz RR. The Toll-like receptor 9 signalling pathway regulates MR1-mediated bacterial antigen presentation in B cells. *Immunology*. (2017) 152 (2):232–42. doi: 10.1111/imm.12759

133. Teng MW, Sharkey J, McLaughlin NM, Exley MA, Smyth MJ. CD1d-based combination therapy eradicates established tumors in mice. *J Immunol* (2009) 183 (3):1911–20. doi: 10.4049/jimmunol.0900796

134. Tazawa H, Irei T, Tanaka Y, Igarashi Y, Tashiro H, Ohdan H. Blockade of invariant TCR-CD1d interaction specifically inhibits antibody production against blood group A carbohydrates. *Blood*. (2013) 122(15):2582–90. doi: 10.1182/blood-2012-02-407452

135. Sampath V, Martinez M, Caplan M, Underwood MA, Cuna A. Necrotizing enterocolitis in premature infants-A defect in the brakes? Evidence from clinical and animal studies. *Mucosal Immunol* (2023) 16(2):208–20. doi: 10.1016/j.mucimm.2023.02.002

frontiers | Frontiers in Immunology

TYPE Brief Research Report
PUBLISHED 13 February 2024
DOI 10.3389/fimmu.2024.1339727

OPEN ACCESS

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RECEIVED 16 November 2023
ACCEPTED 25 January 2024
PUBLISHED 13 February 2024

CITATION
N'guessan KF, Machmach K, Swafford I, Costanzo MC, Wieczorek L, Kim D, Akapirat S, Polonis VR, Pitisuttithum P, Nitayaphan S, Gurunathan S, Sinangil F, Chariyalertsak S, Ake JA, O'connell RJ, Vasan S and Paquin-Proulx D (2024) Innate immune cell activation after HIV-1 vaccine administration is associated with increased antibody production. *Front. Immunol.* 15:1339727. doi: 10.3389/fimmu.2024.1339727

Innate immune cell activation after HIV-1 vaccine administration is associated with increased antibody production

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The RV144 Thai phase III clinical trial's canarypox–protein HIV vaccine regimen showed modest efficacy in reducing infection. We therefore sought to determine the effects of vaccine administration on innate cell activation and subsequent associations with vaccine-induced immune responses. RV306 was a randomized, double-blind clinical trial in HIV-uninfected Thai adults that tested delayed boosting following the RV144 regimen. PBMC collected from RV306 participants prior to and 3 days after the last boost were used to investigate innate immune cell activation. Our analysis showed an increase in CD38+ mucosal associated invariant T (MAIT) cells, CD38+ invariant natural killer T (iNKT) cells, CD38+ γδ T cells, CD38+, CD69+ and HLA-DR+ NK cells 3 days after vaccine administration. An increase in CD14-CD16+ non-classical monocytes and CD14+CD16+ intermediate monocytes accompanied by a decrease in CD14+CD16- classical monocytes was also associated with vaccine administration. Inclusion of ALVAC-HIV in the boost did not further increase MAIT, iNKT, γδ T, and NK cell activation or increase the proportion of non-classical monocytes. Additionally, NK cell activation 3 days after vaccination was positively associated with antibody titers of HIV Env-specific total IgG and IgG1. Vδ1 T cell activation 3 days after vaccine administration was associated with HIV Env-specific IgG3 titers. Finally, we observed trending associations between

MAIT cell activation and Env-specific IgG3 titers and between NK cell activation and TH023 pseudovirus neutralization titers. Our study identifies a potential role for innate cells, specifically NK, MAIT, and γδ T cells, in promoting antibody responses following HIV-1 vaccine administration.

KEYWORDS

MAIT (mucosal-associated invariant T) cell, iNKT cell, NK cell, gamma delta (γδ) T cells, HIV vaccine, immune activation, monocytes

Introduction

The RV144 HIV vaccine regimen consisting of ALVAC-HIV, an attenuated non-replicating canarypox virus vector given at weeks 0, 4, 12, and 24, and AIDSVAX B/E, a bivalent recombinant gp120 protein derived from HIV-1 CRF01_AE and B subtypes given at weeks 12 and 24, demonstrated a modest efficacy of 31.2% in preventing HIV acquisition in a phase III clinical trial in Thailand (1). Subsequent studies identified an inverse correlation between binding of IgG antibodies to variable regions 1 and 2 (V1V2) of HIV-1 envelope proteins (Env) and the rate of HIV-1 acquisition as well as a direct correlation between binding of plasma IgA antibodies to Env and the rate of acquisition (2, 3). In addition, IgG3 antibodies correlated with a decreased risk of infection (3). Furthermore, CD4 T cell functionality and polyfunctionality scores, as calculated by COMPASS, correlated inversely with HIV acquisition (4). RV306 was a follow up phase I trial also conducted in Thailand that administered the RV144 regimen with an additional boost consisting of AIDSVAX B/E gp120 alone or together with ALVAC-HIV (5). The additional boost increased the magnitude and durability of CD4 T cell responses, binding antibodies, as well as pseudovirus neutralization and there was no difference in the primary analysis between the two boosting strategies. The innate immune response to the first ALVAC-HIV administration has been described using samples from a phase 1b trial of the RV144 regimen in HIV-1 uninfected South Africans (HVTN 097) (6). The study found that Type I and II interferon signaling pathways and innate pathways critical for adaptive immune priming were activated 1 day post vaccination. However, the involvement of innate cells' sensing of the vaccine components after subsequent vaccinations remains to be elucidated.

Innate cells play a critical role in shaping adaptive immune responses through the production of immunoregulatory cytokines and through direct-cellular interactions in the case of NK cells and antigen presenting cells (7–11). Peripheral blood NK cells are mostly mature and cytotoxic and are identified as $CD56^{dim}$ NK cells (12). A smaller proportion of NK cells known as immature or early $CD56^{hi}$ can produce IFNγ upon stimulation by cytokines (12). NK cells have been identified as a major source of IFNγ following vaccination with ALVAC-HIV (13). Additionally, studies in a non-human primate (NHP) model indicated that NK cell-induced activation of monocytes following vaccination with ALVAC-SIV decreased risk of SIVmac251 acquisition (14). Recent studies have identified a monocyte-derived gene signature associated with reduced risk of HIV acquisition in RV144 (11). Circulating monocytes can be divided into distinct subtypes based on CD14 and CD16 expression. The classical monocytes, defined as CD14 +CD16-, comprise the majority of monocytes in peripheral blood. Furthermore, studies in NHP have shown that classical monocytes are a strong correlate of decreased risk of SIV acquisition by an RV144 like vaccine regimen (14).

Unconventional T cells include MR1-restricted mucosal-associated invariant T cells (MAIT cells), CD1d-restricted natural killer T cells (NKT cells), and gamma delta (γδ) T cells. MAIT cell have been recently shown to be able to provide B cell help (15, 16) and promote the maturation of dendritic cells (17, 18) via CD40L as well as via the production of cytokines such as IL-21. MAIT cells are activated by vaccination with the ChAdOx01 viral vector, and their activation was essential for induction of antigen specific CD8 T cells (19). Similarly, MAIT cell characteristics have been associated with the adaptive immune response following mRNA vaccination (9, 20). Invariant NKT (iNKT) cells can quickly produce cytokines capable of activating several immune cells, including NK cells, dendritic cells, other unconventional T cells, and B cells (21). These attributes of iNKT cells make them potential immunoregulators in the context of vaccines. Studies have shown that co-injection of iNKT cell agonists, together with antigens promotes humoral and cellular immune responses (22–25). γδ T cells represent a subset of unconventional T cells defined by the expression of T-cell receptors (TCRs) composed of γ and δ chains, differentiating them from classical CD4+ and CD8+ T cells which express αβ TCRs (26). γδ T cells are capable of recognizing markers of cellular stress resulting from infection and display a broad function through the production of cytokines such as IFNγ, TNF-α and IL-17, chemokines such as RANTES and IP-10 and cytolytic proteins such as perforin and granzyme B (27). In humans, γδ T cells can be grouped into two broad subsets characterized by their TCR δ chain usage: Vδ1 T cells are predominantly found in the thymus and peripheral tissues while Vδ2 T cells are the main subset present in blood (27). γδ T cells are capable of regulating B cells by influencing B cell differentiation and promoting CD4 T cells secretion of IL-13 and IL-21 (8, 26, 28). Furthermore, studies in human peripheral

blood have identified a subset of Vγ9/Vδ2 + γδ T cells expressing CXCR5 which can be induced to express costimulatory molecules ICOS and CD40L, secrete IL-2, IL-4, and IL-10, and help B cells in antibody production (29).

Understanding innate immune cell responses to the vaccination could provide us with valuable insights on strategies to improve HIV-1 vaccine regimens. Here, we describe innate immune cell responses in the context of delayed boosting of the RV144 regimen with ALVAC-HIV and AIDSVAX B/E in RV306 (5). In our study, we observe increased NK, MAIT, and γδ T cell activation as well as an increase in CD14+CD16+ monocytes 3 days following late-stage boost vaccination. Importantly, NK cells, MAIT cells, and Vδ1 T cells activation was associated with the promotion of antibody responses while CD14+CD16+ monocytes were negatively associated with CD4 + T cell functionality and polyfunctionality scores.

Materials and methods

Study approval

The RV306 study was approved by ethical review boards at the Walter Reed Army Institute of Research, Thai Ministry of Public Health, Royal Thai Army Medical Department, Faculty of Tropical Medicine, Mahidol University, Chiang Mai University, and Chulalongkorn University Faculty of Medicine. All study participants provided informed consent. The investigators have adhered to the policies for protection of human participants as prescribed in AR 70-25.

Study design and participants

The RV306 clinical trial (ClinicalTrials.gov NCT01931358) was a double-blind, placebo-controlled, randomized clinical trial conducted in healthy Thai volunteers as previously described (5). Study participants included in this analysis received the RV144 vaccine regimen consisting of viral vector ALVAC-HIV prime at weeks 0 and 4 followed by ALVAC-HIV and AIDSVAX B/E protein boost at weeks 12 and 24 and received an additional boost of either ALVAC-HIV and AIDSVAX B/E (group 2) or AIDSVAX B/E alone (group 3) administered at week 48 (Supplementary Figure 1). Peripheral blood mononuclear cells (PBMC) collected from participants in the active arms of groups 2 (n=7) and 3 (n= 9) prior to and 3 days after the last boost were used to investigate innate immune cell activation.

Innate immune cell staining

Cryopreserved PBMC were thawed in 5ml of thawing medium containing benzonase nuclease in DMEM + 20% FBS. Following washes, cells were stained with LIVE/DEAD Fixable Blue Dead (Thermo Fisher, Cat L34957) in PBS for 30 min at room temperature. FcR blocking was conducted for 15 min at room temperature with 10% normal mouse IgG (Thermo Fisher, Cat OB2040-09) in staining buffer (PBS containing 0.1% NaN*3* and BSA). Cell surface staining was conducted using a cocktail of fluorescently labelled antibodies (BD Biosciences unless otherwise indicated): CD69 BB660 (Custom), CD158a/h/g (KIR2DL1/S1/S3/S5) PerCP-Cy5.5 (BioLegend, Cat 339514), CD158e1 (KIR2DL1) PerCP-Cy5.5 (BioLegend, Cat 312718), CXCR5 BB790-P (Cat 755631), Vb11 APC (Beckman, Cat A66905), CD161 R718 (Cat 751652), CD8 APC Cy7 (Cat 557760), CD56 BV421 (Cat 568219), HLA-DR BV480 (Cat 566113), AQUA L/D, Va7.2 BV711 (BioLegend, Cat 351732), CD33 BV786 (Cat 740974), CD19 BV786 (Cat 563325), CD57 BUV395 (Cat 567621), CD16 BUV496 (Cat 612944), TCRd2 BUV563 (Cat 748582), PD-1 BUV661 (Cat 750260), CD38 BUV737 (Cat 612824), CD4 BUV805 (Cat 612887), Va24 PE (Beckman, Cat IM2883), NKG2C PE-Dazzle594 (Miltenyi, Cat 130-123-047), CD3 PECy5.5 (Thermo Fisher, Cat 35-0036-42), TCRd1 PE Cy7 (Thermo Fisher, Cat 25-5679-42), CD80 FITC (BioLegend, Cat 305206), CD83 APC Cy7 (BioLegend, Cat 305330), CD86 BV605 (BioLegend, Cat 305430) in staining buffer with Brilliant Stain Buffer (Thermo Fisher, Cat 00-4409-42) for 15 min at room temperature. Intracellular staining was performed after fixation and permeabilization using FIX & PERM Cell Permeabilization Kit (Invitrogen, Cat GAS003) and a cocktail of fluorescent antibodies against intracellular proteins: FcRg FITC (1:80) (Millipore, Cat FCABS400F) and Ki67 BV750 (1:80) (Custom).

Flow cytometry

Flow cytometry data was collected on a FACSymphony A5 flow cytometer (Becton Dickenson, Franklin Lakes, NJ, USA) and analyzed using FlowJo version 10.8.1 software for Mac OS (Becton Dickenson, Franklin Lakes, NJ, USA). The gating strategy is shown in Supplementary Figure 2.

Statistical analysis and correlates

Statistical analysis was performed using GraphPad Prism version 9.4.1 for Mac OS. Paired comparisons within a group were performed using a Wilcoxon test. Comparisons between groups were performed using the Mann–Whitney test. The median and SD for all group comparisons can be found in the Supplemental Tables File. Env TH023 specific COMPASS functionality scores and polyfunctionality scores for CD4 T cells have been previously reported (5, 30). Antibody titers against gp120 A244 and Env TH023 specific neutralization were previously measured (5, 30, 31). A spearman test was used to calculate correlations between innate cell phenotypes and effector functions, functionality scores and antibody titers. P values below 0.05 were considered significant.

Random forest

A Random Forest algorithm ('randomForest()' function in R) was evaluated. We configured our settings to generate 500 trees. For

each node split, the algorithm considered a subset of predictors equal to the square root of the total number of available variables. The importance of each attribute was quantified using reductions in the Gini Impurity, facilitating a hierarchical feature ranking that underscores their discriminative power in distinguishing between pre- and post-vaccination states.

Results

Conventional T cells and unconventional T cells are activated 3 days post vaccination with ALVAC-HIV and AIDSVAX B/E

To elucidate the function of T cells following the administration of the ALVAC-HIV and AIDSVAX B/E vaccine regimen, we determined the frequency and phenotype of MAIT, iNKT, γδ T cells, and conventional T cells immediately prior to vaccine administration and 3 days post last vaccination in RV306 (Supplementary Figure 1) by flow cytometry (Supplementary Figure 2). There was no change in MAIT cell frequencies 3 days post vaccination (Supplementary Figure 3). We observed an increase in the levels of CD38 expressing MAIT cells 3 days post vaccination (p=0.0084) (Figure 1A and Supplementary Figure 5). The NK cell receptor, CD56, can be used to denote two distinct MAIT cell populations (32). MAIT cells expressing CD56 exhibit a greater ability to respond to IL-12 and IL-18 compared to CD56- MAIT cells (32). Our results show that the increase in CD38 positive MAIT cells occurred in both CD56+ MAIT cells (p=0.0088) and CD56- MAIT cells (p=0.0028) (Supplementary Figure 4B).

Similarly to MAIT cells, we observed an increase in CD38 positive iNKT cells 3 days post vaccination (p=0.0214) (Figure 1B and Supplementary Figure 5) and there was no change in their frequency (Supplementary Figure 3). Functionally, iNKT cells can be categorized into 3 subsets, CD4+ iNKT cells which produce both Th1 and Th2 cytokines, and CD8+ iNKT cells and CD4-CD8- (DN) iNKT cells which exhibit a Th1 response with a greater propensity for cytolytic activity (33). Our data shows an increase in CD8+ iNKT cells following vaccine administration (p=0.0114) (Supplementary Figure 4A). This increase in CD8+ iNKT cells is accompanied by a decrease in DN iNKT cells (0.0010) (Supplementary Figure 4A).

γδ T cell activation post vaccination was also increased, as measured by CD38 positive Vδ1 (p=0.0175) and Vδ2 T cells (p=0.0214), 3 days post vaccination with ALVAC-HIV and AIDSVAX B/E (Figures 1C, D and Supplementary Figure 5). There was no change in the frequency of Vδ1 and Vδ2 T cells 3 days following vaccination (Supplementary Figure 3).

We also observe an increase in frequency of CD4+ T cells (p=0.0126) and a decrease in CD8+ T cells (p=0.0182) 3 days post vaccination (Figure 1E). This was accompanied by an increase in CD38 expression in CD4+ T cells (p=0.0222) and CD8+ T cells (p=0.0182). There was also a trend for an increase in circulating CXCR5+ CD4 T cells post vaccination but it did not reach significance (data not shown). Overall, our results show activation of unconventional and conventional T cells, as measured by CD38 expression, 3 days post last vaccination in RV306.

Natural killer cell are activated by ALVAC-HIV and AIDSVAX B/E vaccine regimen

Next, we investigated the frequency and activation of NK cells following ALVAC-HIV and AIDVAX B/E vaccine administration within total NK cells and the $CD56^{hi}$ and $CD56^{dim}$ subsets (Figure 1F). Overall, there was no change in the frequency of NK cells 3 days post last vaccination (Supplementary Figure 3). We found an increase in the levels of CD38 (p=0.0131), CD69 (p=0.0032) (Figure 1F) and HLA-DR (p=0.0270) in total NK cells three days post vaccination (Figure 1F and Supplementary Figure 4C). Within the $CD56^{hi}$ NK cell compartment, we found an increase in CD69 (p=0.0027) and HLA-DR (p=0.0042) expression following vaccination (Figure 1E and Supplementary Figure 4C). Finally, we observed an increase in levels of CD38 (p=0.0131) and CD69 (p=0.0092) on $CD56^{dim}$ NK cells following vaccination (Figure 1F). Natural killer cells co-expressing NKG2C and CD57 have an adaptive memory-like phenotype (34). Functionally, these adaptive memory-like NK cells have decreased cytolytic capacity and response to cytokines (IL-2 and IL-18) produced by innate cells and increased CD16-mediated antibody-dependent cellular cytotoxicity (ADCC) (34). Our study shows that this NK cell compartment is also activated following vaccination (CD38; p=0.0035) (Supplementary Figure 4C). These results suggest that several subsets of NK cells were activated in response to vaccination in RV306.

Increase in CD16+ monocytes following vaccination with ALVAC-HIV and AIDSVAX B/E

Monocytes play a key role in the protection conferred through vaccination by RV144 based vaccines (11, 14, 35). We therefore measured the frequency of monocyte subsets post-vaccination with ALVAC-HIV and AIDSVAX B/E (Figure 1G). We found a decrease in classical CD14+CD16- monocytes 3 days following vaccination (p=0.0013), this was accompanied by an increase in intermediate CD14+CD16+ monocytes (p=0.0021) and non-classical CD14-CD16+ monocytes (p=0.0443) (Figure 1G). Next, we evaluated expression of costimulatory molecules, CD80, CD83 and CD86 on total monocytes and found no change in expression of any of the markers (data not shown).

Conventional T cell, MAIT, iNKT, and γδ T cell activation three days post-vaccination does not require ALVAC-HIV

In RV306, the last vaccination consisted of both ALVAC-HIV and AIDSVAX B/E for group 2 while group 3 received AIDSVAX B/E alone (5). Alum was used as the adjuvant in both groups. To investigate any group specific effects of the vaccine on innate cell activation, we evaluated activation in each study group (Figure 2). The increase in CD38 expression by MAIT (p=0.0391), iNKT (p=0.0078), γδ T cells (Vδ1, p=0.0703; Vδ2, p=0.0195), CD4 T

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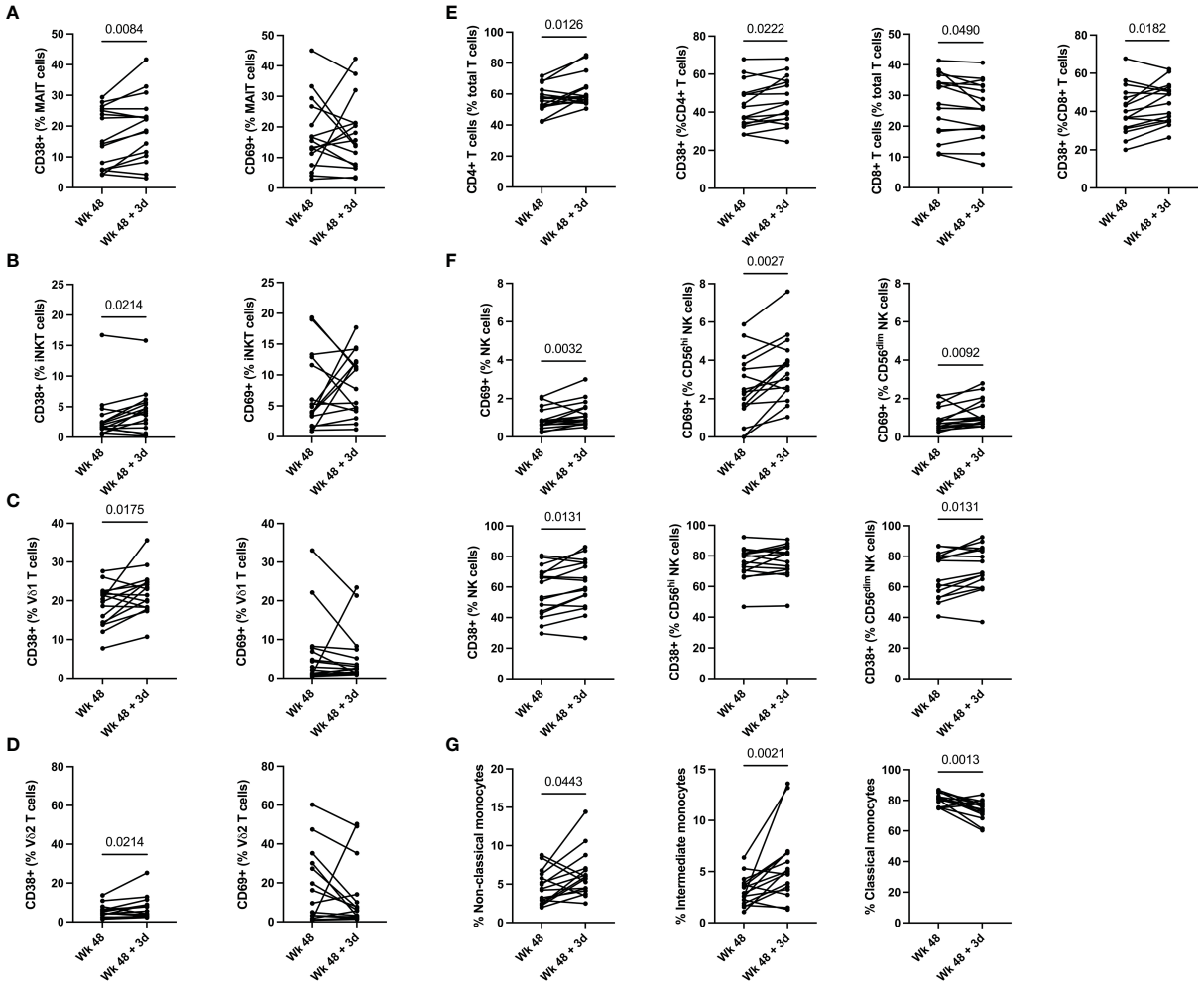

FIGURE 1
Innate and T cell activation 3 days post vaccination. **(A)** MAIT, **(B)** iNKT, **(C)** Vδ1 T, **(D)** Vδ2 T cell, **(E)** conventional T cells and **(F)** NK cell activation pre- and post-vaccination. **(G)** Changes in monocyte frequency pre- and post-vaccination. Classical monocytes are defined as CD14+CD16-, intermediate monocytes as CD14+CD16+ and non-Classical monocytes are defined as CD14-CD16+. Groups 2 (ALVAC-HIV + AIDSVAX B/E) and 3 (AIDSVAX B/E) are combined for analysis (n=16).

cells (p=0.0352), CD8 T cells (0.0391) and CD69 levels on NK cells (p=0.0039) was significant in the AIDSVAX B/E only group. Interestingly, increased expression of CD38 by NK cells was significant in the ALVAC-HIV and AIDSVAX B/E group (p=0.0312) and not in the AIDSVAX B/E only. However, the number of study participants in each group was low and a pattern of increased activation was present in both groups.

Activation of unconventional T cells and NK cells 3 days post vaccination with ALVAC-HIV and AIDSVAX B/E is associated with humoral immune responses

Finally, we looked at associations between immune cell activation and adaptive immune responses measured 14 days post last vaccination previously reported (5) (Figure 3). NK cell activation 3 days after vaccine administration was associated with antibody titers of HIV gp120-specific total IgG (r=0.517; p=0.060), IgG1 (r=0.649; p=0.015) and IgG3 (r=0.548; p=0.045). Vδ1 T cell activation 3 days after vaccine administration associated with HIV gp120-specific IgG3 titers (r=0.604; p=0.024). The frequency of CD14-CD16+ monocytes was inversely associated with HIV Env specific CD4+ T cell functionality score and polyfunctionality score (r=-0.543; p=0.048). Finally, we also observed trending associations between MAIT cell activation and gp120-specific IgG3 titers (r=0.475; p=0.088), between NK cell activation and TH023 pseudovirus neutralization titers (r=0.486; p=0.088), and between CD8+ iNKT and TH023 pseudovirus neutralization titers (r=0.503; p=0.069), HIV Env specific CD4+ T cell functionality score (r=-503; p=0.069) and polyfunctionality score (r=-503; p=0.069). We also observed an association between CD8+ T cell frequency and HIV Env specific CD4+ T cell functionality score and polyfunctionality score (r=0.5598; p=0.040). Overall, this suggests that unconventional T cell and NK cell activation induced by vaccination might have contributed to the development of humoral immune responses in RV306. Lastly, using random forest analysis, we identified classical monocytes as the variable

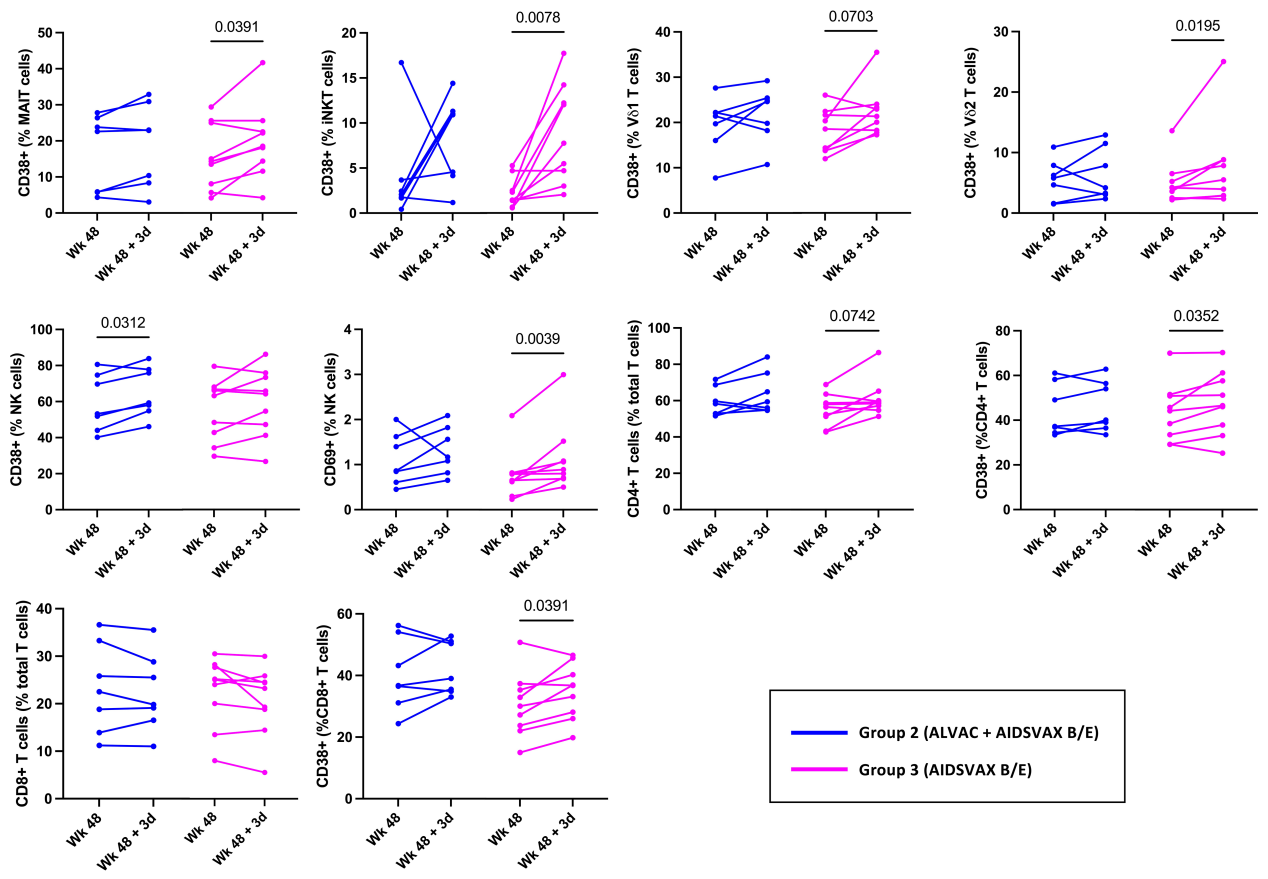

FIGURE 2
Innate and T cell activation in RV306 study participants that received ALVAC-HIV and AIDSVAX B/E or AIDSVAX B/E alone. Activation of MAIT, iNKT, NK, γδ T cells, and conventional T cell was analyzed in 7 participants from group 2 (ALVAC-HIV + AIDSVAX B/E) and 9 participants from group 3 (AIDSVAX B/E).

having the higest ability to discriminate between samples prior to and 3 days post the last boost (Supplementary Figure 6).

Discussion

In this study, we investigated T cell and innate cells' involvement in sensing ALVAC-HIV and AIDVAX B/E and determined associations between our findings and previously identified vaccine induced humoral and cellular responses. Using samples from RV306 which tested delayed boosting following the RV144 regimen, we report increased NK cell, MAIT cell, iNKT, γδ T, CD4+ T cell and CD8+ T cell activation 3 days following late-stage boost vaccination. We also report an increase in CD14+CD16 + monocytes following late-stage boost vaccination. Inclusion of ALVAC-HIV (group 2) was not required for innate and conventional T cell activation 3 days post vaccination. It is possible that any effect conferred by ALVAC-HIV on innate cell activation is no longer detectable after 3 days. Our analysis included additional markers such as PD-1, HLA-DR and CXCR5 in T cells and CD80, CD83 and CD86 costimulatory molecules for monocytes, however we did not see any significant changes in the expression of these markers. Although there was trending increase in circulating CXCR5+ CD4 T cells, there was no change in their expression of activation markers.

Previous work using samples at days 1, 3 and 7 post first vaccination from a phase 1b trial using the RV144 regimen in HIV-1 uninfected South Africans (HVTN097) found that transcriptional responses peaked 1 day post vaccination (6). Type I and II interferon signaling and innate pathways critical for adaptive immune priming were activated. Furthermore, this study reported that innate signatures at day 1 were positively associated with antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP) at 6.5 months (2 weeks post final vaccination). Conversely, the study found that day 3 and day 7 innate immune signatures were inversely associated with Env-specific CD4+ T cell responses at 6.5 months and 12 months (6 months post last vaccination) indicating that a quick resolution of this signature was associated with higher Env-specific CD4+ T cell responses. Similar to our findings, increased CD14+CD16+ monocytes following ALVAC-HIV administration was observed in the HVTN097 study (6).

Interestingly, NK, MAIT and Vδ1 T cell activation 3 days post vaccination was associated with the magnitude of gp120 specific IgG1 and IgG3 antibody responses and the increase in CD14+CD16 + monocytes was negatively associated with HIV-specific CD4+ T

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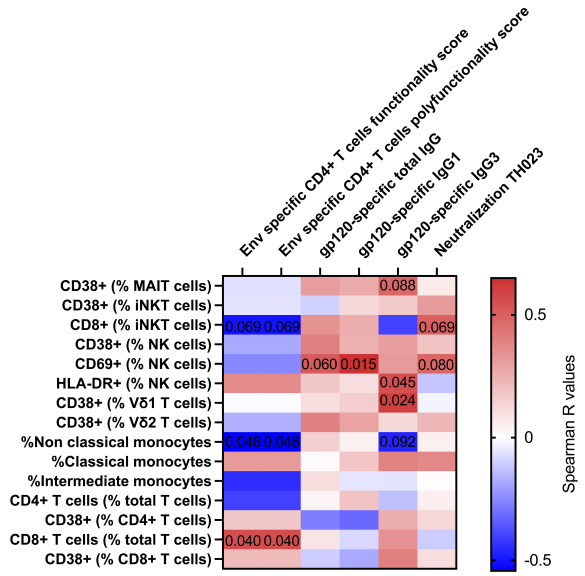

FIGURE 3
Correlation between innate and T cell activation with adaptive immune responses 14 days post-vaccination. Heat map showing the Spearman R values for associations between immune activation at day 3 (Y axis) and adaptive immune responses at day 14 post vaccination (X axis). Significant and trending p-values are denoted on the heatmap. Classical monocytes are defined as CD14+CD16-, intermediate monocytes as CD14+CD16+ and non-Classical monocytes are defined as CD14-CD16+. All participants from group 2 and 3 were included in the heatmap analysis (n=16).

cell functionality and polyfunctionality scores. Production of gp120 specific IgG3 has been associated with a lower risk of acquisition in RV144 (3) and COMPASS CD4 T cell functionality and polyfunctionality correlated inversely with HIV acquisition (4). MAIT cells have been shown to provide B cell help (15, 16) and promote the activation of dendritic cells (17, 18). In addition, cytokines, such as IFNγ, produced by NK cells may promote B cell activation and enhance antibody production (36, 37). γδ T cells are capable of regulating adaptive immune responses (8, 26, 28). Although our study identifies trending correlations between CD8+ iNKT subset and humoral responses, previous studies by Liu et al. (38) showed little functional differences between the CD4- CD8- and CD8+ iNKT cell subsets. In addition, it is unclear whether the changes observed in the iNKT cell subsets are permanent changes or temporal. Additional longitudinal analysis is required to determine this. More work is also needed to understand how NK cell and unconventional T cells activation following vaccination could promote humoral responses. While we also observed conventional CD4 and CD8 T cells activation 3 days post vaccination, it was not associated with the magnitude of humoral immune responses, suggesting a different role for conventional and unconventional T cells.

The mechanism responsible for the innate cell activation that we reported here remains to be investigated. Interestingly, activation of both MAIT and Vδ2 T cells following vaccination with the ChAdOx01 viral vector was dependent on IL-18, TNF, and type I IFN (19, 39). It is possible that a similar pathway was activated following vaccination with ALVAC-HIV and AIDSVAX B/E. One limitation of our study is that there were no samples collected 1 day post vaccination, when innate immune responses are expected to peak. Our results show that 3 days post vaccination, CD69+ NK cells correlated with humoral responses, this was not the case for CD38+ NK cells. This may be due to differing dynamics between CD38 and CD69. As such, it is possible that CD38+ NK cells would also be associated with humoral responses if sampling had occurred at a different time point. The number of study participants was also small when dividing between those who received ALVAC-HIV and AIDSVAX B/E and those who received AIDSVAX B/E alone. Contrasting the innate immune response induced by different adjuvants would be of great interest to better understand how to fine tune adaptive immune responses. Lastly, an additional limitation to this study is that our findings are only correlative. Further investigation is still needed to corroborate the effect of innate cells in modulating adaptive immune responses to ALVAC-HIV and AIDSVAX B/E.

This study suggests that unconventional T cells and NK cell's sensing of vaccine components could contribute to vaccine induced humoral responses. A better understanding of how different boost strategies modulate innate immune responses to adjuvants used in vaccines and modulate the elicitation of differing antibody subtypes could help improve HIV vaccine designs.

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 RV306 study was approved by ethical review boards at the Walter Reed Army Institute of Research, Thai Ministry of Public Health, Royal Thai Army Medical Department, Faculty of Tropical Medicine, Mahidol University, Chiang Mai University, and Chulalongkorn University Faculty of Medicine. 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

KN: Formal Analysis, Investigation, Methodology, Writing – original draft. KM: Investigation, Methodology, Writing – original draft. IS: Investigation, Methodology, Writing – review & editing. MC: Methodology, Resources, Writing – review & editing. LW: Methodology, Resources, Writing – review & editing. DK: Writing – review & editing, Formal analysis. SA: Methodology, Resources, Writing – review & editing. VP: Methodology, Resources, Writing – review & editing. PP: Methodology, Resources, Writing – review & editing. SN: Project administration, Resources, Writing – review & editing. SG: Resources, Writing – review & editing. FS: Resources, Writing – review & editing. SC: Project administration, Resources,

Writing – review & editing. JA: Project administration, Resources, Writing – review & editing. RO: Project administration, Resources, Writing – review & editing. SV: Project administration, Resources, Writing – review & editing. DP: Conceptualization, Supervision, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the US Army Medical Research and Development Command (Military Infectious Diseases Research Program; cooperative agreement W81XWH-18-2-0040 with the Henry M Jackson Foundation for the Advancement of Military Medicine), the US Army Medical Materiel Development Activity, and the National Institute of Allergy and Infectious Diseases (interagency agreement Y1-AI-2642-12 with the US Army Medical Research and Materiel Command).

Acknowledgments

We are deeply grateful to the RV306 participants for their participation and dedication to this study, as well as our Community Advisory Boards for their helpful input.

Conflict of interest

SG is an employee and shareholder of Sanofi Pasteur. FS is an employee of Global Solutions for Infectious Diseases.

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.

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Author disclaimer

Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author and should not be construed as official, or as reflecting true views of the Department of the Army, the Department of Defense, or the Henry M. Jackson Foundation for the Advancement of Military Medicine. The investigators have adhered to the policies for protection of human subjects as prescribed in AR 70-25.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1339727/full#supplementary-material

References

1. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N Engl J Med* (2009) 361(23):2209–20. doi: 10.1056/NEJMoa0908492

2. Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N Engl J Med* (2012) 366(14):1275–86. doi: 10.1056/NEJMoa1113425

3. Yates NL, Liao HX, Fong Y, deCamp A, Vandergrift NA, Williams WT, et al. Vaccine-induced Env V1-V2 IgG3 correlates with lower HIV-1 infection risk and declines soon after vaccination. *Sci Transl Med* (2014) 6(228):228ra39. doi: 10.1126/scitranslmed.3007730

4. Lin L, Finak G, Ushey K, Seshadri C, Hawn TR, Frahm N, et al. COMPASS identifies T-cell subsets correlated with clinical outcomes. *Nat Biotechnol* (2015) 33 (6):610–6. doi: 10.1038/nbt.3187

5. Pitisuttithum P, Nitayaphan S, Chariyalertsak S, Kaewkungwal J, Dawson P, Dhitavat J, et al. Late boosting of the RV144 regimen with AIDSVAX B/E and ALVAC-HIV in HIV-uninfected Thai volunteers: a double-blind, randomised controlled trial. *Lancet HIV* (2020) 7(4):e238–e48. doi: 10.1016/S2352-3018(19)30406-0

6. Andersen-Nissen E, Fiore-Gartland A, Ballweber Fleming L, Carpp LN, Naidoo AF, Harper MS, et al. Innate immune signatures to a partially-efficacious HIV vaccine predict correlates of HIV-1 infection risk. *PLoS Pathog* (2021) 17(3):e1009363. doi: 10.1371/journal.ppat.1009363

7. Downey AM, Kaplonek P, Seeberger PH. MAIT cells as attractive vaccine targets. *FEBS Lett* (2019) 593(13):1627–40. doi: 10.1002/1873-3468.13488

8. Born WK, Huang Y, Reinhardt RL, Huang H, Sun D, O'Brien RL. γδ T cells and B cells. *Adv Immunol* (2017) 134:1–45. doi: 10.1016/bs.ai.2017.01.002

9. Boulouis C, Kammann T, Cuapio A, Parrot T, Gao Y, Mouchtaridi E, et al. MAIT cell compartment characteristics are associated with the immune response magnitude to the BNT162b2 mRNA anti-SARS-CoV-2 vaccine. *Mol Med* (2022) 28(1):54. doi: 10.1186/s10020-022-00484-7

10. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. *Science* (2011) 331(6013):44–9. doi: 10.1126/science.1198687

11. Shangguan S, Ehrenberg PK, Geretz A, Yum L, Kundu G, May K, et al. Monocyte-derived transcriptome signature indicates antibody-dependent cellular phagocytosis as a potential mechanism of vaccine-induced protection against HIV-1. *Elife* (2021) 10. doi: 10.7554/eLife.69577

12. Poli A, Michel T, Thérésine M, Andrès E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology* (2009) 126(4):458–65. doi: 10.1111/j.1365-2567.2008.03027.x

13. Ryan EJ, Harenberg A, Burdin N. The Canarypox-virus vaccine vector ALVAC triggers the release of IFN-gamma by Natural Killer (NK) cells enhancing Th1 polarization. *Vaccine* (2007) 25(17):3380–90. doi: 10.1016/j.vaccine.2006.12.048

14. Gorini G, Fourati S, Vaccari M, Rahman MA, Gordon SN, Brown DR, et al. Engagement of monocytes, NK cells, and CD4+ Th1 cells by ALVAC-SIV vaccination results in a decreased risk of SIVmac251 vaginal acquisition. *PLoS Pathog* (2020) 16(3): e1008377. doi: 10.1371/journal.ppat.1008377

15. Bennett MS, Trivedi S, Iyer AS, Hale JS, Leung DT. Human mucosal-associated invariant T (MAIT) cells possess capacity for B cell help. *J Leukoc Biol* (2017) 102 (5):1261–9. doi: 10.1189/jlb.4A0317-116R

16. Jensen O, Trivedi S, Meier JD, Fairfax KC, Hale JS, Leung DT. A subset of follicular helper-like MAIT cells can provide B cell help and support antibody production in the mucosa. *Sci Immunol* (2022) 7(67):eabe8931. doi: 10.1126/sciimmunol.abe8931

17. Salio M, Gasser O, Gonzalez-Lopez C, Martens A, Veerapen N, Gileadi U, et al. Activation of human mucosal-associated invariant T cells induces CD40L-dependent maturation of monocyte-derived and primary dendritic cells. *J Immunol* (2017) 199 (8):2631–8. doi: 10.4049/jimmunol.1700615

18. Pankhurst TE, Buick KH, Lange JL, Marshall AJ, Button KR, Palmer OR, et al. MAIT cells activate dendritic cells to promote T(FH) cell differentiation and induce humoral immunity. *Cell Rep* (2023) 42(4):112310. doi: 10.1016/j.celrep.2023.112310

19. Provine NM, Amini A, Garner LC, Spencer AJ, Dold C, Hutchings C, et al. MAIT cell activation augments adenovirus vector vaccine immunogenicity. *Science* (2021) 371(6528):521–6. doi: 10.1126/science.aax8819

20. Marzano P, Balin S, Terzoli S, Della Bella S, Cazzetta V, Piazza R, et al. Transcriptomic profile of TNF(high) MAIT cells is linked to B cell response following SARS-CoV-2 vaccination. *Front Immunol* (2023) 14:1208662. doi: 10.3389/fimmu.2023.1208662

21. Hackstein CP, Klenerman P. Emerging features of MAIT cells and other unconventional T cell populations in human viral disease and vaccination. *Semin Immunol* (2022) 61-64:101661. doi: 10.1016/j.smim.2022.101661

22. Li X, Fujio M, Imamura M, Wu D, Vasan S, Wong CH, et al. Design of a potent CD1d-binding NKT cell ligand as a vaccine adjuvant. *Proc Natl Acad Sci U S A* (2010) 107(29):13010–5. doi: 10.1073/pnas.1006662107

23. Cerundolo V, Silk JD, Masri SH, Salio M. Harnessing invariant NKT cells in vaccination strategies. *Nat Rev Immunol* (2009) 9(1):28–38. doi: 10.1038/nri2451

24. Gonzalez-Aseguinolaza G, Van Kaer L, Bergmann CC, Wilson JM, Schmieg J, Kronenberg M, et al. Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines. *J Exp Med* (2002) 195(5):617–24. doi: 10.1084/jem.20011889

25. Padte NN, Boente-Carrera M, Andrews CD, McManus J, Grasperge BF, Gettie A, et al. A glycolipid adjuvant, 7DW8-5, enhances CD8+ T cell responses induced by an adenovirus-vectored malaria vaccine in non-human primates. *PloS One* (2013) 8(10): e78407. doi: 10.1371/journal.pone.0078407

26. de Sousa TR, Victor JR. Natural self-ligand gamma delta T cell receptors (γδTCRs) insight: the potential of induced IgG. *Vaccines (Basel)* (2020) 8(3). doi: 10.3390/vaccines8030436

27. Gao Y, Yang W, Pan M, Scully E, Girardi M, Augenlicht LH, et al. Gamma delta T cells provide an early source of interferon gamma in tumor immunity. *J Exp Med* (2003) 198(3):433–42. doi: 10.1084/jem.20030584

28. Chen Q, Wen K, Lv A, Liu M, Ni K, Xiang Z, et al. Human Vgamma9Vdelta2-T cells synergize CD4(+) T follicular helper cells to produce influenza virus-specific antibody. *Front Immunol* (2018) 9:599. doi: 10.3389/fimmu.2018.00599

29. Caccamo N, Battistini L, Bonneville M, Poccia F, Fournie JJ, Meraviglia S, et al. CXCR5 identifies a subset of Vgamma9Vdelta2 T cells which secrete IL-4 and IL-10 and help B cells for antibody production. *J Immunol* (2006) 177(8):5290–5. doi: 10.4049/jimmunol.177.8.5290

30. Costanzo MC, Paquin-Proulx D, Schuetz A, Akapirat S, Shubin Z, Kim D, et al. ALVAC-HIV and AIDSVAX B/E vaccination induce improved immune responses compared with AIDSVAX B/E vaccination alone. *JCI Insight* (2023) 8(9). doi: 10.1172/jci.insight.167664

31. Shubin Z, Stanfield-Oakley S, Puangkaew J, Pitisutthithum P, Nitayaphan S, Gurunathan S, et al. Additional boosting to the RV144 vaccine regimen increased Fc-mediated effector function magnitude but not durability. *AIDS* (2023) 37(10):1519–24. doi: 10.1097/QAD.0000000000003611

32. Dias J, Leeansyah E, Sandberg JK. Multiple layers of heterogeneity and subset diversity in human MAIT cell responses to distinct microorganisms and to innate cytokines. *Proc Natl Acad Sci U S A* (2017) 114(27):E5434–e43. doi: 10.1073/pnas.1705759114

33. Krovi SH, Gapin L. Invariant natural killer T cell subsets-more than just developmental intermediates. *Front Immunol* (2018) 9:1393. doi: 10.3389/fimmu.2018.01393

34. Kobyzeva PA, Streltsova MA, Erokhina SA, Kanevskiy LM, Telford WG, Sapozhnikov AM, et al. CD56(dim) CD57(-) NKG2C(+) NK cells retaining proliferative potential are possible precursors of CD57(+) NKG2C(+) memory-like NK cells. *J Leukoc Biol* (2020) 108(4):1379–95. doi: 10.1002/JLB.1MA0720-654RR

35. Vaccari M, Fourati S, Gordon SN, Brown DR, Bissa M, Schifanella L, et al. HIV vaccine candidate activation of hypoxia and the inflammasome in CD14(+) monocytes is associated with a decreased risk of SIV(mac251) acquisition. *Nat Med* (2018) 24 (6):847–56. doi: 10.1038/s41591-018-0025-7

36. Jennings P, Yuan D. NK cell enhancement of antigen presentation by B lymphocytes. *J Immunol* (2009) 182(5):2879–87. doi: 10.4049/jimmunol.0803220

37. Snapper CM, Yamaguchi H, Moorman MA, Sneed R, Smoot D, Mond JJ. Natural killer cells induce activated murine B cells to secrete Ig. *J Immunol* (1993) 151 (10):5251–60. doi: 10.4049/jimmunol.151.10.5251

38. Liu J, Hill BJ, Darko S, Song K, Quigley MF, Asher TE, et al. The peripheral differentiation of human natural killer T cells. *Immunol Cell Biol* (2019) 97(6):586–96. doi: 10.1111/imcb.12248

39. Provine NM, Amini A, Garner LC, FitzPatrick MEB, Dold C, Reyes LS, et al. Adenovirus vectors activate Vδ2+ γδT cells in a type I interferon-, TNF-, and IL-18-dependent manner. *Eur J Immunol* (2022) 52(5):835–7. doi: 10.1002/eji.202149367

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 15 February 2024
DOI 10.3389/fimmu.2024.1365843

OPEN ACCESS

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RECEIVED 05 January 2024
ACCEPTED 30 January 2024
PUBLISHED 15 February 2024

CITATION
Satoh M and Iwabuchi K (2024) Contribution of NKT cells and CD1d-expressing cells in obesity-associated adipose tissue inflammation. *Front. Immunol.* 15:1365843. doi: 10.3389/fimmu.2024.1365843

Contribution of NKT cells and CD1d-expressing cells in obesity-associated adipose tissue inflammation

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Natural killer T (NKT) cell are members of the innate-like T lymphocytes and recognizes lipid antigens presented by CD1d-expressing cells. Obesity-associated inflammation in adipose tissue (AT) leads to metabolic dysfunction, including insulin resistance. When cellular communication is properly regulated among AT-residing immune cells and adipocytes during inflammation, a favorable balance of Th1 and Th2 immune responses is achieved. NKT cells play crucial roles in AT inflammation, influencing the development of diet-induced obesity and insulin resistance. NKT cells interact with CD1d-expressing cells in AT, such as adipocytes, macrophages, and dendritic cells, shaping pro-inflammatory or anti-inflammatory microenvironments with distinct characteristics depending on the antigen-presenting cells. Additionally, CD1d may be involved in the inflammatory process independently of NKT cells. In this mini-review, we provide a brief overview of the current understanding of the interaction between immune cells, focusing on NKT cells and CD1d signaling, which control AT inflammation both in the presence and absence of NKT cells. We aim to enhance our understanding of the mechanisms of obesity-associated diseases.

KEYWORDS

NKT cells, CD1d, macrophages, obesity, insulin resistance, adipose tissue inflammation

Introduction

Inflammation/immune response control obesity in the adipose tissue (AT) of the body (1, 2). Obesity is a low-grade chronic inflammatory disease that contributes to metabolic dysfunction and insulin resistance (3, 4). Initially, hypertrophied adipocytes secrete inflammatory cytokines and chemokines, thereby recruiting immune cells that promote AT inflammation, including macrophages (Mϕs), T cells, B cells, and neutrophils (5–11). While various immune cells contribute to AT homeostasis and inflammation, natural killer T (NKT) cells play a crucial role in developing obesity and insulin resistance (12). NKT cells are a unique subset of T cells that recognize lipid antigens on MHC class I-like CD1d

molecules (13–15). NKT cells are further classified into two subsets depending on their T cell receptor (TCR) expression: type I NKT (invariant NKT; iNKT) cells that harbor an invariant TCR α-chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) and type II NKT (variant NKT; vNKT) cells that express diverse TCRs. iNKT cells recognize a prototypical ligand, α-galactosylceramide (α-GalCer), whereas vNKT cells recognize various lipid antigens, including sulfatide (16–18). NKT cells secrete various cytokines/chemokines/cytocidal molecules, activating and recruiting immune cells to eliminate target cells (19). Furthermore, iNKT cells can be categorized based on the expression of transcription factors such as T-bet for NKT1 cells, GATA3 and PLZF for NKT2 cells, and RORγt for NKT17 cells (20, 21), which may contribute to the development of inflammatory diseases, including obesity (22–24).

The contribution of CD1d itself to cellular signaling, aside from its role in ligand presentation to NKT cells in inflammatory and immune responses, should be considered. Recent reports have demonstrated that CD1d can modulate critical responses through its cytoplasmic portion (25–30). In this review, we discuss how NKT cells contribute to inflammation through interaction with CD1d-expressing cells in AT and how the intrinsic function of CD1d itself may influence the response in an NKT cell-independent manner.

Roles of NKT cells in diet-induced obesity using global CD1d knockout mice

Numerous studies have revealed that AT-resident and infiltrating immune cells control obesity-associated AT inflammation in a DIO model by feeding mice a high-fat diet (HFD; 60% fat kcal). T helper type 1 (Th1)-immune responses formed by M1-Mϕs, $CD8^+$ T cells, NK cells, and ILC1s exacerbate AT inflammation and insulin resistance (5, 7, 31–34). In contrast, Th2-immune responses induced by M2-Mϕs, regulatory T cells, eosinophils, and ILC2s improve insulin sensitivity (8, 35, 36). Additionally, the ILC2s-eosinophils-M2-Mϕs circuit plays a central role in differentiating beige fat from white fat (beiging), where beige fat expresses thermogenic genes to defend against cold and obesity (37–39). Thus, in the steady state, a lean Th2-immune environment is maintained by the AT-resident cells.

NKT cells appear to play dual roles in the development of obesity. Reports have shown that both iNKT and vNKT cells promote AT inflammation and insulin resistance (40–43), whereas other studies have indicated that iNKT cells play either protective or neutral roles against obesity in DIO experiments using CD1d KO and Jα18 KO mice (44–49) (Table 1). Jα18 KO mice exhibit a significant reduction in T-cell receptor (TCR) diversity (52), thus mucosal-associated invariant T (MAIT) cells which utilize Jα33 are lost (53). Considering that MAIT cells also impact the development of obesity (54–57), Traj18 KO mice (distinct from Jα18 KO mice here) generated by depleting only the Traj18 locus, essentially represent iNKT cell KO mice (42). AT-resident iNKT cells express E4BP4 but not PLZF, reflecting their anti-inflammatory phenotype, and IL-10-producing NKT cells (NKT10) are enriched in subcutaneous white AT (58, 59). Moreover, when the F108Y substitution was artificially induced in TCRβ (Vβ8.2), thereby reducing the interaction of mutated iTCR with CD1d by a partial disruption of the hydrophobic patch formation with TCR α and β chain pairing, it altered iNKT cell development to an E4BP4-expressing, AT-resident-like phenotype (60). Thus, AT-resident iNKT cells may differentiate and exhibit unique functions compared to iNKT cells located in other tissues (61).

NKT cell-deficient mice do not show any pathogenic phenotype in comparison with WT mice, either in an obese or lean state under normal dietary conditions. However, when they are fed an HFD, NKT cells function as either pro-obese or pro-lean (40, 41, 44, 45, 49). This implies that NKT cells in HFD-fed mice are activated by unknown endogenous ligands presented by $CD1d^+$ APCs, including flora-derived ligands (62–64), presumably even during obesity. Schipper et al. showed that CD1d KO mice exhibited adipocyte dysfunction and insulin resistance even under steady-state conditions (46), suggesting that NKT cells function in both obesity and a lean state. Alternatively, the dysfunction may be primarily attributed to the deficiency of CD1d molecules in adipocytes, as discussed later. Moreover, the phenotype of CD1d KO and Jα18 KO mice fed an HFD varied among laboratories (12), ranging from lean to obese, including a neutral state, compared to WT mice. Although contradictory results have been reported, presumably due to differences in the lipid composition of the HFD and intestinal microbiota for different murine strains, the critical reason for the discrepancy remains elusive. Selvanantham et al. have reported that CD1d KO mice exhibit altered gut microbiome profiles, characterized by increase in segmented filamentous bacteria and decrease of *Akkermansia*, which exacerbate intestinal inflammation (65). *Bacteroides fragilis* produces the glycosphingolipid α-$GalCer_{Bf}$, which is structurally related to the prototypic ligand α-GalCer or KRN7000 (62), exhibiting regulatory activity by iNKT cells. This indicates that some bacteria in the intestine are involved in the functional modulation of NKT cells, however, whether it indeed affects the function of NKT cells during obesity has not been examined. Meanwhile, the composition and fatty acid concentration in sera, altered and elevated in obese subjects, are determined based on endogenous synthesis rates and dietary fat characteristics (66, 67). Although the ligand for NKT cells in adipocytes remains elusive, it appears possible that dietary-derived lipid components are involved in their ligand production. At least, alteration in serum fatty acid composition affects Mϕs, via TLR4 and other receptors, which may modulate NKT cell activation in an indirect fashion (24). Additionally, single-cell analysis have unveiled distinct subsets of AT-resident iNKT cells, including AT-iNKT10 (induction of Tregs and M2-Mϕs), AT-iNKT1 (killing pathogenic Mϕs via NK cells and clearance of dead adipocytes via Mϕs) and AT-iNKT17 (via induction of adipose stem cell proliferation by amphiregulin) (68, 69). Of significance is the inquiry into how AT-iNKT cells are generated and activated, representing a likely major contribution of the adipose tissue microenvironment. Additionally, from the perspective of the CD1d molecule itself, the activation of the CD1d is differentially regulated by endogenous/exogenous lipid ligands which are biosynthesized during obesity, or through the

TABLE 1 Role of NKT cells in a murine DIO model.

Strain	KO/Tg mice, NKT cell agonist	Diet (fat source)	Compared to control		NKT cell responses in DIO	Ref
			Body weight	Insulin resistance		
C57BL/6	CD1d KO	HFD (soybean oil, lard)	↓	improved		(41)
C57BL/6	Jα18 KO	HFD (soybean oil, lard)	↓	improved		
C57BL/6	αGC administration	HFD (soybean oil, lard)	→	worsened	produce TNF-α, IFN-γ	
C57BL/6	CD1d KO	HFD (safflower oil, beef tallow)	↓	improved		(40)
C57BL/6	Jα18 KO	HFD (safflower oil, beef tallow)	→	not changed		
C57BL/6	αGC administration	HFD (safflower oil, beef tallow)	→	worsened		
C57BL/6	CD1d KO	HFD (soybean oil, lard)	↓	no data		(42)
C57BL/6	Traj18 KO	HFD (soybean oil, lard)	↓	improved		
C57BL/6	Vα14Tg/Ldlr KO	high fat, high sucrose, 0.15% cholesterol	↑	worsened		(43)
Balb/c	CD1d KO	HFD (soybean oil, lard)	→	not changed		(48)
C57BL/6	CD1d KO	HFD (soybean oil, lard)	→	worsened		(49)
C57BL/6	Jα18 KO	HFD (soybean oil, lard)	→	not changed		
C57BL/6	CD1d KO	HFD (soybean oil, lard)	↑	worsened	produce IL-10	(44)
C57BL/6	Jα18 KO	HFD (soybean oil, lard)	↑	worsened		
C57BL/6	αGC administration	HFD (soybean oil, lard)	↓	improved		
C57BL/6	CD1d KO	HFD (soybean oil, lard)	no data	not changed		(45)
C57BL/6	αGC administration	HFD (soybean oil, lard)	no data	improved	IL-4/STAT6 dependent	
C57BL/6	CD1d KO	both LFD and HFD (soybean oil, lard)	→	worsened		(46)
C57BL/6	Jα18 KO	LFD	no data	worsened		
C57BL/6	αGC administration	LFD	no data	not changed	produce IL-4, IL-13	
C57BL/6	αGC administration	HFD (soybean oil, lard)	↓	improved	produce IL-13	(47)
C57BL/6	sulfatide	HFD (soybean oil, lard)	↓	improved		
C57BL/6	Adipoq-cre-Cd1d1$^{f/f}$	HFD (safflower oil, beef tallow)	↓	improved	produce IFN-γ	(50)
C57BL/6	Adipoq-cre-Cd1d1$^{f/f}$	HFD (soybean oil, lard)	→	worsened	produce IL-4	(51)
C57BL/6	LysM-cre-Cd1d1$^{f/f}$	HFD (safflower oil, beef tallow)	→	worsened	produce IFN-γ	(24)
C57BL/6	CD11c-cre-Cd1d1$^{f/f}$	HFD (safflower oil, beef tallow)	→	improved		(24)

modulation of the CD1d signaling cascade, as indicated by reports suggesting that endogenous ligands switch on CD1d activation (28, 29). Hence, further investigation is necessary to reconcile this contradiction and establish a therapeutic strategy for obesity-associated diseases.

NKT cell-adipocyte interactions in AT using tissue-specific CD1d KO mice

NKT cells are activated in AT by interacting with CD1d-expressing cells, including Mϕs, dendritic cells, adipocytes, and leukocytes, such as eosinophils. AT functions as an endocrine organ, as adipocytes secrete adipokines and store triglycerides for energy (70). Several studies have demonstrated that adipocytes activate both T and NKT cells through antigen presentation (50, 51, 71–73). CD1d expressed on the surface of adipocytes can induce iNKT cell activation depending on the expression of microsomal triglyceride transfer protein (MTP) and CCAAT/enhancer-binding protein (C/EBP)-β and -δ, even in the absence of exogenous ligands, suggesting that adipocytes express endogenous ligands recognized by NKT cells (71). The inhibition of UDP-glucose ceramide glucosyltransferase (UGCG), the first rate-limiting step in the glucosylceramide biosynthesis pathway, resulted in decreased iNKT cell activity (74). This finding suggests that the amount of glucosylceramide influences the biosynthetic pathway of lipid self-

antigen presentation by adipocytes in iNKT cells. Additionally, during their interaction with adipocytes within a lipid-rich microenvironment, the cytokine output of NKT cells skews towards IFN-γ rather than IL-4 (75), indicating that lipid conditions, such as the balance of fatty acids, are important factors for NKT cell activation by adipocytes. Furthermore, the LDL-α-GalCer complex elicits a stronger iNKT cell response than α-GalCer alone; while, LDL receptor mutation impairs their activation. Thus, lipoproteins can form complexes with lipid antigens to facilitate LDL receptor-mediated uptake by APCs, leading to enhanced iNKT cell activation (76).

Our group has demonstrated the role of NKT cell-adipocyte interactions *in vivo* using adipocyte-specific *Cd1d1*-deficient (AdipoqCre-*Cd1d1*$^{f/f}$) mice during obesity. In comparison to control mice fed a HFD, AdipoqCre-Cd1d1$^{f/f}$ mice exhibited suppressed body weight gain and insulin resistance, suggesting that the interaction between iNKT cells and adipocytes plays a pro-inflammatory role in AT. iNKT cells activated by adipocytes secrete IFN-γ, which enhances the expression of CD1d and CCL2 in adipocytes, thereby promoting a positive loop for AT inflammation (50).

In contrast, other reports have shown that a similar conditional knockout mouse, adipocyte-specific CD1d-KO (CD1d^{ADKO}), exhibited reduced IL-4 expression in adipose iNKT cells, resulting in aggravated AT inflammation and insulin resistance in HFD-fed mice. This implies that iNKT cells stimulated by CD1d-expressing adipocytes induce anti-inflammatory responses in the AT (51). Furthermore, the results observed in CD1d^{ADKO} mice recapitulated those in whole-body CD1d KO mice, demonstrating the opposite conclusion.

Recently, Xiao et al. re-expressed CD1d by transferring the *Cd1d* gene into the visceral AT (VAT) of CD1d KO mice using an adeno-associated viral (AAV) vector to investigate the interactions between adipocytes and immune cells. The mice with the *Cd1d* gene transferred showed massive expansion of CD8$^+$ T cells in the VAT, leading to the dysregulation of adipocyte functions through the activation of the NLRP3 inflammasome (77). Although the transduced mice exhibited a 25% reduction in VAT weight, there was no significant change in other parts of the AT. Since the mice were NKT cell-deficient and *CD1d*-gene in AAV appeared not to be expressed in either CD4$^+$CD8$^+$ thymocytes or thymic epithelial cells, CD1d expressed on adipocytes served as a neoantigen introduced like an allograft, inducing responses by CD8$^+$ T cells along with an increase in CD4$^+$ and CD4$^-$8$^-$ T cells. It would be of an interest whether iNKT cells or vNKT cells respond in CD1d-AAV transduced WT or Jα18 KO mice fed on HFD.

NKT cell-macrophage interactions in AT using cell lineage-specific CD1d KO mice

Mϕs are abundantly present in AT and play a crucial role in maintaining AT homeostasis. Mϕs are phenotypically and functionally classified into two types: pro-inflammatory M1 and anti-inflammatory M2, based on gene expression and markers. Adipose iNKT cells have been shown to interact with Mϕ, which act as antigen-presenting cells that polarize Mϕs towards M2 under the influence of IL-10 (44). To examine the role of the interaction between NKT cells and Mϕs during obesity, we utilized myeloid-specific Cd1d1-deficient (LysMCre-*Cd1d1*$^{f/f}$) mice. If the interaction is beneficial, its disruption between iNKT cells and Mϕ should result in AT inflammation and obesity. However, LysMCre-*Cd1d1*$^{f/f}$ mice gained body and VAT weights similar to those of control mice and exhibited enhanced insulin resistance, which was associated with M1-Mϕ and a bias toward NKT1/Th1 in the AT. The insulin resistance result aligns with the findings of a previous study (44), while the weight gain was not significantly greater than that of the control mice. These results may be primarily interpreted as defective M2 polarization due to the lack of interaction between iNKT cells and Mϕ via CD1d. Additionally, CD1d-deficient Mϕs expressed more IL-12p40 than control Mϕs in response to palmitic acid, and the inflammatory phenotype was enhanced by direct contact with iNKT cells (24).

Alternatively, these results may indicate that CD1d-deficient Mϕs themselves exhibit an inflammatory phenotype upon TLR stimulation (palmitic acid as a TLR4 ligand), and the interaction with iNKT cells strengthens the TLR responses, however, the mechanism of interaction remains elusive. A previous report by Zhang et al. suggested that the iNKT cell-Mϕ interactions are important in controlling AT inflammation during obesity. They employed LysMCre-*Cd1d1*$^{f/f}$ mice as M2-specific CD1d-deleted mice, induced inflammation and insulin resistance during obesity due to the inhibition of M2-Mϕ and iNKT cell interactions (78). M2-Mϕ express more CD1d than M1-Mϕ in murine VAT, facilitating the induction of IL-4 and IL-13 by interacting with iNKT cells (44, 78).

In humans with diabetes, CD11c$^+$ VAT Mϕs laden with several lipids express more CD1d than CD206$^+$ VAT Mϕs, although functional profiling such as M1 or M2 classification is not clear based on surface markers, thus implying that the signatures of human ATMϕ subtypes are unique (79, 80). In the presentation of antigens via CD1d, Th1-biasing glycolipids such as α-GC C26:0 (both Th1+Th2) and α-C-GC C26:0, which stimulate much lower IL-4 and relatively higher and more prolonged IFN-γ secretion, have consistently been observed to form complexes with CD1d that preferentially localize to cholesterol-rich membrane rafts. Contrarily, CD1d with Th2-biasing glycolipids such as α-GC C20:2, α-GC C20:1, α-GC C18:3, and α-GC C10:0, which stimulate strong IL-4 secretion relative to IFN-γ, are more evenly distributed throughout the cell membrane. Neutralization of lysosomal pH enhanced the localization of the CD1d-Th2-biasing glycolipid complex to lipid rafts on the plasma membrane, although the presentation of Th1-biasing glycolipids was drastically reduced. These results suggest that lysosomal pH controls the stability and localization of CD1d-glycolipid complexes in lipid rafts by modulating the cytokine output of iNKT cells (81, 82). Lysosomes accumulate in lipid-laden ATMϕs and appear to be important for lipid metabolism (83). However, it is unclear whether lysosomal

activation and pH are involved in M1/M2 Mϕ polarization and obesity development.

In addition to Mϕs, DCs contribute to the control of AT homeostasis and insulin sensitivity. Flt3 KO mice lacking DCs or $CD11c^+$ cell-depleted mice treated with diphtheria toxin failed to induce obesity, insulin resistance, and liver steatosis compared to that in control mice during HFD feeding (84, 85). In contrast, Batf3 KO mice lacking cDC1 showed increased body weight and adiposity during aging, partially mediated by the cDC-iNKT cell axis. However, these data did not show the actual interaction between cDC and iNKT cells (86). In our model, inhibition of the interaction between iNKT cells and DC in CD11cCre-*Cd1d1*$^{f/f}$ mice demonstrated that insulin sensitivity was improved in obese mice compared to that in control mice (24). iNKT cells activated by adipose DCs produced more IFN-γ than those activated by adipose Mϕs, and different phenotypes were observed between LysMCre-*Cd1d1*$^{f/f}$ and CD11cCre-*Cd1d1*$^{f/f}$ mice. These findings suggest that NKT cells play different roles in the development of obesity by interacting with adipose Mϕs or DCs. However, it is unclear whether the lack of CD1d affects the functions of CD1d-expressing cells.

The function of CD1d signaling in CD1d-expressing cells

Although the NKT cell-Mϕ interaction seems beneficial in obesity (24), CD1d-deficient Mϕs may exhibit pro-inflammatory functions independent of their lack of cellular interaction with iNKT cells. Given our results that BMMϕs derived from LysMCre-*Cd1d1*$^{f/f}$ mice express more IL-12p40 in a cell-autonomous manner, it is the deficiency (or downregulation) of CD1d molecules on the cell surface that causes Mϕs to produce more IL-12p40 in response to TLR4 stimulation. The cytoplasmic domain of CD1d transduces signals for the trafficking and regulation of inflammatory responses. The CD1d molecule contains a tyrosine-based signal (YXXZ) (where Y represents tyrosine, X represents any amino acid, and Z is a hydrophobic amino acid) that mediates intracellular trafficking, antigen presentation, NKT cell development (87–90), and a leucine-based basolateral sorting signal in the cytoplasmic tail (91). Another threonine residue (T322) and a serine residue (S323) in the cytoplasmic tail of CD1d control its transport to the cell surface and lysosomal degradation; thus, these motifs can regulate the functional expression of CD1d (92, 93). A few components for sorting CD1d into lysosomes, such as the AP-3 adaptor protein complex and MTP, are necessary to functionally express CD1d (94, 95).

Moreover, phosphorylation of tyrosine residues in the cytoplasmic tail is induced by CD1d crosslinking with an anti-CD1d antibody, which leads to upregulated expression of IL-10 in intestinal epithelial cells (25, 26). Conversely, CD1d crosslinking induces IL-12 production in monocytes and DC via NF-κB activation (27), indicating that the output of the CD1d crosslinking may vary depending on the cell type where CD1d is expressed. Similarly, CD1d in endosomal compartments binds isoglobotrihexosylceramide (iGb_3), an endogenous ligand for iNKT cells, induces Tyr^{332} phosphorylation of the CD1d cytoplasmic domain, and synergizes with TLR signaling in Mϕs and DC (28). Cui et al. have reported that CD1d stimulated with iGb_3 induces Ser^{330} dephosphorylation of CD1d cytoplasmic residue, followed by the downregulation NF-κB activation through the reduction of Peroxiredoxin 1 (PRDX1)-associated AKT-STAT1 phosphorylation in Mϕ (29). The short cytoplasmic tail of CD1d might mediate inhibitory signals for NF-κB activation, depending on Ser^{330} rather than Tyr^{332} residue. A similar signaling pathway has been studied as reverse signaling by MHC class Ia in immune and non-immune cells (96).

Notably, a recent study has shown that CD1d-deficient Mϕs amplify TLR signaling by increasing lipid uptake via CD36, independent of CD1d cytoplasmic signaling. This is demonstrated by the fact that the expression of CD1d with a deficient cytoplasmic tail (7 residues) or the tail mutant (^{332}Y -> A) showed similar activity as that of the WT CD1d molecule (30).

According to above reports, the inhibitory effect of CD1d on TLR signaling is weakened in the Mϕs of LysMCre-Cd1d1$^{f/f}$ mice, thereby potentiating the IL-12-IFN-γ circuit leading to AT inflammation (24). However, whether the signaling via CD1d influences only TLR responses or other factors, such as C-type lectins and scavenger receptors, remains unclear. These data suggest that it is difficult to distinguish whether the phenotype observed in CD1d-deficient mice is due to the lack of NKT cells or the CD1d deletion in the respective studies. Caution should be exercised when interpreting the results of studies using mice with either whole-body or conditional deletions of CD1d.

Conclusion

NKT cells control obesity-associated AT inflammation by interacting with CD1d-expressing cells such as adipocytes, Mϕs, and DCs. In the interactions among these cells, unknown endogenous lipid ligands are presented via CD1d. Different ligands are possibly expressed depending on the cell type, leading to the respective immune microenvironment and phenotypes in the development of obesity (Figure 1). Additionally, NKT cells may contribute to both lean and obese phase, and their interaction with the predominant APCs at the time, such as M2-Mϕs in steady state AT or hypertrophied adipocytes in obesity, can affect NKT cell function. However, if CD1d molecules had a regulatory function that either enhances or suppresses the inflammatory process in Mϕs, this pathway could function independently of NKT cells. To understand the chronic inflammatory process during obesity, it will

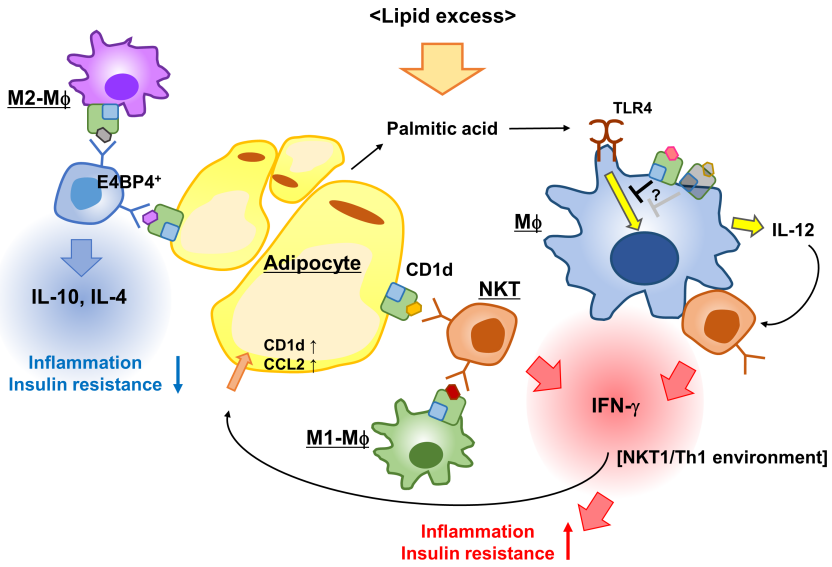

FIGURE 1
iNKT cells and CD1d control AT inflammation. Adipose iNKT cells interact with adipocytes, predominantly secreting IFN-γ and activating adipocytes to enhance the expression of CD1d and CCL2. The iNKT-adipocyte interaction forms an inflammatory circuit leading to AT inflammation and insulin resistance. The interaction between M2-Mϕ and iNKT cells induces Th2 cytokines, while the interaction with M1-Mϕ leads to Th1 cytokines, thereby modulating AT microenvironment. CD1d-deficient (or reduced) Mϕs secreted more IL-12 in response to palmitic acid than WT Mϕs to induce IFN-γ production in iNKT cells, because CD1d molecules presumably suppressed TLR responses in WT Mϕs. This scheme indicates that the activation process of adipose iNKT cells is APC-specific and that CD1d molecules contribute to immune responses.

be important to further investigate the regulatory effect of CD1d, endogenous glycolipids, and NKT cells on immune responses in the AT. Thus, we obtained new insights into the strategies for overcoming obesity and obesity-associated diseases.

Author contributions

MS: Writing – original draft, Writing – review & editing. KI: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Takeda Science Foundation and JSPS KAKENHI Grant Numbers JP22K08679.

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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References

1. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* (2006) 444 (7121):860–7. doi: 10.1038/nature05485

2. Gliniak CM, Pedersen L, Scherer PE. Adipose tissue fibrosis: the unwanted houseguest invited by obesity. *J Endocrinol* (2023) 259(3):e230180. doi: 10.1530/JOE-23-0180

3. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* (2006) 444(7121):840–6. doi: 10.1038/nature05482

4. Kahn CR, Wang G, Lee KY. Altered adipose tissue and adipocyte function in the pathogenesis of metabolic syndrome. *J Clin Invest* (2019) 129(10):3990–4000. doi: 10.1172/JCI129187

5. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* (2003) 112(12):1796–808. doi: 10.1172/JCI19246

6. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, et al. CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest* (2006) 116(1):115–24. doi: 10.1172/JCI24335

7. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, et al. $CD8^+$ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. *Nat Med* (2009) 15(8):914–20. doi: 10.1038/nm.1964

8. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med* (2009) 15(8):930–9. doi: 10.1038/nm.2002

9. Winer DA, Winer S, Shen L, Wadia PP, Yantha J, Paltser G, et al. B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. *Nat Med* (2011) 17(5):610–7. doi: 10.1038/nm.2353

10. Talukdar S, Oh DY, Bandyopadhyay G, Li D, Xu J, McNelis J, et al. Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. *Nat Med* (2012) 18(9):1407–12. doi: 10.1038/nm.2885

11. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol* (2011) 11:85–97. doi: 10.1038/nri2921

12. Satoh M, Iwabuchi K. Role of natural killer T cells in the development of obesity and insulin resistance: insights from recent progress. *Front Immunol* (2018) 9:1314. doi: 10.3389/fimmu.2018.01314

13. Van Kaer L. NKT cells: T lymphocytes with innate effector functions. *Curr Opin Immunol* (2007) 19:354–64. doi: 10.1016/j.coi.2007.03.001

14. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi: 10.1146/annurev.immunol.25.022106.141711

15. Canchis PW, Bhan AK, Landau SB, Yang L, Balk SP, Blumberg RS. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. *Immunology* (1993) 80(4):561–5.

16. Godfrey DI, Stankovic S, Baxter AG. Raising the NKT cell family. *Nat Immunol* (2010) 11:197–206. doi: 10.1038/ni.1841

17. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. *Science* (1997) 278(5343):1626–9. doi: 10.1126/science.278.5343.1626

18. Halder RC, Aguilera C, Maricic I, Kumar V. Type II NKT cell-mediated anergy induction in type I NKT cells prevents inflammatory liver disease. *J Clin Invest* (2007) 117:2302–12. doi: 10.1172/JCI31602

19. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol* (2008) 20(3):358–68. doi: 10.1016/j.coi.2008.03.018

20. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-specific distribution of iNKT cells impacts their cytokine response. *Immunity* (2015) 43 (3):566–78. doi: 10.1016/j.immuni.2015.06.025

21. Kwon DI, Lee YJ. Lineage differentiation program of invariant natural killer T cells. *Immune Netw* (2017) 17(6):365–77. doi: 10.4110/in.2017.17.6.365

22. Lee B, Jo Y, Kim G, Ali LA, Sohn DH, Lee SG, et al. Specific inhibition of soluble γc receptor attenuates collagen-induced arthritis by modulating the inflammatory T cell responses. *Front Immunol* (2019) 10:209. doi: 10.3389/fimmu.2019.00209

23. Tumes D, Hirahara K, Papadopoulos M, Shinoda K, Onodera A, Kumagai J, et al. Ezh2 controls development of natural killer T cells, which cause spontaneous asthma-like pathology. *J Allergy Clin Immunol* (2019) 144(2):549–60.e10. doi: 10.1016/j.jaci.2019.02.024

24. Satoh M, Iizuka M, Majima M, Ohwa C, Hattori A, Van Kaer L, et al. Adipose invariant NKT cells interact with CD1d-expressing macrophages to regulate obesity-related inflammation. *Immunology* (2022) 165(4):414–27. doi: 10.1111/imm.13447

25. Colgan SP, Hershberg RM, Furuta GT, Blumberg RS. Ligation of intestinal epithelial CD1d induces bioactive IL-10: critical role of the cytoplasmic tail in autocrine signaling. *Proc Natl Acad Sci USA* (1999) 96(24):13938–43. doi: 10.1073/pnas.96.24.13938

26. Olszak T, Neves JF, Dowds CM, Baker K, Glickman J, Davidson NO, et al. Protective mucosal immunity mediated by epithelial CD1d and IL-10. *Nature* (2014) 509(7501):497–502. doi: 10.1038/nature13150

27. Yue SC, Shaulov A, Wang R, Balk SP, Exley MA. CD1d ligation on human monocytes directly signals rapid NF-kappaB activation and production of bioactive IL-12. *Proc Natl Acad Sci U.S.A.* (2005) 102(33):11811–6. doi: 10.1073/pnas.0503366102

28. Liu X, Zhang P, Zhang Y, Wang Z, Xu S, Li Y, et al. Glycolipid iGb3 feedback amplifies innate immune responses *via* CD1d reverse signaling. *Cell Res* (2019) 29 (1):42–53. doi: 10.1038/s41422-018-0122-7

29. Cui S, Wang C, Bai W, Li J, Pan Y, Huang X, et al. CD1d1 intrinsic signaling in macrophages controls NLRP3 inflammasome expression during inflammation. *Sci Adv* (2020) 6(43):eaaz7290. doi: 10.1126/sciadv.aaz7290

30. Brailey PM, Evans L, López-Rodríguez JC, Sinadinos A, Tyrrel V, Kelly G, et al. CD1d-dependent rewiring of lipid metabolism in macrophages regulates innate immune responses. *Nat Commun* (2022) 13(1):6723. doi: 10.1038/s41467-022-34532-x

31. Lee BC, Kim MS, Pae M, Yamamoto Y, Eberlé D, Shimada T, et al. Adipose natural killer cells regulate adipose tissue macrophages to promote insulin resistance in obesity. *Cell Metab* (2016) 23(4):685–98. doi: 10.1016/j.cmet.2016.03.002

32. Wensveen FM, Jelenčić V, Valentić S, Šestan M, Wensveen TT, Theurich S, et al. NK cells link obesity-induced adipose stress to inflammation and insulin resistance. *Nat Immunol* (2015) 16(4):376–85. doi: 10.1038/ni.3120

33. Boulenouar S, Michelet X, Duquette D, Alvarez D, Hogan AE, Dold C, et al. Adipose type one innate lymphoid cells regulate macrophage homeostasis through targeted cytotoxicity. *Immunity* (2017) 46(2):273–86. doi: 10.1016/j.immuni.2017.01.008

34. O'Sullivan TE, Rapp M, Fan X, Weizman OE, Bhardwaj P, Adams NM, et al. Adipose-resident group 1 innate lymphoid cells promote obesity-associated insulin resistance. *Immunity* (2016) 45(2):428–41. doi: 10.1016/j.immuni.2016.06.016

35. Wu D, Molofsky AB, Liang HE, Ricardo-Gonzalez RR, Jouihan HA, Bando JK, et al. Eosinophils sustain adipose alternatively activated macrophages associated with glucose homeostasis. *Science* (2011) 332(6026):243–7. doi: 10.1126/science.1201475

36. Sasaki T, Moro K, Kubota T, Kubota N, Kato T, Ohno H, et al. Innate lymphoid cells in the induction of obesity. *Cell Rep* (2019) 28(1):202–17.e7. doi: 10.1016/j.celrep.2019.06.016

37. Brestoff JR, Kim BS, Saenz SA, Stine RR, Monticelli LA, Sonnenberg GF, et al. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature* (2015) 519(7542):242–6. doi: 10.1038/nature14115

38. Qiu Y, Nguyen KD, Odegaard JI, Cui X, Tian X, Locksley RM, et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell* (2014) 157(6):1292–308. doi: 10.1016/j.cell.2014.03.066

39. Rao RR, Long JZ, White JP, Svensson KJ, Lou J, Lokurkar I, et al. Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. *Cell* (2014) 157(6):1279–91. doi: 10.1016/j.cell.2014.03.065

40. Satoh M, Andoh Y, Clingan CS, Ogura H, Fujii S, Eshima K, et al. Type II NKT cells stimulate diet-induced obesity by mediating adipose tissue inflammation, steatohepatitis and insulin resistance. *PloS One* (2012) 7(2):e30568. doi: 10.1371/journal.pone.0030568

41. Wu L, Parekh VV, Gabriel CL, Bracy DP, Marks-Shulman PA, Tamboli RA, et al. Activation of invariant natural killer T cells by lipid excess promotes tissue inflammation, insulin resistance, and hepatic steatosis in obese mice. *Proc Natl Acad Sci USA* (2012) 109(19):E1143–52. doi: 10.1073/pnas.1200498109

42. Ren Y, Sekine-Kondo E, Shibata R, Kato-Itoh M, Umino A, Yanagida A, et al. A Novel Mouse Model of iNKT Cell-deficiency Generated by CRISPR/Cas9 Reveals a Pathogenic Role of iNKT Cells in Metabolic Disease. *Sci Rep* (2017) 7(1):12765. doi: 10.1038/s41598-017-12475-4

43. Subramanian S, Turner MS, Ding Y, Goodspeed L, Wang S, Buckner JH, et al. Increased levels of invariant natural killer T lymphocytes worsen metabolic abnormalities and atherosclerosis in obese mice. *J Lipid Res* (2013) 54:2831–41. doi: 10.1194/jlr.M041020

44. Lynch L, Nowak M, Varghese B, Clark J, Hogan AE, Toxavidis V, et al. Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. *Immunity* (2012) 37(3):574–87. doi: 10.1016/j.immuni.2012.06.016

45. Ji Y, Sun S, Xu A, Bhargava P, Yang L, Lam KS, et al. Activation of natural killer T cells promotes M2 Macrophage polarization in adipose tissue and improves systemic glucose tolerance *via* interleukin-4 (IL-4)/STAT6 protein signaling axis in obesity. *J Biol Chem* (2012) 287(17):13561–71. doi: 10.1074/jbc.M112.350066

46. Schipper HS, Rakhshandehroo M, van de Graaf SF, Venken K, Koppen A, Stienstra R, et al. Natural killer T cells in adipose tissue prevent insulin resistance. *J Clin Invest* (2012) 122(9):3343–54. doi: 10.1172/JCI62739

47. Hams E, Locksley RM, McKenzie AN, Fallon PG. Cutting edge: IL-25 elicits innate lymphoid type 2 and type II NKT cells that regulate obesity in mice. *J Immunol* (2013) 191(11):5349–53. doi: 10.4049/jimmunol.1301176

48. Mantell BS, Stefanovic-Racic M, Yang X, Dedousis N, Sipula IJ, O'Doherty RM. Mice lacking NKT cells but with a complete complement of $CD8^+$ T-cells are not protected against the metabolic abnormalities of diet-induced obesity. *PloS One* (2011) 6:e19831. doi: 10.1371/journal.pone.0019831

49. Kotas ME, Lee HY, Gillum MP, Annicelli C, Guigni BA, Shulman GI, et al. Impact of CD1d deficiency on metabolism. *PloS One* (2011) 6(9):e25478. doi: 10.1371/journal.pone.0025478

50. Satoh M, Hoshino M, Fujita K, Iizuka M, Fujii S, Clingan CS, et al. Adipocyte-specific CD1d-deficiency mitigates diet-induced obesity and insulin resistance in mice. *Sci Rep* (2016) 6:28473. doi: 10.1038/srep28473

51. Huh JY, Park J, Kim JI, Park YJ, Lee YK, Kim JB. Deletion of CD1d in adipocytes aggravates adipose tissue inflammation and insulin resistance in obesity. *Diabetes* (2017) 66(4):835–47. doi: 10.2337/db16-1122

52. Bedel R, Matsuda JL, Brigl M, White J, Kappler J, Marrack P, et al. Lower TCR repertoire diversity in Traj18-deficient mice. *Nat Immunol* (2012) 13:705–6. doi: 10.1038/ni.2347

53. Xie J, Pan Y, Tao H, Wang P, Chen Y, Gao J, et al. Deficiency of mucosal-associated invariant T cells in TCRJα18 germline knockout mice. *Immunohorizons* (2019) 3:203–7. doi: 10.4049/immunohorizons.1900035

54. Toubal A, Kiaf B, Beaudoin L, Cagninacci L, Rhimi M, Fruchet B, et al. Mucosal-associated invariant T cells promote inflammation and intestinal dysbiosis leading to metabolic dysfunction during obesity. *Nat Commun* (2020) 11(1):3755. doi: 10.1038/s41467-020-17307-0

55. Bergin R, Kinlen D, Kedia-Mehta N, Hayes E, Cassidy FC, Cody D, et al. Mucosal-associated invariant T cells are associated with insulin resistance in childhood obesity, and disrupt insulin signalling *via* IL-17. *Diabetologia* (2022) 65:1012–7. doi: 10.1007/s00125-022-05682-w

56. Carolan E, Tobin LM, Mangan BA, Corrigan M, Gaoatswe G, Byrne G, et al. Altered distribution and increased IL-17 production by mucosal-associated invariant T cells in adult and childhood obesity. *J Immunol* (2015) 194:5775–80. doi: 10.4049/jimmunol.1402945

57. Magalhaes I, Pingris K, Poitou C, Bessoles S, Venteclef N, Kiaf B, et al. Mucosal-associated invariant T cell alterations in obese and type 2 diabetic patients. *J Clin Invest* (2015) 125:1752–62. doi: 10.1172/JCI78941

58. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124 (9):3725–40. doi: 10.1172/JCI72308

59. Lynch L, Michelet X, Zhang S, Brennan PJ, Moseman A, Lester C, et al. Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T(reg) cells and macrophages in adipose tissue. *Nat Immunol* (2015) 16(1):85–95. doi: 10.1038/ni.3047

60. Vieth JA, Das J, Ranaivoson FM, Comoletti D, Denzin LK, Sant'Angelo DB. TCRα-TCRβ pairing controls recognition of CD1d and directs the development of adipose NKT cells. *Nat Immunol* (2017) 18(1):36–44. doi: 10.1038/ni.3622

61. Aguiar CF, Corrêa-da-Silva F, Gonzatti MB, Angelim MK, Pretti MA, Davanzo GG, et al. Tissue-specific metabolic profile drives iNKT cell function during obesity and liver injury. *Cell Rep* (2023) 42(1):112035. doi: 10.1016/j.celrep.2023.112035

62. Wieland Brown LC, Penaranda C, Kashyap PC, Williams BB, Clardy J, Kronenberg M, et al. Production of α-galactosylceramide by a prominent member of human gut microbiota. *PloS Biol* (2013) 11(7):e1001610. doi: 10.1371/journal.pbio.1001610

63. An D, Oh SF, Olszak T, Neves JF, Avci FY, Erturk-Hasdemir D, et al. Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. *Cell* (2014) 156(1-2):123–33. doi: 10.1016/j.cell.2013.11.042

64. Oh SF, Praveena T, Song H, Yoo JS, Jung DJ, Erturk-Hasdemir D, et al. Host immunomodulatory lipids created by symbionts from dietary amino acids. *Nature* (2022) 601(7894):E32. doi: 10.1038/s41586-021-04276-7

65. Selvanantham T, Lin Q, Guo CX, Surendra A, Fieve S, Escalante NK, et al. NKT cell-deficient mice harbor an altered microbiota that fuels intestinal inflammation during chemically induced colitis. *J Immunol* (2016) 197:4464–72. doi: 10.4049/jimmunol.1601410

66. Warensjö E, Risérus U, Vessby B. Fatty acid composition of serum lipids predicts the development of the metabolic syndrome in men. *Diabetologia* (2005) 48:1999–2005. doi: 10.1007/s00125-005-1897-x

67. Hodson L, Skeaff CM, Fielding BA. Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Prog Lipid Res* (2008) 47:348–80. doi: 10.1016/j.plipres.2008.03.003

68. LaMarche NM, Kane H, Kohlgruber AC, Dong H, Lynch L, Brenner MB. Distinct iNKT cell populations use IFNγ or ER stress-induced IL-10 to control adipose tissue homeostasis. *Cell Metab* (2020) 32:243–58.e6. doi: 10.1016/j.cmet.2020.05.017

69. Han SM, Park ES, Park J, Nahmgoong H, Choi YH, Oh J, et al. Unique adipose tissue invariant natural killer T cell subpopulations control adipocyte turnover in mice. *Nat Commun* (2023) 14:8512. doi: 10.1038/s41467-023-44181-3

70. Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol* (2006) 6(10):772–83. doi: 10.1038/nri1937

71. Rakhshandehroo M, Gijzel SM, Siersbæk R, Broekema MF, de Haar C, Schipper HS, et al. CD1d-mediated presentation of endogenous lipid antigens by adipocytes requires microsomal triglyceride transfer protein. *J Biol Chem* (2014) 289(32):22128–39. doi: 10.1074/jbc.M114.551242

72. Huh JY, Kim JI, Park YJ, Hwang IJ, Lee YS, Sohn JH, et al. A novel function of adipocytes in lipid antigen presentation to iNKT cells. *Mol Cell Biol* (2013) 33(2):328–39. doi: 10.1128/MCB.00552-12

73. Deng T, Lyon CJ, Minze LJ, Lin J, Zou J, Liu JZ, et al. Class II major histocompatibility complex plays an essential role in obesity-induced adipose inflammation. *Cell Metab* (2013) 17(3):411–22. doi: 10.1016/j.cmet.2013.02.009

74. Rakhshandehroo M, van Eijkeren RJ, Gabriel TL, de Haar C, Gijzel SMW, Hamers N, et al. Adipocytes harbor a glucosylceramide biosynthesis pathway involved in iNKT cell activation. *Biochim Biophys Acta Mol Cell Biol Lipids* (2019) 1864 (8):1157–67. doi: 10.1016/j.bbalip.2019.04.016

75. van Eijkeren RJ, Morris I, Borgman A, Markovska A, Kalkhoven E. Cytokine output of adipocyte-iNKT cell interplay is skewed by a lipid-rich microenvironment. *Front Endocrinol (Lausanne)* (2020) 11:479. doi: 10.3389/fendo.2020.00479

76. Engelen SE, Ververs FA, Markovska A, Lagerholm BC, Kraaijenhof JM, Yousif LI, et al. Lipoproteins act as vehicles for lipid antigen delivery and activation of invariant natural killer T cells. *JCI Insight* (2023) 8(9):e158089. doi: 10.1172/jci.insight.158089

77. Xiao R, Mansour AG, Huang W, Hassan QN 2nd, Wilkins RK, Komatineni SV, et al. Adipocyte CD1d gene transfer induces T cell expansion and adipocyte inflammation in CD1d knockout mice. *J Immunol* (2022) 208(9):2109–21. doi: 10.4049/jimmunol.2100313

78. Zhang H, Xue R, Zhu S, Fu S, Chen Z, Zhou R, et al. M2-specific reduction of CD1d switches NKT cell-mediated immune responses and triggers metaflammation in adipose tissue. *Cell Mol Immunol* (2018) 15(5):506–17. doi: 10.1038/cmi.2017.11

79. Muir LA, Cho KW, Geletka LM, Baker NA, Flesher CG, Ehlers AP, et al. Human $CD206^+$ macrophages associate with diabetes and adipose tissue lymphoid clusters. *JCI Insight* (2022) 7(3):e146563. doi: 10.1172/jci.insight.146563

80. Wentworth JM, Naselli G, Brown WA, Doyle L, Phipson B, Smyth GK, et al. Pro-inflammatory $CD11c^+CD206^+$ adipose tissue macrophages are associated with insulin resistance in human obesity. *Diabetes* (2010) 59(7):1648–56. doi: 10.2337/db09-0287

81. Arora P, Kharkwal SS, Ng TW, Kunnath-Velayudhan S, Saini NK, Johndrow CT, et al. Endocytic pH regulates cell surface localization of glycolipid antigen loaded CD1d complexes. *Chem Phys Lipids* (2016) 194:49–57. doi: 10.1016/j.chemphyslip.2015.10.006

82. Morris I, Croes CA, Boes M, Kalkhoven E. Advanced omics techniques shed light on CD1d-mediated lipid antigen presentation to iNKT cells. *Biochim Biophys Acta Mol Cell Biol Lipids* (2023) 1868(5):159292. doi: 10.1016/j.bbalip.2023.159292

83. Xu X, Grijalva A, Skowronski A, van Eijk M, Serlie MJ, Ferrante AW Jr. Obesity activates a program of lysosomal-dependent lipid metabolism in adipose tissue macrophages independently of classic activation. *Cell Metab* (2013) 18(6):816–30. doi: 10.1016/j.cmet.2013.11.001

84. Stefanovic-Racic M, Yang X, Turner MS, Mantell BS, Stolz DB, Sumpter TL, et al. Dendritic cells promote macrophage infiltration and comprise a substantial proportion of obesity-associated increases in $CD11c^+$ cells in adipose tissue and liver. *Diabetes* (2012) 61(9):2330–9. doi: 10.2337/db11-1523

85. Patsouris D, Li PP, Thapar D, Chapman J, Olefsky JM, Neels JG. Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. *Cell Metab* (2008) 8(4):301–9. doi: 10.1016/j.cmet.2008.08.015

86. Hernández-García E, Cueto FJ, Cook ECL, Redondo-Urzainqui A, Charro-Zanca S, Robles-Vera I, et al. Conventional type 1 dendritic cells protect against age-related adipose tissue dysfunction and obesity. *Cell Mol Immunol* (2022) 19(2):260–75. doi: 10.1038/s41423-021-00812-7

87. Rodionov DG, Nordeng TW, Pedersen K, Balk SP, Bakke O. A critical tyrosine residue in the cytoplasmic tail is important for CD1d internalization but not for its basolateral sorting in MDCK cells. *J Immunol* (1999) 162(3):1488–95. doi: 10.4049/jimmunol.162.3.1488

88. Roberts TJ, Sriram V, Spence PM, Gui M, Hayakawa K, Bacik I, et al. Recycling CD1d1 molecules present endogenous antigens processed in an endocytic compartment to NKT cells. *J Immunol* (2002) 168(11):5409–14. doi: 10.4049/jimmunol.168.11.5409

89. Lawton AP, Prigozy TI, Brossay L, Pei B, Khurana A, Martin D, et al. The mouse CD1d cytoplasmic tail mediates CD1d trafficking and antigen presentation by adaptor protein 3-dependent and -independent mechanisms. *J Immunol* (2005) 174(6):3179–86. doi: 10.4049/jimmunol.174.6.3179

90. Chiu YH, Park SH, Benlagha K, Forestier C, Jayawardena-Wolf J, Savage PB, et al. Multiple defects in antigen presentation and T cell development by mice expressing cytoplasmic tail-truncated CD1d. *Nat Immunol* (2002) 3(1):55–60. doi: 10.1038/ni740

91. Rodionov DG, Nordeng TW, Kongsvik TL, Bakke O. The cytoplasmic tail of CD1d contains two overlapping basolateral sorting signals. *J Biol Chem* (2000) 275 (12):8279–82. doi: 10.1074/jbc.275.12.8279

92. Liu J, Shaji D, Cho S, Du W, Gervay-Hague J, Brutkiewicz RR. A threonine-based targeting signal in the human CD1d cytoplasmic tail controls its functional expression. *J Immunol* (2010) 184(9):4973–81. doi: 10.4049/jimmunol.0901448

93. Liu J, Glosson NL, Du W, Gervay-Hague J, Brutkiewicz RR. A Thr/Ser dual residue motif in the cytoplasmic tail of human CD1d is important for the down-regulation of antigen presentation following a herpes simplex virus 1 infection. *Immunology* (2013) 140(2):191–201. doi: 10.1111/imm.12127

94. Cernadas M, Sugita M, van der Wel N, Cao X, Gumperz JE, Maltsev S, et al. Lysosomal localization of murine CD1d mediated by AP-3 is necessary for NK T cell development. *J Immunol* (2003) 171(8):4149–55. doi: 10.4049/jimmunol.171.8.4149

95. Sagiv Y, Bai L, Wei DG, Agami R, Savage PB, Teyton L, et al. A distal effect of microsomal triglyceride transfer protein deficiency on the lysosomal recycling of CD1d. *J Exp Med* (2007) 204(4):921–8. doi: 10.1084/jem.20061568

96. Muntjewerff EM, Meesters LD, van den Bogaart G, Revelo NH. Reverse signaling by MHC-I molecules in immune and non-immune cell types. *Front Immunol* (2020) 11:605958. doi: 10.3389/fimmu.2020.605958

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 19 February 2024
DOI 10.3389/fimmu.2024.1349184

OPEN ACCESS

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RECEIVED 04 December 2023
ACCEPTED 06 February 2024
PUBLISHED 19 February 2024

CITATION
Cui G, Abe S, Kato R and Ikuta K (2024) Insights into the heterogeneity of iNKT cells: tissue-resident and circulating subsets shaped by local microenvironmental cues. *Front. Immunol.* 15:1349184. doi: 10.3389/fimmu.2024.1349184

Insights into the heterogeneity of iNKT cells: tissue-resident and circulating subsets shaped by local microenvironmental cues

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Invariant natural killer T (iNKT) cells are a distinct subpopulation of innate-like T lymphocytes. They are characterized by semi-invariant T cell receptors (TCRs) that recognize both self and foreign lipid antigens presented by CD1d, a non-polymorphic MHC class I-like molecule. iNKT cells play a critical role in stimulating innate and adaptive immune responses, providing an effective defense against infections and cancers, while also contributing to chronic inflammation. The functions of iNKT cells are specific to their location, ranging from lymphoid to non-lymphoid tissues, such as the thymus, lung, liver, intestine, and adipose tissue. This review aims to provide insights into the heterogeneity of development and function in iNKT cells. First, we will review the expression of master transcription factors that define subsets of iNKT cells and their production of effector molecules such as cytokines and granzymes. In this article, we describe the gene expression profiles contributing to the kinetics, distribution, and cytotoxicity of iNKT cells across different tissue types. We also review the impact of cytokine production in distinct immune microenvironments on iNKT cell heterogeneity, highlighting a recently identified circulating iNKT cell subset. Additionally, we explore the potential of exploiting iNKT cell heterogeneity to create potent immunotherapies for human cancers in the future.

KEYWORDS

iNKT cell, heterogeneity, immune microenvironment, IL-15, immunotherapy

Introduction

Invariant natural killer T (iNKT) cells are a subset of T lymphocytes with innate-like properties that detect self or foreign lipid antigens presented on non-polymorphic major histocompatibility complex (MHC) class I-like molecule CD1d (1–3). They express semi-invariant T cell receptors (TCRs) encoded by Vα14-Jα18 in mice and Vα24-Jα18 in humans, paired with a limited number of TCRβ chains Vβ8.2, Vβ7, or Vβ2 in mice and Vβ11 in humans (4). iNKT cells become activated rapidly at the onset of immune responses

by signals from CD1d-restricted semi-invariant TCRs and cytokine receptors such as the interleukin-12 receptor (IL-12R) and IL-18R (5). Activated iNKT cells exert their effector functions indirectly by inducing immune responses in other immune cells and directly through cytotoxicity against target cells.

Upon activation, iNKT cells rapidly secrete significant levels of cytokines, such as interferon-γ (IFN-γ) and IL-4. These cytokines promote the maturation of antigen-presenting cells (APCs), NK cells, and cytotoxic T lymphocytes (CTLs) (6, 7). Additional cytokines such as IL-2, IL-5, IL-6, IL-10, IL-13, IL-17, and IL-22, along with growth factors and chemokines (CCL3, CCL4, and CCL5), are produced by iNKT cells to enhance immune responses (8). Additionally, iNKT cells can stimulate the maturation and activation of APCs through their interaction between CD40 and CD40L. Regarding direct cytotoxicity, activated iNKT cells release cytotoxic proteins, such as granzymes and perforin, which collaborate to provoke apoptosis of target cells. Furthermore, iNKT cells express Fas ligand (FASL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), which can trigger target cell apoptosis by activating the death receptor pathway (3, 9).

Due to their numerous effector functions, iNKT cells link innate and adaptive immunity, rendering them vital for host defense against cancer and infection. This versatility makes them an attractive target for immunotherapy (10). However, as most iNKT cells are tissue-resident, their regulation and effector functions vary across tissues. Several studies have demonstrated that iNKT cells in mice exhibit heterogeneity and perform diverse functions and that the composition of iNKT cell subsets can alter disease outcomes (11–14). This article reviews the tissue residency and heterogeneity of iNKT cells across different tissues, focusing on a newly identified circulating iNKT cell subset that differs from conventional tissue-resident iNKT cells.

Developmental and functional subsets of iNKT cells

Like conventional T cells, most iNKT cells arise from $CD4^+CD8^+$ double-positive (DP) thymocytes in the thymus and are regulated by CD1d-expressing DP thymocytes, as well as the crucial cytokine IL-7 (1, 15–18). Alternatively, it has been reported that a fraction of $CD4^-CD8^-$ double-negative (DN) iNKT cells develop from late DN-stage thymocytes (19). The development of iNKT cells is regulated by the transcription factors PLZF (encoded by *Zbtb16*) and EGR2 (20, 21). Specifically, the absence of PLZF results in a significant decrease in the number of iNKT cells, and bone marrow chimeric mice deficient in EGR2 show an arrest of iNKT cell differentiation.

Mouse iNKT cells have been categorized into four developmental subsets, namely stage 0, 1, 2, and 3 iNKT cells, and three effector subsets, namely NKT1, NKT2, and NKT17 cells (2, 7, 22–25). During thymic development in mice, iNKT cells can be categorized into four subsets using CD24, CD44, and NK1.1 markers. iNKT cells undergo positive selection during the earliest stage 0 precursor cells ($CD24^{high}CD44^-NK1.1^-$) in response to agonistic interactions with $CD4^+CD8^+$ double-positive (DP) thymocytes in the thymic cortex. Then, CD24 is downregulated in iNKT cells to transit into the immature stage 1 ($CD24^{low}CD44^-NK1.1^-$) cells. Subsequently, the cells progress to stage 2 ($CD24^{low}CD44^+NK1.1^-$) cells with upregulation of CD44 and eventually differentiate into mature stage 3 ($CD24^{low}CD44^+NK1.1^+$) cells. The mature population of iNKT cells displays functional heterogeneity. These cells have been categorized into three effector subsets, NKT1, NKT2, and NKT17 cells, based on differential expression of cytokines and transcription factors associated with distinct functions similar to Th1, Th2, and Th17 cells in helper T cells, respectively (7, 24–27). Generally, NKT1 cells are $T\text{-}bet^+PLZF^{low}$ and produce IFN-γ, NKT2 cells are $GATA\text{-}3^+PLZF^{high}$ and produce IL-4, and NKT17 cells are $ROR\gamma t^+PLZF^{int}$ and produce IL-17. Notably, NKT1, NKT2, and NKT17 cells are found in different fractions of stage 3, stage 1 or 2, and stage 2, respectively, suggesting that markers of conventional iNKT cell developmental stages in the thymus, CD44 and NK1.1, are expressed heterogeneously across these distinct effector subsets (7). Functional subsets of iNKT cells in C57BL/6 mice can be distinguished by NK1.1, CD4, and IL-17RB cell surface markers. Specifically, $NK1.1^+$ iNKT cells typically produce IFN-γ and are categorized as NKT1 cells. NKT2 and NKT17 cells, distinguished by $CD4^+$ and $CD4^-$ expression, respectively, express IL-17RB, while NKT1 cells do not express this cytokine receptor. CD122 (IL-2Rβ) and CXCR3 are useful surface markers for NKT1 cells (7, 25), as other mouse strains, except for C57BL/6, do not express NK1.1. Furthermore, CD138 is uniquely expressed in NKT17 cells among effector subsets of iNKT cells (28–30). In addition to NKT1, NKT2, and NKT17, a population of iNKT cells has been identified as follicular helper NKT (NKT_{FH}) cells that produce IL-21 and provide cognate help to antigen-specific B cells (31). These iNKT cells express BCL-6, CXCR5, and PD-1, similar to follicular helper T cells (T_{FH}). Moreover, IL-10-producing NKT10 cells express E4BP4 but not FoxP3 (32). Upon stimulation, they exhibit regulatory properties akin to Treg cells.

Certain iNKT cells acquire various NK cell receptors and generate cytotoxic molecules similar to NK cells. The development of iNKT cells, especially NKT1 cells, is regulated by IL-15, an essential cytokine for NK cells (11, 33). Since medullary thymic epithelial cells (mTECs) are the primary source of IL-15 and the diversity of mTECs controls intrathymic iNKT cell development and maturation, it has been proposed that the thymic medulla is crucial for the continued development and maturation of iNKT cells (34–36). Subsequently, it was discovered that $CCR7^+PLZF^{high}$ iNKT cells in the mouse thymus serve as progenitors for the three effector subsets of NKT1, NKT2, and NKT17 cells in the mouse. Furthermore, CCR7 enables the progenitors to migrate from the cortex to the medulla, and iNKT cells expressing CCR7 leave the thymus in a Klf2- and S1PR1-dependent manner, followed by subsequent maturation and expansion in peripheral tissues (12, 37).

Human iNKT cells are typically divided into two subsets: $CD4^+$ and $CD4^-$ iNKT cells (38, 39). $CD4^-$ iNKT cells include $CD4^-CD8^-$ DN and $CD8^+$ iNKT cells. $CD4^+$ iNKT cells produce Th1 and Th2 cytokines, such as IFN-γ, IL-4 and IL-13, while $CD4^-$ iNKT cells

mainly produce Th1 cytokines, such as IFN-γ and TNF-α. Upon activation, human $CD4^-$ iNKT cells, particularly DN iNKT cells, exhibit greater effector functions and cytolytic activity, with increased IFN-γ expression similar to NK cells. Additionally, in the presence of TGF-β and rapamycin, some human iNKT cells express Foxp3 and exhibit Treg-like features. They are functionally capable of suppressing the proliferation of conventional $CD4^+$ T cells by producing IL-10 (40).

Tissue-residency and heterogeneity of iNKT cells

Based on the results of thymus transplantation and parabiosis experiments in mice, iNKT cells are considered tissue-resident lymphocytes (11, 41–43). These cells reside permanently within various lymphoid and non-lymphoid organs, such as the thymus, spleen, lymph nodes, liver, lung, intestine, and adipose tissue. iNKT cells play a crucial role in both systemic and local immunity. RNA-seq analysis revealed the differences in tissue location between effector subsets of iNKT cells (44). A recent study utilizing single-cell RNA sequencing (scRNA-seq) investigated iNKT cells in different organs (12). The study integrated thymic iNKT cells with peripheral iNKT cells and distinguished iNKT cells at various developmental stages in the thymus and across different peripheral tissues using the uniform manifold approximation and projection (UMAP) plot. The iNKT cells isolated from different organs exhibit unique tissue-specific features dependent on their origin. Furthermore, while iNKT cells are scarce in each organ under normal conditions, their numbers significantly increase during inflammation and infection (45, 46). Therefore, iNKT cells are molded by diverse tissue microenvironments and display phenotypic variety influenced by their resident tissues and effector subtypes (Table 1).

Although effector subsets of iNKT cells, namely NKT1, NKT2, and NKT17, differentiated in the thymus, most mature iNKT cells found in the mouse thymus are thymus-resident (11, 43, 68, 69). A recent scRNA-seq study revealed that NKT1 and NKT2 cells exhibit considerable phenotypic and functional heterogeneity, while NKT17 cells are relatively uniform (47). Apart from differences among effector subsets of iNKT cells, thymus-resident iNKT cells require CD28 co-stimulation during antigenic activation (48). In addition, local signals seem to influence the TCR repertoire and antigen specificity of tissue-resident iNKT cells. These iNKT cells exhibit diverse TCR Vβ usage and experience clonal expansion in the thymus, spleen, and lymph nodes. Furthermore, distinct TCRβ repertoires in iNKT cells from different tissues are associated with their different ability to recognize lipid antigens (70).

In mice, iNKT cells are most abundant in the liver compared to other tissues, while in humans, they are also enriched but much less abundant. Hepatic iNKT cells conduct random and crawling patrols along the liver sinusoids. In mice, the patrolling iNKT cells in the liver can be arrested through TCR signals, inflammatory cytokines like IL-12 and IL-18, or microbial antigens during infections (71, 72). Sinusoidal endothelial cells, Kupffer cells, stellate cells, and hepatocytes all express CD1d, presenting lipid antigens to hepatic iNKT cells, and CD1d expression on hepatocytes contributes to maintaining hepatic iNKT cells (49). Besides CD1d, hepatocyte-derived IL-7 is crucial for iNKT cell survival in the liver but does not affect iNKT cells in the spleen (50). The anti-apoptotic gene *Bcl2* and suppressor of cytokine signaling 2 (Socs2) are highly expressed in hepatic iNKT cells, when compared to iNKT cells in the spleen and lymph nodes. In addition, hepatic NKT1 cells show a higher cell proliferation status (12). By producing IFN-γ, hepatic iNKT cells defend against infections of *B. burgdorferi*, hepatitis B virus (HBV), and hepatitis C virus (HCV) (73–76).

Activated hepatic iNKT cells have been implicated in liver damage and fibrosis in pathological conditions like hepatitis, chronic inflammation, and sterile liver injury, in addition to their immunomodulatory roles. For instance, hepatic iNKT cells can

TABLE 1 Heterogeneity of tissue-resident iNKT cells in mice.

Tissue	Localization	Feature
Thymus	Matured cells distribute in the thymic medulla	• NKT1 and NKT2: phenotypic and functional heterogeneity (47) • NKT17: relatively uniform (47) • Require CD28 co-stimulation (48)
Liver	Patrol along the liver sinusoids	• Activated by CD1d-dependent lipid antigen presentation (49) • CD1d and IL-7 dependent cell maintenance (49, 50) • Higher expression of Bcl-2 and Socs2 (12) • Higher proliferation status (12) • Related to hepatocyte cell death and liver damage (14, 49) • LFA-1, CXCR6, and CD69 for residence and maintenance (41, 51–54) • NKT17: promote cancer cell extravasation (55)
Lung	Vasculature and interstitial tissue	• Exacerbate inflammation in allergies (45, 56) • Th2 cytokine expression (45, 56) • ICOS-ICOSL dependent cytokine production (56) • Enriched transcripts of bZIP and NF-kB family (44) • CCR4, CCR9, and CXCR6 for migration and maintenance (41, 57)
Gut	Prevalent in the lamina propria than in the epithelial layer	• Activated by dietary components, with microbial metabolites containing lipid molecules (58) • Modulate gut inflammation, tissue homeostasis, and microbiota (59–61) • Impacted by commensal microbiota (62–64)
Adipose tissue	-	• Frequency of NKT10 cells is significantly high (32) • Regulate the anti-inflammatory immune cells, such as M2 macrophages and Treg cells (65, 66) • Higher expression of GLUT1 and CD36 for glycolytic and fatty acid metabolism (67) • Metabolically active and require AMPK pathway to regulate adipose tissue homeostasis during inflammation (67)

induce FASL expression and release TNF, perforin, and granzymes, leading to hepatocyte cell death (14, 49). Liver-resident NKT17 cells secrete IL-22, which has been found to promote cancer cell extravasation during liver metastasis (55). Additionally, iNKT cells found in the liver, spleen, and mucosal tissues express P2RX7, an ATP-sensitive purinergic receptor that plays a crucial role in the survival of tissue-resident lymphocytes (77, 78). In the liver, the depletion of iNKT cells, dependent on P2RX7, functions as a feedback mechanism to limit tissue damage related to iNKT cells, leading to a tolerogenic microenvironment.

iNKT cells are present in the lung's vasculature and interstitial tissues in mice, contributing to an effective immune response against pulmonary infections (79–81). They are essential in protecting against viral and bacterial infections, often activated in a CD1d-dependent manner, with cytokine production, such as IFN-γ or IL-17. Conversely, activated pulmonary iNKT cells have also been shown to exacerbate inflammation in allergies. Studies utilizing the OVA-induced model of allergic asthma have reported that iNKT cells produce IL-4, IL-5, IL-10, IL-13, and IFN-γ in an inducible T cell costimulator (ICOS)-ICOS ligand (ICOSL)-dependent manner (45, 56). Transcripts encoding AP-1 and other members of the basic leucine zipper (bZIP) family, as well as some members of the NF-κB family, are enriched in lung iNKT cell subsets, as are transcripts encoding CTLA-4, CD69, and Nur77 (44).

In mouse and human gut, iNKT cells are more prevalent in the lamina propria than in the epithelial layer (62, 82). These cells can be activated and impacted by dietary components, with microbial metabolites containing lipid molecules presented by CD1d (58). Intestinal iNKT cells emerge prior to microbial colonization and contribute to enhancing or suppressing immune responses. They modulate gut inflammation, tissue homeostasis, and microbiota by binding with CD1d and expressing IFN-γ (59–61). On the other hand, the commensal microbiota impacts iNKT cells (62, 63). For example, intestinal iNKT cell numbers are brought back to normal levels in neonatal germ-free mice through monocolonization with *B. fragilis* or exposure to the purified glycolipid antigen of this bacterium (64). Furthermore, environmental differences can modulate the frequency, Vβ usage, and cytokine production of intestinal iNKT cells in mice, despite expressing CD69, CD44, and CD122, similarly to their splenic counterparts (62).

iNKT cells are enriched in the visceral adipose tissue (VAT) in mice and humans (32, 65, 66). The frequency of IL-10-producing NKT10 cells is significantly high in the iNKT cells in the adipose tissue. Therefore, the tissue-resident iNKT cells in the adipose tissue regulate the homeostasis of anti-inflammatory immune cells, such as M2 macrophages and Treg cells, and maintain tissue quiescence. Furthermore, stimulating iNKT cells in the adipose tissue *in vivo* leads to the expansion of iNKT cells and the production of IL-10 and IL-4, which can induce M2 macrophages (65). iNKT cells interact with M2 macrophages, resulting in IL-4 and IL-13 production, while interaction with M1 macrophages triggers IFN-γ production by iNKT cells. A recent study showed that proteins necessary for glycolytic and fatty acid metabolism, such as GLUT1 and CD36, are higher in the adipose tissue iNKT cells than in hepatic and spleen iNKT cells (67). Additionally, adipose tissue iNKT cells are metabolically active and require the AMP-activated protein kinase (AMPK) pathway to regulate adipose tissue homeostasis during inflammation induced by obesity.

In addition, chemoattractants, integrins, and lectins drive tissue-specific differences in mouse iNKT cells. The CXC chemokine receptor 5 (CXCR5) is expressed by a subset of iNKT cells in the spleen, leading to homing to lymphoid organs in response to CXCL13 (83). For iNKT cells in the lung, their migration or maintenance heavily depends on several chemokine receptors, including CCR4, CCR9, and CXCR6 (41, 57). PLZF, a critical transcription factor for iNKT cell development, upregulates LFA-1 in the liver for the intravascular residence of hepatic iNKT cells (41). Furthermore, CXCR6 and Id2 play a critical role in the accumulation and maintenance of hepatic iNKT cells (51, 52). CD69, a C-type lectin receptor, which inhibits S1PR1 and is vital for tissue residency of T cells, is increased on non-circulating iNKT cells within the liver (53, 54). More recently, a study examined the establishment of tissue residency following lineage commitment of iNKT cells. The study classified iNKT cells into three populations: resident memory ($iNKT_{RM}$, $CD69^{+}CD62L^{-}$), effector memory ($iNKT_{EM}$, $CD69^{-}CD62L^{-}$), and central memory ($iNKT_{CM}$, $CD69^{-}CD62L^{+}$) cells using the C-type lectin receptor CD69 and the lymph node homing receptor CD62L (84). Hobit and its homolog Blimp-1 function as major regulators of tissue retention across various tissue-resident lymphocytes. Hobit expression is increased in $iNKT_{RM}$ cells with high expression of tissue-resident molecules, such as CD49a, CXCR6, and P2RX7. This implies that Hobit and Blimp-1 direct not only iNKT cell differentiation but also tissue-resident features.

Furthermore, studies have examined the significance of metabolism in T cell development, proliferation, and effector functions (85). Concerning iNKT cells, maintaining elevated levels of oxidative phosphorylation (OXPHOS) seems to be crucial for sustaining iNKT cell survival, while glutaminolysis may contribute to the regulation of activation-induced iNKT cell expansion in mice (86). Additionally, the regulation of tissue-specific immunometabolism in mouse iNKT cells has been found to impact liver injury and inflammation caused by obesity (67). Nevertheless, the effects of nutrient availability in the tissue microenvironment on the metabolic programming of tissue-resident iNKT cells at steady state remain largely unknown. Although iNKT cells are infrequent *in vivo*, a new flow cytometry-based single-cell metabolism profiling technique used to study γδ T cell and ILC2 metabolism may be able to analyze the metabolic needs of diverse tissue-resident iNKT cells in the future (87, 88). Consequently, various local tissue factors related to cell distribution, differentiation, survival, activation, and metabolism contribute to the heterogeneity of iNKT cells.

Thymic IL-15 niche-dependent circulating iNKT cells

Coordinated cytokine expression by diverse stromal cells in tissue-specific microenvironments regulates the distinct

lymphocyte development and immune response in various tissues. Specifically, IL-15 is a crucial cytokine for the development, maintenance, and function of iNKT cells in the thymus and peripheral tissues. IL-15-expressing cells have been identified in primary and secondary lymphoid organs using several IL-15 reporter mouse lines, such as IL-15-cyan fluorescent protein (CFP) knock-in mice and IL-15 bacterial artificial chromosome (BAC)-emerald green fluorescent protein (EmGFP) mice (34, 89–91). Various stromal cells with a unique distribution highly express IL-15 *in vivo*, in addition to hematopoietic cells such as macrophages and dendritic cells. IL-15-expressing cells are mainly situated in the thymic medulla, the primary source of which is MHC class II high mTECs. In the bone marrow, IL-15-expressing cells can be found dispersed throughout the marrow cavity, with a fraction of CXCL12-abundant reticular (CAR) cells expressing high levels of IL-15. Within the T-cell zone of the spleen, IL-15-expressing cells are also distributed. In lymph nodes, IL-15 is expressed in all high endothelial venules (HEVs) and a fraction of fibroblastic reticular cells (FRCs) in the T-cell zone and medulla. Intestinal epithelial cells express IL-15 in the intestine. Additionally, blood vascular endothelial cells (BECs) and lymphatic endothelial cells (LECs) upregulate IL-15 expression during inflammatory conditions.

To determine whether distinct IL-15 niches regulate iNKT cell heterogeneity, Cui et al. established an IL-15-floxed mouse model that enables dissection of the local function of IL-15 in various immune microenvironments through crossing with multiple tissue-specific Cre reporter mice (11). The FoxN1-Cre IL-15 conditional knockout (cKO) mice, deficient in IL-15 expression in mTECs, were utilized to elucidate the thymic IL-15 niche for iNKT cells. The FoxN1-Cre IL-15 cKO mice exhibit a reduction in NKT1 cells, but not NKT2 or NKT17 cells, which aligns with a preceding study confirming the indispensability of TEC-derived IL-15 for type 1 innate-like T cells, including iNKT cells (92). Subsequently, mature stage 3 iNKT cells, which are nearly identical to NKT1 cells in mice, can be classified into three subpopulations based on the presence of the NK cell receptor CD244 (also known as 2B4 or SLAMF4) and the chemokine receptor CXCR6: $CD244^{+}CXCR6^{+}$ double-positive C2 iNKT cells, $CD244^{-}CXCR6^{+}$ single-positive C1 iNKT cells, and $CD244^{-}CXCR6^{-}$ double-negative C0 iNKT cells (11). Notably, C2 iNKT cells have nearly vanished, while C1 and C0 iNKT cells have only slightly decreased or stayed the same in the thymus of FoxN1-Cre IL-15 cKO mice. Additionally, although C1 iNKT cells recuperate, C2 iNKT cells remain significantly reduced in peripheral tissues, including the spleen, lung, and liver of FoxN1-Cre IL-15 cKO mice. Furthermore, IL-15 production by mTECs leads to the development and maturation of C0 iNKT cells into C2 and C1 iNKT cells in the thymus. The findings suggest that the development and maturation of C2 iNKT cells rely significantly on the thymic epithelial IL-15 niche. Moreover, a recent study showed that thymic NKT1 cell heterogeneity necessitates TGF-β and IL-15 (93).

Digital RNA-seq showed differential gene expression between two mature thymic iNKT cell subsets, C1 iNKT cells and C2 iNKT cells (11). Besides the NK cell receptor CD244, C2 iNKT cells express KLF2, a transcription factor for NK cell homeostasis (94). KLF2 expression is high in $CCR7^{+}$ iNKT cell progenitors but diminishes during their differentiation into iNKT cell subsets (37). Functionally, C2 iNKT cells express high levels of cytotoxicity-related genes such as IFN-γ and granzymes (*Gzma* and *Gzmb*) at steady state. They also express high levels of chemokines (*Ccl5*), integrins (*Itga1*, also known as *Cd49a*), galectins (*Lgals1*), and multiple killer cell lectin-like receptors (KLRs). Consequently, C2 iNKT cells exhibit properties similar to NK cells. On the other hand, C1 iNKT cells exhibit more T cell-like properties and express genes related to TCR signal strength or T cell activation, including *Icos*, *Lef1*, *Zap70*, and *Cd5*. The heterogeneity of NKT1 in the thymus has recently been identified through scRNA-seq (47), with the NK cell-related signature SLAMF4 (CD244) distinguishing the terminally differentiated NKT1 cell cluster. The $SLAMF4^{+}$ iNKT cell cluster showed high levels of granzyme A and IFN-γ cytotoxic mediators, but not IL-4. Additionally, these $Gzma^{+}SLAMF4^{+}$ iNKT cells are detected in peripheral organs.

The dynamic heterogeneity between C2 and C1 iNKT cells in peripheral tissues has been further elucidated (11). Parabiosis experiments revealed that C2 iNKT cells are a newly identified circulating subset of iNKT cells, while C1 iNKT cells are a conventional tissue-resident subset of iNKT cells. The regulation of C2 iNKT cell migration and retention in peripheral tissues is governed by the integrin α1 (CD49a) and S1P receptors S1PR1 and S1PR4, while C1 iNKT cells require CXCR6 for their tissue residency and express high levels of P2RX7. Interestingly, C2 iNKT cells express KLF2, a transcription factor regulating T cell migration by directly controlling cell surface receptors S1PR1 and CCR7 (94, 95). A recent study showed the circulatory signature of iNKT cells, such as *Klf2*, *Cd62l*, *S1pr1*, and *S1pr4* expression, identified through scRNA-seq. Additionally, the study demonstrated that KLF2 regulates the migration and differentiation of iNKT cells by using KLF2-deficient mice (12).

Functionally, C2 iNKT cells play a role in anti-tumor and antiviral immunity (11). Lung metastasis of melanoma cells is exacerbated in mice without C2 iNKT cells, suggesting that conventional tissue-resident C1 iNKT cells alone may be insufficient for mediating anti-tumor immunity. Moreover, rapid immune responses by circulating C2 iNKT cells may be essential for initiating anti-tumor immunity. C2 iNKT cells protect mice from lung metastasis of melanoma cells and show elevated direct tumor-killing capacity in cultures with elevated expression levels of cytotoxic molecules such as IFN-γ, granzyme B, perforin, and TRAIL. Consequently, these C2 iNKT cells circulate throughout the periphery to carry out cancer immunosurveillance and work as a powerful tumor-suppressing subset of iNKT cells. Peripheral C2 iNKT cells express TLR2, IL-1β, and galectin-1 at high levels, which play a crucial role in the immune response against bacteria and influenza viruses (96, 97). Furthermore, they secrete significant amounts of chemokines such as CCL2, CCL5, and CXCL2, which are associated with the recruitment of immune cells and are increased in influenza and coronavirus infections (98). In addition, due to their ability to reduce influenza type A virus (IAV)-induced MDSCs and

the restricted maintenance of MDSCs by apolipoprotein E (ApoE), it is presumed that C2 iNKT cells function as a potent MDSC-suppressing subset during IAV infection, facilitated by their high expression of ApoE (81, 99). Consequently, C2 iNKT cells accelerate virus clearance during influenza A virus infection. However, FoxN1-Cre IL-15 cKO mice showed less body weight loss and lower mortality following a sublethal dose of IAV infection. This suggests that circulating C2 iNKT cells play a significant role in regulating the development of IAV-induced acute lung injury, leading to disease progression and increased mortality.

Human CD244$^+$ C2 iNKT cells have also been found in peripheral blood as counterparts to mouse C2 iNKT cells (11). Human C2 iNKT cells are part of the CD4$^-$ iNKT cell fraction that highly expresses genes related to cytotoxicity, such as granzymes, perforin, and granulysin, which are essential for iNKT cell-mediated effector functions in anti-tumor and antiviral immunity. In contrast, human C1 iNKT cells can be found in CD4$^+$ and CD4$^-$ iNKT cell fractions. In addition, human C2 iNKT cells express CCR5, a chemokine receptor involved in human T cell trafficking (100). Interestingly, in the peripheral blood of aging humans, the frequency of C2 iNKT cells decreases, likely due to their reliance on the thymic microenvironment. Overall, IL-15 expression by mTECs in the thymic microenvironment tightly regulates the development of circulating C2 iNKT cells, while tissue-resident C1 iNKT cells are less reliant on this thymic epithelial IL-15 niche. mTEC-derived IL-15 is important for conferring high cytotoxicity and NK cell-like properties to C2 iNKT cells and contributes to the generation of iNKT cell heterogeneity (Figure 1).

Numerous studies utilize agonists such as α-galactosylceramide or PMA/ionomycin to stimulate iNKT cells and evaluate their functional potential. However, the regulation of activation and functional heterogeneity of iNKT cells *in vivo* remains unresolved. As the circulating and tissue-resident iNKT cells seem to have different dependencies on the IL-15 niche in individual tissue, combining the IL-15-floxed mouse model with multiple tissue-specific Cre reporter mice may be a useful tool for dissecting the functional heterogeneity of tissue-resident iNKT cells, especially NKT1 cells *in vivo*.

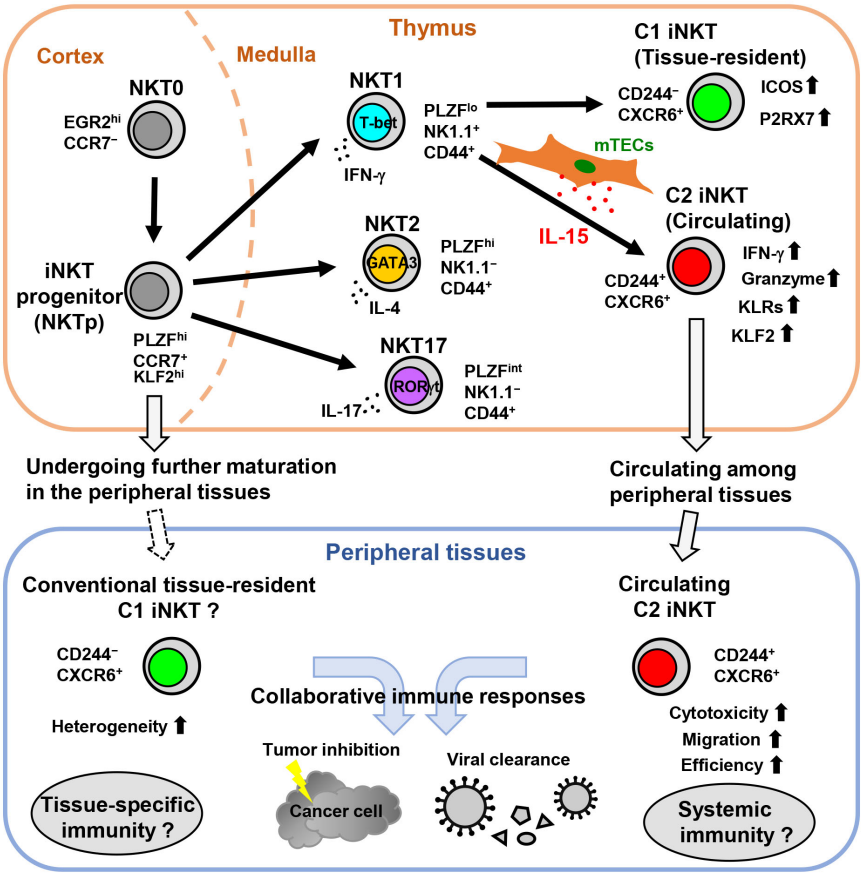

FIGURE 1
The development of circulating C2 iNKT cells is driven by the thymic epithelial IL-15 niche in mice. In the thymus, NKT0 cells differentiate into CCR7$^+$ iNKT cell progenitors (NKTp). NKTp cells can then emigrate from the thymus to peripheral tissues and undergo further maturation or continue their differentiation into effector subsets, including NKT1, NKT2, or NKT17. IL-15 produced by mTECs drives the development and terminal maturation of C2 iNKT cells in the NKT1 cell population. Although most mature iNKT cells in the thymus are tissue-resident, the C2 iNKT cells exhibit high cytotoxicity with NK cell-like features and circulate among peripheral tissues. Subsequently, C2 iNKT cells serve as the circulating iNKT cells in peripheral tissues, whereas C1 iNKT cells are conventional tissue-resident iNKT cells.

Heterogeneity of iNKT cells in cancer immunotherapy

Due to their diverse immunoregulatory functions in innate and adaptive immunity against cancer and infection, iNKT cells are considered an attractive candidate for cancer immunotherapy. In addition, iNKT cells offer a novel platform and approach to enhance chimeric antigen receptor (CAR)- or engineered TCR-based cancer immunotherapy. An iNKT cell-targeted immunotherapy has been reported to successfully treat advanced stages of non-small cell lung cancer and head and neck cancer (101). Clinical trials using activated iNKT cell adoptive transfer in non-small cell lung cancer have reported no serious adverse effects and have shown an increase in circulating iNKT cell numbers. However, few patients experienced a reduction in tumor progression, likely due to the functional defects and absent long-term efficacy of iNKT cells in cancer patients. Subsequently, patients who receive combined therapy with α-GalCer-loaded DCs, which can enhance iNKT cell activation, show increases in circulating iNKT cell numbers and IFN-γ production, with some patients achieving a partial anti-tumor response (102–104). Prior studies have also demonstrated the effective redirection of human iNKT cells towards hematologic or solid malignancies by engineering these cells to express tumor-specific CARs or TCRs (105, 106). TCR-engineered iNKT (TCR-iNKT) cells that use a second TCR for a tumor-associated peptide create bispecific effectors for CD1d- and MHC-restricted antigens and show higher efficacy in inhibiting the progression of multiple tumors expressing the cognate antigen compared to $CD8^+$ T cells engineered with the same TCR (107).

To date, iNKT cells have been isolated from peripheral blood mononuclear cells (PBMCs) of patients and expanded ex vivo before adoptive transfer into the patient. However, iNKT cell-based immunotherapy faces several significant challenges, such as the limited number of iNKT cells obtained from peripheral blood, weak activity, and low tissue specificity of ex vivo expanded iNKT cells. To enhance the effectiveness and tissue-specificity of iNKT cell-based immunotherapy across various tissues, it is necessary to examine the diversity of tissue-resident and circulating iNKT cells. For instance, the human $CD244^+$ C2 iNKT cells, which correspond to the circulating C2 iNKT cells in mice, exhibit robust cytotoxicity owing to their high expression of granzyme B, perforin, and granulysin. Utilizing this subset of iNKT cells may amplify the therapeutic efficacy of iNKT cell-based immunotherapy. Thus, depending on the type of target cancer, chemokine receptors for migration, cytokines, and co-stimulatory factors for survival, proliferation, and activation of tissue-resident or circulating iNKT cells can be utilized.

Graft-versus-host disease (GVHD) is a common and serious complication of allogeneic transplantation and CAR-T cell immunotherapies. Recipient tissue cells express high levels of MHC class I and II molecules, resulting in broad binding of donor T cells with highly variable TCRs. iNKT cells, on the other hand, can escape MHC recognition because they are not restricted by MHC, unlike T cells. iNKT cells have been shown to modulate and reduce the GVHD response in transplant patients. For instance, it has been proposed that $CD4^+$ iNKT cells control GVHD via their immunoregulatory function through IL-4 (108). A recent study used scRNA-seq to analyze iNKT cells from healthy individuals and GVHD patients (109). The findings revealed that $CD4^-CD94^+$ iNKT cells with substantial cytotoxic potency are associated with GVHD control, possibly by inducing DC death. Notably, all human C2 iNKT cells are $CD4^-$, and substantial cytotoxicity is observed due to elevated CD94 (also known as KLRD1) levels, similar to the $CD4^-CD94^+$ iNKT cells. These cells show potential as a platform for CAR-NKT or engineered TCR-NKT cells.

Immunotoxicity is an adverse effect of drugs like immune checkpoint inhibitors on the immune system. It frequently arises in organs with abundant tissue-resident lymphocytes, such as the liver, lung, and intestine (110, 111). The mechanism underlying tissue-specific immunotoxicity is largely unknown, making it challenging to predict. Because tissue-resident iNKT cells are enriched in these tissues and change with age, understanding the heterogeneity of iNKT cells may offer a new approach to evaluate and reduce tissue-specific drug-induced immunotoxicity.

A phase I clinical trial investigated autologous iNKT cells that co-express a GD2-specific CAR with IL-15 (GD2-CAR-NKT) against neuroblastoma. The trial showed that CAR-NKT cells express elevated levels of CD62L, and responders have a higher abundance of CAR-NKT cells with elevated KLF2 expression than non-responders (112). Furthermore, GD2-CAR-NKT cells exhibit similar or reduced toxicity, such as cytokine release syndrome (CRS), compared to conventional GD2-CAR T cells targeting the same antigen in neuroblastoma patients. These results suggest that a specific subset of iNKT cells may primarily impact the efficacy of CAR NKT cell immunotherapy. Besides the highly cytotoxic nature of human C2 iNKT cells, they express high levels of KLF2. Therefore, understanding the heterogeneity of iNKT cells could assist the direction of CAR-NKT cell engineering for various cancer types and offer valuable biomarkers for predicting the efficacy and immunotoxicity of iNKT cell-based immunotherapies.

Conclusion

iNKT cells exhibit heterogeneity during their developmental stages and depending on their location within various tissues. This heterogeneity arises from the differential expression of transcription factors, as well as distinctive gene expression profiles linked to iNKT cell kinetics, distribution, and effector functions within different tissues. IL-15 production by mTECs in the thymic microenvironment contributes to the acquisition of heterogeneity among iNKT cells. In mice, the thymic epithelial IL-15 niche strictly controls the development of a novel circulating subset of NKT1 cells called C2 iNKT cells (Figure 1). These C2 iNKT cells express high levels of cytotoxic molecules and exhibit more NK cell-like characteristics, while the conventional tissue-resident C1 iNKT cells exhibit more T cell-like characteristics. In addition, human C2 iNKT cells with high cytotoxicity have also been identified in peripheral blood as counterparts to mouse C2 iNKT cells. Understanding the heterogeneity of iNKT cells could offer a new strategy to enhance and predict the efficacy of iNKT cell-based

immunotherapies and to assess and reduce tissue-specific immunotoxicity.

Author contributions

GC: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. SA: Writing – review & editing. RK: Writing – review & editing. KI: Conceptualization, Funding acquisition, Writing – review & editing, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grants 21K07067 and 19K16687 (GC), and 23H02735 and 20H03501 (KI); Grants from the Takeda Science Foundation (GC), and the Shimizu Foundation for Immunology and Neuroscience (GC). It was also supported by the Joint Usage Research Center program of the Institute for Life and Medical Sciences, Kyoto University.

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

References

1. Gapin L, Matsuda JL, Surh CD, Kronenberg M. NKT cells derive from double-positive thymocytes that are positively selected by CDId. *Nat Immunol* (2001) 2:971–8. doi: 10.1038/ni710

2. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* (2007) 25:297–336. doi: 10.1146/annurev.immunol.25.022106.141711

3. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* (2013) 13:101–17. doi: 10.1038/nri3369

4. Pellicci DG, Clarke AJ, Patel O, Mallevaey T, Beddoe T, Le Nours J, et al. Recognition of β-linked self glycolipids mediated by natural killer T cell antigen receptors. *Nat Immunol* (2011) 12:827–33. doi: 10.1038/ni.2076

5. Reilly EC, Wands JR, Brossay L. Cytokine dependent and independent iNKT cell activation. *Cytokine* (2010) 51:227–31. doi: 10.1016/j.cyto.2010.04.016

6. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-γ-producing NKT response induced with α-galactosylceramide-loaded DCs. *Nat Immunol* (2002) 3:867–74. doi: 10.1038/ni827

7. Lee YJ, Holzapfel KL, Zhu JF, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol* (2013) 14:1146–54. doi: 10.1038/ni.2731

8. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol* (2008) 20:358–68. doi: 10.1016/j.coi.2008.03.018

9. Diaz-Basabe A, Strati F, Facciotti F. License to kill: when iNKT cells are granted the use of lethal cytotoxicity. *Int J Mol Sci* (2020) 21:3909. doi: 10.3390/ijms21113909

10. Look A, Burns D, Tews I, Roghanian A, Mansour S. Towards a better understanding of human iNKT cell subpopulations for improved clinical outcomes. *Front Immunol* (2023) 14:1176724. doi: 10.3389/fimmu.2023.1176724

11. Cui G, Shimba A, Jin J, Ogawa T, Muramoto Y, Miyachi H, et al. A circulating subset of iNKT cells mediates antitumor and antiviral immunity. *Sci Immunol* (2022) 7: eabj8760. doi: 10.1126/sciimmunol.abj8760

12. Wang J, Loveless I, Adrianto I, Liu T, Subedi K, Wu X, et al. Single-cell analysis reveals differences among iNKT cells colonizing peripheral organs and identifies Klf2 as a key gene for iNKT emigration. *Cell Discovery* (2022) 8:75. doi: 10.1038/s41421-022-00432-z

13. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-specific distribution of iNKT cells impacts their cytokine response. *Immunity* (2015) 43:566–78. doi: 10.1016/j.immuni.2015.06.025

14. Crosby CM, Kronenberg M. Tissue-specific functions of invariant natural killer T cells. *Nat Rev Immunol* (2018) 18:559–74. doi: 10.1038/s41577-018-0034-2

15. Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, Hennighausen L, et al. Genetic evidence supporting selection of the Vα14i NKT cell lineage from double-positive thymocyte precursors. *Immunity* (2005) 22:705–16. doi: 10.1016/j.immuni.2005.03.011

16. Bezbradica JS, Hill T, Stanic AK, Van Kaer L, Joyce S. Commitment toward the natural T (iNKT) cell lineage occurs at the $CD4^+8^+$ stage of thymic ontogeny. *Proc Natl Acad Sci USA* (2005) 102:5114–9. doi: 10.1073/pnas.0408449102

17. Tani-ichi S, Shimba A, Wagatsuma K, Miyachi H, Kitano S, Imai K, et al. Interleukin-7 receptor controls development and maturation of late stages of thymocyte subpopulations. *Proc Natl Acad Sci USA* (2013) 110:612–7. doi: 10.1073/pnas.1219242110

18. Cui G, Shimba A, Ma GY, Takahara K, Tani-ichi S, Zhu YB, et al. IL-7R-dependent phosphatidylinositol 3-kinase competes with the STAT5 signal to modulate T cell development and homeostasis. *J Immunol* (2020) 204:844–57. doi: 10.4049/jimmunol.1900456

19. Dashtsoodol N, Shigeura T, Aihara M, Ozawa R, Kojo S, Harada M, et al. Alternative pathway for the development of $V\alpha14^+$ NKT cells directly from $CD4^-CD8^-$ thymocytes that bypasses the $CD4^+CD8^+$ stage. *Nat Immunol* (2017) 18:274–82. doi: 10.1038/ni.3668

20. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li BF, et al. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* (2008) 29:391–403. doi: 10.1016/j.immuni.2008.07.011

21. Seiler MP, Mathew R, Liszewski MK, Spooner CJ, Barr K, Meng F, et al. Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling. *Nat Immunol* (2012) 13:264–71. doi: 10.1038/ni.2230

22. Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A. A thymic precursor to the NK T cell lineage. *Science* (2002) 296:553–5. doi: 10.1126/science.1069017

23. Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DI. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent $NK1.1^-CD4^+$ CD1d-dependent precursor stage. *J Exp Med* (2002) 195:835–44. doi: 10.1084/jem.20011544

24. Engel I, Seumois G, Chavez L, Samaniego-Castruita D, White B, Chawla A, et al. Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat Immunol* (2016) 17:728–39. doi: 10.1038/ni.3437

25. Georgiev H, Ravens I, Benarafa C, Forster R, Bernhardt G. Distinct gene expression patterns correlate with developmental and functional traits of iNKT subsets. *Nat Commun* (2016) 7:13116. doi: 10.1038/ncomms13116

26. Lee YJ, Starrett GJ, Lee ST, Yang R, Henzler CM, Jameson SC, et al. Lineage-specific effector signatures of invariant NKT cells are shared amongst γδ T, innate lymphoid, and Th cells. *J Immunol* (2016) 197:1460–70. doi: 10.4049/jimmunol.1600643

27. Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-

producing CD4$^-$NK1.1$^-$ NKT cell population. *Proc Natl Acad Sci USA* (2008) 105:11287–92. doi: 10.1073/pnas.0801631105

28. Dai H, Rahman A, Saxena A, Jaiswal AK, Mohamood A, Ramirez L, et al. Syndecan-1 identifies and controls the frequency of IL-17-producing naive natural killer T (NKT17) cells in mice. *Eur J Immunol* (2015) 45:3045–51. doi: 10.1002/eji.201545532

29. Georgiev H, Ravens I, Shibuya A, Forster R, Bernhardt G. CD155/CD226-interaction impacts on the generation of innate CD8$^+$ thymocytes by regulating iNKT-cell differentiation. *Eur J Immunol* (2016) 46:993–1003. doi: 10.1002/eji.201546073

30. Klibi J, Li S, Amable L, Joseph C, Brunet S, Delord M, et al. Characterization of the developmental landscape of murine RORγt$^+$ iNKT cells. *Int Immunol* (2020) 32:105–16. doi: 10.1093/intimm/dxz064

31. Chang PP, Barral P, Fitch J, Pratama A, Ma CS, Kallies A, et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol* (2011) 13:35–43. doi: 10.1038/ni.2166

32. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* (2014) 124:3725–40. doi: 10.1172/JCI72308

33. Gordy LE, Bezbradica JS, Flyak AI, Spencer CT, Dunkle A, Sun JC, et al. IL-15 regulates homeostasis and terminal maturation of NKT cells. *J Immunol* (2011) 187:6335–45. doi: 10.4049/jimmunol.1003965

34. Cui G, Hara T, Simmons S, Wagatsuma K, Abe A, Miyachi H, et al. Characterization of the IL-15 niche in primary and secondary lymphoid organs. *vivo. Proc Natl Acad Sci USA* (2014) 111:1915–20. doi: 10.1073/pnas.1318281111

35. Lucas B, White AJ, Cosway EJ, Parnell SM, James KD, Jones ND, et al. Diversity in medullary thymic epithelial cells controls the activity and availability of iNKT cells. *Nat Commun* (2020) 11:2198. doi: 10.1038/s41467-020-16041-x

36. White AJ, Jenkinson WE, Cowan JE, Parnell SM, Bacon A, Jones ND, et al. An essential role for medullary thymic epithelial cells during the intrathymic development of invariant NKT cells. *J Immunol* (2014) 192:2659–66. doi: 10.4049/jimmunol.1303057

37. Wang H, Hogquist KA. CCR7 defines a precursor for murine iNKT cells in thymus and periphery. *eLife* (2018) 7:e34793. doi: 10.7554/eLife.34793

38. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human Vα24 natural killer T cells. *J Exp Med* (2002) 195:637–41. doi: 10.1084/jem.20011908

39. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* (2002) 195:625–36. doi: 10.1084/jem.20011786

40. Moreira-Teixeira L, Resende M, Devergne O, Herbeuval JP, Hermine O, Schneider E, et al. Rapamycin combined with TGF-β converts human invariant NKT cells into suppressive Foxp3$^+$ regulatory cells. *J Immunol* (2012) 188:624–31. doi: 10.4049/jimmunol.1102281

41. Thomas SY, Scanlon ST, Griewank KG, Constantinides MG, Savage AK, Barr KA, et al. PLZF induces an intravascular surveillance program mediated by long-lived LFA-1-ICAM-1 interactions. *J Exp Med* (2011) 208:1179–88. doi: 10.1084/jem.20102630

42. Lynch L, Michelet X, Zhang S, Brennan PJ, Moseman A, Lester C, et al. Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T_{reg} cells and macrophages in adipose tissue. *Nat Immunol* (2015) 16:85–95. doi: 10.1038/ni.3047

43. Berzins SP, McNab FW, Jones CM, Smyth MJ, Godfrey DI. Long-term retention of mature NK1.1$^+$ NKT cells in the thymus. *J Immunol* (2006) 176:4059–65. doi: 10.4049/jimmunol.176.7.4059

44. Murray MP, Engel I, Seumois G, Herrera-De la Mata S, Rosales SL, Sethi A, et al. Transcriptome and chromatin landscape of iNKT cells are shaped by subset differentiation and antigen exposure. *Nat Commun* (2021) 12:1446. doi: 10.1038/s41467-021-21574-w

45. Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med* (2003) 9:582–8. doi: 10.1038/nm851

46. Kim EY, Battaile JT, Patel AC, You Y, Agapov E, Grayson MH, et al. Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. *Nat Med* (2008) 14:633–40. doi: 10.1038/nm1770

47. Wang J, Adrianto I, Subedi K, Liu T, Wu X, Yi Q, et al. Integrative scATAC-seq and scRNA-seq analyses map thymic iNKT cell development and identify Cbfβ for its commitment. *Cell Discovery* (2023) 9:61. doi: 10.1038/s41421-023-00547-x

48. Shissler SC, Singh NJ, Webb TJ. Thymic resident NKT cell subsets show differential requirements for CD28 co-stimulation during antigenic activation. *Sci Rep* (2020) 10:8218. doi: 10.1038/s41598-020-65129-3

49. Gu X, Chu Q, Ma X, Wang J, Chen C, Guan J, et al. New insights into iNKT cells and their roles in liver diseases. *Front Immunol* (2022) 13:1035950. doi: 10.3389/fimmu.2022.1035950

50. Liang B, Hara T, Wagatsuma K, Zhang J, Maki K, Miyachi H, et al. Role of hepatocyte-derived IL-7 in maintenance of intrahepatic NKT cells and T cells and development of B cells in fetal liver. *J Immunol* (2012) 189:4444–50. doi: 10.4049/jimmunol.1201181

51. Wehr A, Baeck C, Heymann F, Niemietz PM, Hammerich L, Martin C, et al. Chemokine receptor CXCR6-dependent hepatic NK T Cell accumulation promotes inflammation and liver fibrosis. *J Immunol* (2013) 190:5226–36. doi: 10.4049/jimmunol.1202909

52. Monticelli LA, Yang Y, Knell J, D'Cruz LM, Cannarile MA, Engel I, et al. Transcriptional regulator Id2 controls survival of hepatic NKT cells. *Proc Natl Acad Sci USA* (2009) 106:19461–6. doi: 10.1073/pnas.0908249106

53. Mackay LK, Minnich M, Kragten NA, Liao Y, Nota B, Seillet C, et al. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science* (2016) 352:459–63. doi: 10.1126/science.aad2035

54. Shiow LR, Rosen DB, Brdickova N, Xu Y, An J, Lanier LL, et al. CD69 acts downstream of interferon-α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* (2006) 440:540–4. doi: 10.1038/nature04606

55. Giannou AD, Kempski J, Shiri AM, Lucke J, Zhang T, Zhao L, et al. Tissue resident iNKT17 cells facilitate cancer cell extravasation in liver metastasis via interleukin-22. *Immunity* (2023) 56:125–42. doi: 10.1016/j.immuni.2022.12.014

56. Akbari O, Stock P, Meyer EH, Freeman GJ, Sharpe AH, Umetsu DT, et al. ICOS/ICOSL interaction is required for CD4$^+$ invariant NKT cell function and homeostatic survival. *J Immunol* (2008) 180:5448–56. doi: 10.4049/jimmunol.180.8.5448

57. Meyer EH, Wurbel MA, Staton TL, Pichavant M, Kan MJ, Savage PB, et al. iNKT cells require CCR4 to localize to the airways and to induce airway hyperreactivity. *J Immunol* (2007) 179:4661–71. doi: 10.4049/jimmunol.179.7.4661

58. Rossjohn J, Pellicci DG, Patel O, Gapin L, Godfrey DI. Recognition of CD1d-restricted antigens by natural killer T cells. *Nat Rev Immunol* (2012) 12:845–57. doi: 10.1038/nri3328

59. Saez de Guinoa J, Jimeno R, Gaya M, Kipling D, Garzon MJ, Dunn-Walters D, et al. CD1d-mediated lipid presentation by CD11c$^+$ cells regulates intestinal homeostasis. *EMBO J* (2018) 37:e97537. doi: 10.15252/embj.201797537

60. Dowds CM, Blumberg RS, Zeissig S. Control of intestinal homeostasis through crosstalk between natural killer T cells and the intestinal microbiota. *Clin Immunol* (2015) 15:128–33. doi: 10.1016/j.clim.2015.05.008

61. Nieuwenhuis EE, Matsumoto T, Lindenbergh D, Willemsen R, Kaser A, Simons-Oosterhuis Y, et al. Cd1d-dependent regulation of bacterial colonization in the intestine of mice. *J Clin Invest* (2009) 119:1241–50. doi: 10.1172/JCI36509

62. Wingender G, Stepniak D, Krebs P, Lin L, McBride S, Wei B, et al. Intestinal microbes affect phenotypes and functions of invariant natural killer T cells in mice. *Gastroenterology* (2012) 143:418–28. doi: 10.1053/j.gastro.2012.04.017

63. Olszak T, An DD, Zeissig S, Vera MP, Richter J, Franke A, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science* (2012) 336:489–93. doi: 10.1126/science.1219328

64. An DD, Oh SF, Olszak T, Neves JF, Avci FY, Erturk-Hasdemir D, et al. Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. *Cell* (2014) 156:123–33. doi: 10.1016/j.cell.2013.11.042

65. Lynch L, Nowak M, Varghese B, Clark J, Hogan AE, Toxavidis V, et al. Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. *Immunity* (2012) 37:574–87. doi: 10.1016/j.immuni.2012.06.016

66. Huh JY, Kim JI, Park YJ, Hwang IJ, Lee YS, Sohn JH, et al. A novel function of adipocytes in lipid antigen presentation to iNKT cells. *Mol Cell Biol* (2013) 33:328–39. doi: 10.1128/MCB.00552-12

67. Aguiar CF, Correa-da-Silva F, Gonzatti MB, Angelim MK, Pretti MA, Davanzo GG, et al. Tissue-specific metabolic profile drives iNKT cell function during obesity and liver injury. *Cell Rep* (2023) 42:112035. doi: 10.1016/j.celrep.2023.112035

68. White AJ, Baik S, Parnell SM, Holland AM, Brombacher F, Jenkinson WE, et al. A type 2 cytokine axis for thymus emigration. *J Exp Med* (2017) 214:2205–16. doi: 10.1084/jem.20170271

69. Miller CN, Proekt I, von Moltke J, Wells KL, Rajpurkar AR, Wang H, et al. Thymic tuft cells promote an IL-4-enriched medulla and shape thymocyte development. *Nature* (2018) 559:627–31. doi: 10.1038/s41586-018-0345-2

70. Jimeno R, Lebrusant-Fernandez M, Margreitter C, Lucas B, Veerapen N, Kelly G, et al. Tissue-specific shaping of the TCR repertoire and antigen specificity of iNKT cells. *eLife* (2019) 8:e51663. doi: 10.7554/eLife.51663

71. Velazquez P, Cameron TO, Kinjo Y, Nagarajan N, Kronenberg M, Dustin ML. Activation by innate cytokines or microbial antigens can cause arrest of natural killer T cell patrolling of liver sinusoids. *J Immunol* (2008) 180:2024–8. doi: 10.4049/jimmunol.180.4.2024

72. Geissmann F, Cameron TO, Sidobre S, Manlongat N, Kronenberg M, Briskin MJ, et al. Intravascular immune surveillance by CXCR6$^+$ NKT cells patrolling liver sinusoids. *PloS Biol* (2005) 3:e113. doi: 10.1371/journal.pbio.0030113

73. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C, Zhou D, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* (2005) 434:525–9. doi: 10.1038/nature03408

74. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR, et al. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol* (2006) 7:978–86. doi: 10.1038/ni1380

75. Zeissig S, Murata K, Sweet L, Publicover J, Hu Z, Kaser A, et al. Hepatitis B virus-induced lipid alterations contribute to natural killer T cell-dependent protective immunity. *Nat Med* (2012) 18:1060–8. doi: 10.1038/nm.2811

76. Miyaki E, Hiraga N, Imamura M, Uchida T, Kan H, Tsuge M, et al. Interferon alpha treatment stimulates interferon gamma expression in type I NKT cells and

enhances their antiviral effect against hepatitis C virus. *PloS One* (2017) 12:e0172412. doi: 10.1371/journal.pone.0172412

77. Liu QY, Kim CH. Control of tissue-resident invariant NKT cells by vitamin A metabolites and P2X7-mediated cell death. *J Immunol* (2019) 203:1189–97. doi: 10.4049/jimmunol.1900398

78. Bovens AA, Wesselink TH, Behr FM, Kragten NAM, van Lier RAW, van Gisbergen K, et al. Murine iNKT cells are depleted by liver damage via activation of P2RX7. *Eur J Immunol* (2020) 50:1515–24. doi: 10.1002/eji.201948509

79. Scanlon ST, Thomas SY, Ferreira CM, Bai L, Krausz T, Savage PB, et al. Airborne lipid antigens mobilize resident intravascular NKT cells to induce allergic airway inflammation. *J Exp Med* (2011) 208:2113–24. doi: 10.1084/jem.20110522

80. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li XM, et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat Immunol* (2011) 12:966–74. doi: 10.1038/ni.2096

81. De Santo C, Salio M, Masri SH, Lee LYH, Dong T, Speak AO, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. *J Clin Invest* (2008) 118:4036–48. doi: 10.1172/Jci36264

82. Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, et al. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* (2000) 192:741–54. doi: 10.1084/jem.192.5.741

83. Johnston B, Kim CH, Soler D, Emoto M, Butcher EC. Differential chemokine responses and homing patterns of murine TCRαβ NKT cell subsets. *J Immunol* (2003) 171:2960–9. doi: 10.4049/jimmunol.171.6.2960

84. Kragten NA, Taggenbrock RL, Parga Vidal L, van Lier RA, Stark R, van Gisbergen KP. Hobit and Blimp-1 instruct the differentiation of iNKT cells into resident-phenotype lymphocytes after lineage commitment. *Eur J Immunol* (2022) 52:389–403. doi: 10.1002/eji.202149360

85. Rangel Rivera GO, Knochelmann HM, Dwyer CJ, Smith AS, Wyatt MM, Rivera-Reyes AM, et al. Fundamentals of T cell metabolism and strategies to enhance cancer immunotherapy. *Front Immunol* (2021) 12:645242. doi: 10.3389/fimmu.2021.645242

86. Kumar A, Pyaram K, Yarosz EL, Hong H, Lyssiotis CA, Giri S, et al. Enhanced oxidative phosphorylation in NKT cells is essential for their survival and function. *Proc Natl Acad Sci USA* (2019) 116:7439–48. doi: 10.1073/pnas.1901376116

87. Lopes N, McIntyre C, Martin S, Raverdeau M, Sumaria N, Kohlgruber AC, et al. Distinct metabolic programs established in the thymus control effector functions of γδ T cell subsets in tumor microenvironments. *Nat Immunol* (2021) 22:179–92. doi: 10.1038/s41590-020-00848-3

88. Cui G, Shimba A, Jin J, Hojo N, Asahi T, Abe S, et al. CD45 alleviates airway inflammation and lung fibrosis by limiting expansion and activation of ILC2s. *Proc Natl Acad Sci USA* (2023) 120:e2215941120. doi: 10.1073/pnas.2215941120

89. Colpitts SL, Stoklasek TA, Plumlee CR, Obar JJ, Guo C, Lefrancois L. The role of IFN-α receptor and MyD88 signaling in induction of IL-15 expression. *vivo. J Immunol* (2012) 188:2483–7. doi: 10.4049/jimmunol.1103609

90. Zhu Y, Cui G, Miyauchi E, Nakanishi Y, Mukohira H, Shimba A, et al. Intestinal epithelial cell-derived IL-15 determines local maintenance and maturation of intra-epithelial lymphocytes in the intestine. *Int Immunol* (2020) 32:307–19. doi: 10.1093/intimm/dxz082

91. Abe S, Asahi T, Hara T, Cui G, Shimba A, Tani-Ichi S, et al. Hematopoietic cell-derived IL-15 supports NK cell development in scattered and clustered localization within the bone marrow. *Cell Rep* (2023) 42:113127. doi: 10.1016/j.celrep.2023.113127

92. Tao HS, Li L, Liao NS, Schluns KS, Luckhart S, Sleasman JW, et al. Thymic epithelial cell-derived IL-15 and IL-15 receptor α chain foster local environment for type 1 innate like T cell development. *Front Immunol* (2021) 12:623280. doi: 10.3389/fimmu.2021.623280

93. Morgan RC, Frank C, Greger M, Attaway M, Sigvardsson M, Bartom ET, et al. TGF-β promotes the postselection thymic development and peripheral function of IFN-γ-producing invariant NKT cells. *J Immunol* (2023) 211:1376–84. doi: 10.4049/jimmunol.2200809

94. Rabacal W, Pabbisetty SK, Hoek KL, Cendron D, Guo Y, Maseda D, et al. Transcription factor KLF2 regulates homeostatic NK cell proliferation and survival. *Proc Natl Acad Sci USA* (2016) 113:5370–5. doi: 10.1073/pnas.1521491113

95. Skon CN, Lee JY, Anderson KG, Masopust D, Hogquist KA, Jameson SC. Transcriptional downregulation of S1pr1 is required for the establishment of resident memory $CD8^+$ T cells. *Nat Immunol* (2013) 14:1285–93. doi: 10.1038/ni.2745

96. Iwasaki A, Pillai PS. Innate immunity to influenza virus infection. *Nat Rev Immunol* (2014) 14:315–28. doi: 10.1038/nri3665

97. Komai-Koma M, Jones L, Ogg GS, Xu D, Liew FY. TLR2 is expressed on activated T cells as a costimulatory receptor. *Proc Natl Acad Sci USA* (2004) 101:3029–34. doi: 10.1073/pnas.0400171101

98. Teijaro JR, Walsh KB, Cahalan S, Fremgen DM, Roberts E, Scott F, et al. Endothelial cells are central orchestrators of cytokine amplification during influenza virus infection. *Cell* (2011) 146:980–91. doi: 10.1016/j.cell.2011.08.015

99. Tavazoie MF, Pollack I, Tanqueco R, Ostendorf BN, Reis BS, Gonsalves FC, et al. LXR/ApoE activation restricts innate immune suppression in cancer. *Cell* (2018) 172:825–40. doi: 10.1016/j.cell.2017.12.026

100. Yang YF, Tomura M, Iwasaki M, Mukai T, Gao P, Ono S, et al. IL-12 as well as IL-2 upregulates CCR5 expression on T cell receptor-triggered human $CD4^+$ and $CD8^+$ T cells. *J Clin Immunol* (2001) 21:116–25. doi: 10.1023/a:1011059906777

101. Takami M, Ihara F, Motohashi S. Clinical application of iNKT cell-mediated anti-tumor activity against lung cancer and head and neck cancer. *Front Immunol* (2018) 9:2021. doi: 10.3389/fimmu.2018.02021

102. Yamasaki K, Horiguchi S, Kurosaki M, Kunii N, Nagato K, Hanaoka H, et al. Induction of NKT cell-specific immune responses in cancer tissues after NKT cell-targeted adoptive immunotherapy. *Clin Immunol* (2011) 138:255–65. doi: 10.1016/j.clim.2010.11.014

103. Kunii N, Horiguchi S, Motohashi S, Yamamoto H, Ueno N, Yamamoto S, et al. Combination therapy of in *vitro*-expanded natural killer T cells and α-galactosylceramide-pulsed antigen-presenting cells in patients with recurrent head and neck carcinoma. *Cancer Sci* (2009) 100:1092–8. doi: 10.1111/j.1349-7006.2009.01135.x

104. Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, et al. A phase I study of in *vitro* expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* (2006) 12:6079–86. doi: 10.1158/1078-0432.CCR-06-0114

105. Rowan AG, Ponnusamy K, Ren H, Taylor GP, Cook LBM, Karadimitris A. CAR-iNKT cells targeting clonal TCRVβ chains as a precise strategy to treat T cell lymphoma. *Front Immunol* (2023) 14:1118681. doi: 10.3389/fimmu.2023.1118681

106. Simon B, Wiesinger M, Marz J, Wistuba-Hamprecht K, Weide B, Schuler-Thurner B, et al. The Generation of CAR-transfected natural killer T cells for the immunotherapy of melanoma. *Int J Mol Sci* (2018) 19:2365. doi: 10.3390/ijms19082365

107. Delfanti G, Cortesi F, Perini A, Antonini G, Azzimonti L, de Lalla C, et al. TCR-engineered iNKT cells induce robust antitumor response by dual targeting cancer and suppressive myeloid cells. *Sci Immunol* (2022) 7:eabn6563. doi: 10.1126/sciimmunol.abn6563

108. Schneidawind D, Baker J, Pierini A, Buechele C, Luong RH, Meyer EH, et al. Third-party $CD4^+$ invariant natural killer T cells protect from murine GVHD lethality. *Blood* (2015) 125:3491–500. doi: 10.1182/blood-2014-11-612762

109. Erkers T, Xie BJ, Kenyon LJ, Smith B, Rieck M, Jensen KP, et al. High-parametric evaluation of human invariant natural killer T cells to delineate heterogeneity in allo- and autoimmunity. *Blood* (2020) 135:814–25. doi: 10.1182/blood.2019001903

110. Fessas P, Possamai LA, Clark J, Daniels E, Gudd C, Mullish BH, et al. Immunotoxicity from checkpoint inhibitor therapy: clinical features and underlying mechanisms. *Immunology* (2020) 159:167–77. doi: 10.1111/imm.13141

111. Gerussi A, Natalini A, Antonangeli F, Mancuso C, Agostinetto E, Barisani D, et al. Immune-mediated drug-induced liver injury: immunogenetics and experimental models. *Int J Mol Sci* (2021) 22:4557. doi: 10.3390/ijms22094557

112. Heczey A, Xu X, Courtney AN, Tian G, Barragan GA, Guo L, et al. Anti-GD2 CAR-NKT cells in relapsed or refractory neuroblastoma: updated phase 1 trial interim results. *Nat Med* (2023) 29:1379–88. doi: 10.1038/s41591-023-02363-y

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 19 February 2024
DOI 10.3389/fimmu.2024.1341180

OPEN ACCESS

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RECEIVED 20 November 2023
ACCEPTED 29 January 2024
PUBLISHED 19 February 2024

CITATION
Fukui C, Yamana S, Xue Y, Shirane M, Tsutsui H, Asahara K, Yoshitomi K, Ito T, Lestari T, Hasegawa E, Yawata N, Takeda A, Sonoda K-H and Shibata K (2024) Functions of mucosal associated invariant T cells in eye diseases.
Front. Immunol. 15:1341180.
doi: 10.3389/fimmu.2024.1341180

Functions of mucosal associated invariant T cells in eye diseases

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Mucosal-associated invariant T (MAIT) cells are a unique subset of T cells that recognizes metabolites derived from the vitamin B2 biosynthetic pathway. Since the identification of cognate antigens for MAIT cells, knowledge of the functions of MAIT cells in cancer, autoimmunity, and infectious diseases has been rapidly expanding. Recently, MAIT cells have been found to contribute to visual protection against autoimmunity in the eye. The protective functions of MAIT cells are induced by T-cell receptor (TCR)-mediated activation. However, the underlying mechanisms remain unclear. Thus, this mini-review aims to discuss our findings and the complexity of MAIT cell-mediated immune regulation in the eye.

KEYWORDS

eye, autoimmune disease, metabolite, T cells, immunotherapy

1 Introduction

The eye is the organ responsible for visual function, which is associated with quality of life. Therefore, it is essential that harmful immune responses in the eye are strictly regulated. The unique immunoregulatory features of the eye were first recognized by Peter Medawar in the mid-20th century. He showed that tissue from nongenetically identical animals was successfully grafted to the anterior chamber of the eye (1). T cells play a central role in graft rejection. Thus, most studies have focused on understanding how their functions are regulated in the eye. Under normal conditions, the immune-privileged environment in the eye is established through physical compartmentalization by the blood–retinal barrier, which is composed of retinal pigment epithelial cells and retinal vascular endothelial cells (2). The blood–retinal barrier sequesters retinal T-cell antigens within the eye (3). T cells in the eye are not only functionally regulated through TCR-mediated signals upon recognition of their cognate antigens. They are also regulated by other interactions with retinal glial Müller, retinal pigment epithelial, corneal endothelial and ciliary body

epithelial cells. These interactions are mediated through inhibitory cell-surface molecules, such as membrane-bound transforming growth factor-β (TGF-β), Fas ligand, cytotoxic T-lymphocyte-associated protein 4, galectin-1 and thrombospondin (4). In addition to cell-contact dependent mechanisms, ocular fluids contain various immunoregulatory molecules such as TGF-β2, interleukin (IL)-10 and a series of neuropeptides. Notably, immunosuppressive factors in the eye lead to systemic tolerance. The administration of foreign antigens to the anterior chamber of the eye induces the migration of antigen-capturing macrophages from the eye to peripheral tissues such as the spleen, thereby dampening antigen-specific inflammatory responses in the periphery (5, 6). This phenomenon is known as anterior chamber-associated immune deviation.

In addition to the eye, the thymus plays a role in maintaining homeostasis by eliminating pathogenic T cells that recognize self-antigens. After T cell progenitors enter the thymus, functional T cells undergo positive selection. Among the selected functional T cell repertoires, most self-antigen-specific T cells are deleted or are functionally energized via negative selection. In this process, self-antigen-presenting medullary thymic epithelial cells dictate T cell fates via high affinity binding with the self-antigen-specific T cells. Arrays of gene expressions that encode tissue-specific self-antigens are regulated by a transcriptional regulator protein, Autoimmune regulator (Aire) (7). However, some self-antigen-specific T cells with potential to cause autoimmunity escape this checkpoint. Autoimmune uveitis is an autoimmune disorder of the eye that is thought to be induced by such escapee T cells. Indeed, using experimental autoimmune uveitis (EAU) models, T cells recognizing self-antigens such as arrestin, interphotoreceptor retinoid-binding protein (IRBP), rhodopsin, recoverin and phosducin have been identified (8). Using EAU mouse models, the breakdown of central tolerance in the thymus was reported to worsen clinical symptoms of EAU, as genetic depletion of the gene encoding the retinal T-cell antigen, IRBP, augmented the antigen-specific T-cell response to IRBP and thereby enhanced ocular inflammation in the retina (9). In support of this, mice lacking the *Aire* gene responsible for expressing tissue-specific self-antigens including IRBP in the thymus spontaneously developed retinal autoimmunity (7, 10). Furthermore, after identification of the regulatory T (Treg) cells responsible for maintenance of immunological self-tolerance and homeostasis (11), accumulating studies have shown that Treg cells play a role in regulating the pathogenesis of autoimmune disorders including autoimmune uveitis (12, 13). Depletion of Treg cells leads to increased susceptibility to EAU (9, 14, 15).

Other T cell subsets, such as natural killer T (NKT) and mucosal-associated invariant (MAIT) cells, have been reported to be involved in ocular immunity through the recognition of nonpeptide antigens such as lipids and metabolites. In an EAU mouse model, NKT cell activation by alpha-galactosylceramide (α-GalCer), a prototype antigen, conferred mitigation of clinical symptoms partly through innate interferon-gamma (IFNγ) production that reduced pathogenic IFNγ and IL-17A production (16, 17). This finding was further supported by a study that showed administration of RCAI-56, a Th1-biased NKT cell ligand, to EAU mice had higher therapeutic efficacy than α-GalCer (18). More recently, protective effects against EAU have been observed upon activation of MAIT cells with the authentic antigen, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) by IL-22 secretion (19). This data suggest that the inflammatory responses triggered by retinal antigen-specific T cells during EAU induction are counteracted not only by Treg cells but also by other T-cell subsets including NKT and MAIT cells.

Among these T cell subsets involved in ocular immunity, we focus on MAIT cells in the following sections.

2 MAIT cells

MAIT cells are a subset of innate-like T cells that require major histocompatibility complex (MHC) class I-related molecule 1 (MR1) for their development (20). MR1 is an antigen-presenting molecule that captures metabolites such as 5-OP-RU and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil, derived from intermediates in the vitamin B2 biosynthetic pathway, which is present in bacteria and fungi but not in mammals (21, 22). MAIT cells recognize the antigen-MR1 complex through semi-invariant αβ T-cell receptors (TCRs). In humans, these are typically TRAV1-2-TRAJ33/12/20 α chains paired preferentially with limited β chains such as TRBV6-1, TRBV6-4, and TRBV20, and Trav1-Traj33 α chains paired with β chains, such as Trbv13 and Trbv19 in mice. Germ-free mice had a severely reduced MAIT cell number, but monocolonization with vitamin B2-producing bacteria recovered MAIT cell development in the thymus (23–26). These findings suggest that MAIT cells develop or expand in response to 5-OP-RU derived from symbiotic bacteria (26). Consistent with these studies, it has been reported that human MAIT cell frequency is very low from the fetal to perinatal period and rapidly expands after birth when the individual is exposed to symbiotic bacteria (27, 28). Thus, the development and function of MAIT cells are regulated by symbiotic bacteria during early ontogeny. Because this developmental program is evolutionally conserved among mammals, MAIT cells may have nonredundant functions in adult life (29, 30). In support of this, MAIT cells have been shown to play an important role in infection, autoimmunity, and cancer through the production of various inflammatory mediators such as cytokines, cytotoxic molecules and growth factors. MAIT cells were activated after co-culture with bone marrow-derived dendritic cells infected with wide variety of bacteria including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, *Staphylococcus aureus* and *Streptococcus epidermidis* (23). Protective roles of MAIT cells have been reported in infection against *Streptococcus pneumoniae* (31), *Klebsiella pneumoniae* (32), *Francisella tularensis* (33) and *Legionella longbeachae* (34). Among these bacteria, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* are the major causal agents of eye diseases such as bacterial conjunctivitis, keratitis and endophthalmitis (35), although the involvement of MAIT cells has not been reported. In the tumor environment, MAIT cells have not only cytotoxic activity against tumors (36, 37) but also tumor-promoting activity (38). Recently, a new function of MAIT

cells related to tissue homeostasis has been proposed (39). In a wound-healing mouse model after a skin-punch biopsy, MAIT cells had tissue repair functions that were inducible by the administration of MAIT cell antigen 5-OP-RU (25). In the brain, MAIT cells maintain tissue integrity through the production of antioxidant molecules. MAIT cell deficiency in mice accumulates reactive oxygen species around the meninges, impairing meningeal barrier function (40). Such context dependent multifaceted MAIT cell functions raise the question of how they are regulated *in vivo*. MR1-dependent MAIT cell activation is required for host defense against *Legionella* and *Francisella* infection (34, 41, 42). In contrast, in a wound-healing mouse model, the migration and tissue repair function of MAIT cells were MR1-independent (43). These findings have been summarized in previous review articles (44–47). However, in these articles, the role of MAIT cells in the eye has not been well described. Nevertheless, association of MAIT cell frequencies in particular autoimmune diseases such as ankylosing spondylitis and Sjogren syndrome with ocular manifestations suggests the potential role in the eye (48–50). Therefore, we aimed to introduce the role of MAIT cells in autoimmune uveitis and further discuss other eye diseases, such as age-related macular degeneration (AMD), allergic conjunctivitis and acute anterior uveitis (AAU), in which MAIT cells may have an immunoregulatory role (Figure 1).

3 MAIT cells and eye diseases

3.1 MAIT cells in autoimmune uveitis

Autoimmune uveitis is a leading cause of blindness among patients with uveitis in the United States and Asia (51, 52). Autoimmune uveitis has two types: eye-specific inflammation, such as Sympathetic ophthalmia and Birdshot retinochoroidopathy, and systemic inflammation that also affects the eye, such as Behcet's disease, Sarcoidosis and Vogt-Koyanagi Harada (VKH) disease (4). In these patients, severe inflammation is frequently observed in the retina, located in the posterior compartment of the eye where neural and photoreceptor cells responsible for the transmission of visual information to the brain are present (Figure 1 and Figure 2A). Recognition of retinal antigens by T cells is thought to induce chronic inflammation in the retina and adjacent structures including optic nerves, leading to impairment of visual functions resulting from cell damage. In contrast to the pathogenic roles of T cells, their protective functions remain less understood. Dr. R. R. Caspi's group showed the protective functions of IL-22-producing T cells using a mouse model of EAU induced by the retinal antigen IRBP (53). IL-22 bound to the IL-22 receptor on retinal ganglion cells and prevented their cell death (53) (Figure 1). Another possible protective mechanism could be that IL-22 can suppress MHC class II expression responsible for the development of retinal antigen-specific T cells (54). MAIT cell frequency in the peripheral blood has been found to be inversely correlated with disease activity in patients with VKH disease, which is representative of autoimmune uveitis with chronic inflammation in the retina (19). These findings motivated us to explore MAIT cell functions in a mouse model of EAU induced by the retinal antigen IRBP. MAIT cells also secrete IL-22 and contribute to the reduction of EAU clinical symptoms after EAU induction (19). MAIT cells were hardly detected in the retina under normal conditions and gradually increased after EAU induction. This finding suggests that MAIT cells migrate from the draining lymph nodes, where preceding MAIT cell expansion was observed (19). The involvement of TCR-mediated signaling in MAIT cell expansion during EAU induction has not been experimentally proven. However, TCR-mediated activation of MAIT cells was observed in the eyes and draining lymph nodes of EAU-induced mice. The MAIT TCR-mediated signal has a therapeutic potential. Administration of the cognate antigen 5-OP-RU improved clinical symptoms and visual function, although the underlying mechanism need to be further explored (19).

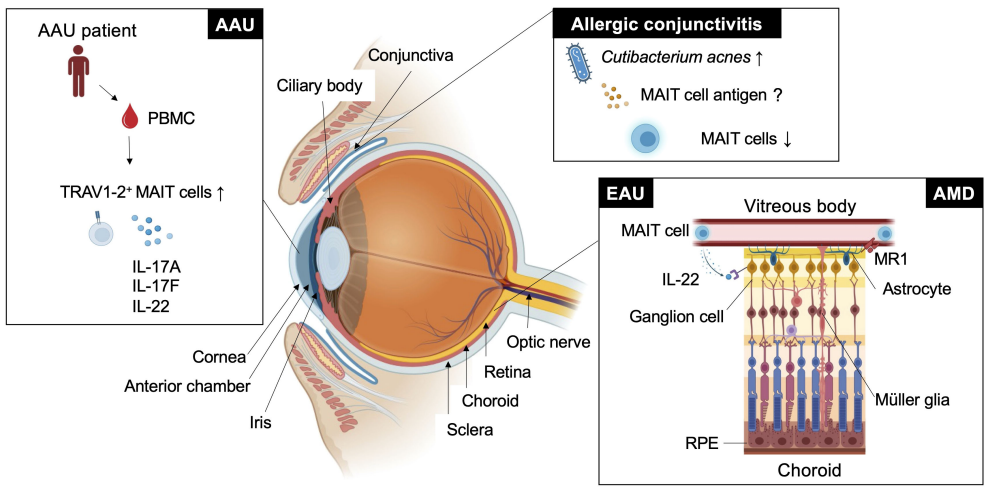

FIGURE 1
MAIT cell functions in eye diseases. This figure shows how MAIT cells or TRAV1-2$^+$ cells are involved in ocular diseases such as experimental autoimmune uveitis (EAU), age-related macular degeneration (AMD), acute anterior uveitis (AAU), and allergic conjunctivitis in different locations.

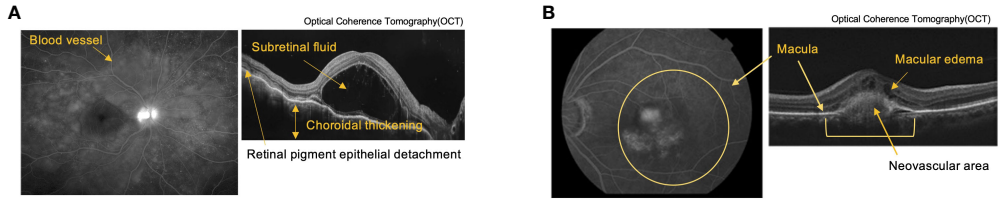

FIGURE 2
Pathological tissue images of autoimmune uveitis and Age-related Macular Degeneration (AMD). **(A)** Fluorescein Angiography (FA) (left) and Optical Coherence Tomography (OCT) (right) images of Vogt-Koyanagi-Harada (VKH) disease. FA shows multiple fluorescent leaks around the blood vessel. OCT shows retina layers with subretinal fluid, choroidal thickening, and retinal pigment detachment. **(B)** Indocyanine Green Angiography (left) and OCT (right) images of AMD. Images shows layers of retina with choroidal neovascularization associated with macular edema due to exudative changes.

Previous studies have implicated TCR-mediated MAIT cell activation by the gut microbiota in EAU. *Bacteroides* and *Parabacteroides* species belonging to *Bacteroidetes* were significantly increased in patients with VKH disease compared with healthy controls (55), and *Bacteroidetes* contained species that produce metabolites with higher MAIT cell agonistic activity (56). The activation of MAIT cells by metabolites from symbiotic bacteria can occur in the eyes of individuals with autoimmune uveitis, as 5-OP-RU has been reported to travel between distal organs (26). Furthermore, MAIT cells may be primed in the gut and infiltrate the eye, as in the case of retina-specific T cells (57). However, a more precise analysis to investigate how and where MAIT cell functions are regulated *in vivo* is required.

3.2 Implication of the immunoregulatory functions of MAIT cells in other eye diseases

AMD is a neurodegenerative disorder with a similar pathogenicity to Alzheimer's and Parkinson's disease. The similarity in anatomy and cellular composition between the retina and brain prompted the consideration of similar immunological roles of MAIT cells. AMD causes progressive photoreceptor degeneration in the macula, leading to vision loss alongside with aging (Figure 2B). In a previous study, single-cell RNA sequencing analysis using human retinal cells demonstrated that the expression of AMD-associated genes identified by a genome-wide association study (GWAS) was highly biased toward particular retinal cell populations, including Müller glia and astrocytes (58). Another study showed that the highest expression of *MR1* was observed in the retinal astrocytes of patients with AMD (59) (Figure 1). Astrocytes are resident neural cells present in the brain and retina. Brain astrocytes have been shown to express MR1 and have the potential to activate MAIT cells (60). MAIT cells have been reported to play a protective role in neuroinflammation by maintaining tissue integrity through the productions of IL-10 and antioxidative molecules in the brain (40, 61). However, MAIT cell functions in AMD remain unclear.

The conjunctiva is a mucosal tissue in the anterior eye with barrier functions against pathogenic insults. These functions are mediated by resident immune cells, and dysregulated activation of immune cells can cause allergic conjunctivitis. Allergic conjunctivitis is associated with chronic inflammation at the ocular surface where there is immunoregulatory interplay between commensal microbiota, conjunctival intraepithelial lymphocytes and epithelial cells (62). Approximately 16% of $CD45^+$ leukocytes in the upper tarsal conjunctiva of healthy individuals were MAIT cells (63). MAIT cell frequency was not increased at the ocular surface in patients with chronic allergic conjunctivitis, in whom *Cutibacterium acnes* became the predominant commensal bacterial population (63) (Figure 1). This suggests that *C. acnes* does not induce MAIT cell expansion as observed in human volunteers infected with *Salmonella* Paratyphi A (64). Although *C. acnes* has a vitamin B2 synthetic pathway, it has a low MAIT cell-stimulating ability (56). Thus, it is possible that *C. acnes* has evolved to escape adaptive immunity by reducing MAIT cell antigen production, as described in *Salmonella* and *Francisella* sp. (41, 42). Thus, examining whether MAIT cell functions are regulated by *C. acnes* in allergic conjunctivitis in future studies would be interesting.

AAU is the most common form of uveitis (65) and frequently accompanies HLA-B27-related inflammatory diseases, such as ankylosing spondylitis and spondyloarthropathies (66). AAU manifests as an acute onset of nongranulomatous uveitis, characterized by cellular and protein extravasation into the aqueous humor. GWAS between patients with AAU and healthy donors using peripheral blood revealed several AAU-associated loci, including *IL6R*, *IL10*, *IL19* and *IL18R* (67). IL-18R is one of the authentic markers for MAIT cells (23). The frequency of MAIT cell-enriched TRAV1-2^+ cells producing IL-17A, IL-17F and IL-22 was increased in the peripheral blood compared with the healthy control (68) (Figure 1). It remains to be determined whether enrichment of immunoregulatory MAIT cells also occurs in the anterior chamber of the eye.

4 Discussion for future direction

The retina converts light stimuli into signals and transmits visual images to the brain. Layered nerve cell populations transmit signals one after another through intercellular interactions to accomplish their complex functions, thus maintaining visual function (Figure 1). Degeneration or dysfunction of only one of the component cells can destroy this sophisticated interaction (69). Under inflammatory

conditions, infiltrating inflammatory cells, such as monocytes, macrophages, and T cells, and resident glial cells, such as astrocytes and microglial cells, have immunoregulatory functions to maintain tissue homeostasis in the retina and uvea. Therefore, understanding spatiotemporal cell functions in the eye is essential for understanding the pathogenesis of certain diseases. Recent advances in single-cell technology with spatial information allows us to understand their functions and mutual interactions at the single-cell level. Elucidation of the regulatory network could open a new avenue to clarify the full picture of ocular immunity and how MAIT cells are involved in this process.

Accumulating evidence has shown that MAIT cells play an immunoregulatory role in autoimmune diseases targeting various organs, including the eye. In the case of autoimmune uveitis, recent findings on the therapeutic potential of TCR-mediated MAIT cell activation have motivated us to identify putative MAIT cell antigens in EAU mice. More detailed analysis of metabolites using highly sensitive mass spectrometry (70–72) and single metabolite-probing technology (73) will allow us to test this possibility.

Author contributions

CF: Writing – review & editing. SY: Writing – review & editing. YX: Writing – review & editing. MS: Writing – review & editing. HT: Writing – review & editing. KA: Writing – review & editing. KY: Writing – review & editing. TI: Writing – review & editing. TL: Writing – review & editing. EH: Writing – review & editing. NY: Writing – review & editing. AT: Writing – review & editing. KHS: Supervision, Writing – review & editing. KS: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Japan Society for the Promotion of Science KAKENHI (JP23H04784, JP23K18330 to KS).

Acknowledgments

We thank Machiko Shirahase and Atsuko Sakota for secretarial support; Kei Sakamoto and Makoto Furutani-Seiki for discussion; Brenda Luong, Joseph Leon Maurice Barlier and James Richard Wright for paper editing.

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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References

1. Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Br J Exp Pathol* (1948) 29:58–69.

2. Cunha-Vaz J, Bernardes R, Lobo C. Blood-retinal barrier. *Eur J Ophthalmol* (2011) 21 Suppl 6:S3–9. doi: 10.5301/EJO.2010.6049

3. Dullforce PA, Garman KL, Seitz GW, Fleischmann RJ, Crespo SM, Planck SR, et al. APCs in the anterior uveal tract do not migrate to draining lymph nodes. *J Immunol* (2004) 172:6701–8. doi: 10.4049/jimmunol.172.11.6701

4. Caspi RR. Ocular autoimmunity: the price of privilege? *Immunol Rev* (2006) 213:23–35. doi: 10.1111/j.1600-065X.2006.00439.x

5. Kaplan HJ, Streilein JW. Do immunologically privileged sites require a functioning spleen? *Nature* (1974) 251:553–4. doi: 10.1038/251553a0

6. Streilein JW, Niederkorn JY. Induction of anterior chamber-associated immune deviation requires an intact, functional spleen. *J Exp Med* (1981) 153:1058–67. doi: 10.1084/jem.153.5.1058

7. Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, et al. Projection of an immunological self shadow within the thymus by the aire protein. *Science* (2002) 298:1395–401. doi: 10.1126/science.1075958

8. Agarwal RK, Silver PB, Caspi RR. Rodent models of experimental autoimmune uveitis. *Methods Mol Biol* (2012) 900:443–69. doi: 10.1007/978-1-60761-720-4_22

9. Avichezer D, Grajewski RS, Chan CC, Mattapallil MJ, Silver PB, Raber JA, et al. An immunologically privileged retinal antigen elicits tolerance: major role for central selection mechanisms. *J Exp Med* (2003) 198:1665–76. doi: 10.1084/jem.20030413

10. Devoss J, Hou Y, Johannes K, Lu W, Liou GI, Rinn J, et al. Spontaneous autoimmunity prevented by thymic expression of a single self-antigen. *J Exp Med* (2006) 203:2727–35. doi: 10.1084/jem.20061864

11. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* (1995) 155:1151–64. doi: 10.4049/jimmunol.155.3.1151

12. Gery I, Caspi RR. Tolerance induction in relation to the eye. *Front Immunol* (2018) 9:2304. doi: 10.3389/fimmu.2018.02304

13. Sakaguchi S, Mikami N, Wing JB, Tanaka A, Ichiyama K, Ohkura N. Regulatory T cells and human disease. *Annu Rev Immunol* (2020) 38:541–66. doi: 10.1146/annurev-immunol-042718-041717

14. Grajewski RS, Silver PB, Agarwal RK, Su SB, Chan CC, Liou GI, et al. Endogenous IRBP can be dispensable for generation of natural CD4+CD25+ regulatory T cells that protect from IRBP-induced retinal autoimmunity. *J Exp Med* (2006) 203:851–6. doi: 10.1084/jem.20050429

15. Silver PB, Horai R, Chen J, Jittayasothorn Y, Chan CC, Villasmil R, et al. Retina-specific T regulatory cells bring about resolution and maintain remission of autoimmune uveitis. *J Immunol* (2015) 194:3011–9. doi: 10.4049/jimmunol.1402650

16. Grajewski RS, Hansen AM, Agarwal RK, Kronenberg M, Sidobre S, Su SB, et al. Activation of invariant NKT cells ameliorates experimental ocular autoimmunity by a mechanism involving innate IFN-gamma production and dampening of the adaptive Th1 and Th17 responses. *J Immunol* (2008) 181:4791–7. doi: 10.4049/jimmunol.181.7.4791

17. Luger D, Silver PB, Tang J, Cua D, Chen Z, Iwakura Y, et al. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *J Exp Med* (2008) 205:799–810. doi: 10.1084/jem.20071258

18. Satoh M, Namba KI, Kitaichi N, Endo N, Kitamei H, Iwata D, et al. Invariant natural killer T cells play dual roles in the development of experimental autoimmune uveoretinitis. *Exp Eye Res* (2016) 153:79–89. doi: 10.1016/j.exer.2016.10.003

19. Yamana S, Shibata K, Hasegawa E, Arima M, Shimokawa S, Yawata N, et al. Mucosal-associated invariant T cells have therapeutic potential against ocular autoimmunity. *Mucosal Immunol* (2022) 15:351–61. doi: 10.1038/s41385-021-00469-5

20. Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* (2003) 422:164–9. doi: 10.1038/nature01433

21. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* (2012) 491:717–23. doi: 10.1038/nature11605

22. Corbett AJ, Eckle SB, Birkinshaw RW, Liu L, Patel O, Mahony J, et al. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* (2014) 509:361–5. doi: 10.1038/nature13160

23. Le Bourhis L, Martin E, Peguillet I, Guihot A, Froux N, Core M, et al. Antimicrobial activity of mucosal-associated invariant T cells. *Nat Immunol* (2010) 11:701–8. doi: 10.1038/ni.1890

24. Koay HF, Gherardin NA, Enders A, Loh L, Mackay LK, Almeida CF, et al. A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage. *Nat Immunol* (2016) 17:1300–11. doi: 10.1038/ni.3565

25. Constantinides MG, Link VM, Tamoutounour S, Wong AC, Perez-Chaparro PJ, Han SJ, et al. MAIT cells are imprinted by the microbiota in early life and promote tissue repair. *Science* (2019) 366. doi: 10.1126/science.aax6624

26. Legoux F, Bellet D, Daviaud C, El Morr Y, Darbois A, Niort K, et al. Microbial metabolites control the thymic development of mucosal-associated invariant T cells. *Science* (2019) 366:494–9. doi: 10.1126/science.aaw2719

27. Ben Youssef G, Tourret M, Salou M, Ghazarian L, Houdouin V, Mondot S, et al. Ontogeny of human mucosal-associated invariant T cells and related T cell subsets. *J Exp Med* (2018) 215:459–79. doi: 10.1084/jem.20171739

28. Gherardin NA, Souter MN, Koay HF, Mangas KM, Seemann T, Stinear TP, et al. Human blood MAIT cell subsets defined using MR1 tetramers. *Immunol Cell Biol* (2018) 96:507–25. doi: 10.1111/imcb.12021

29. Tsukamoto K, Deakin JE, Graves JA, Hashimoto K. Exceptionally high conservation of the MHC class I-related gene, MR1, among mammals. *Immunogenetics* (2013) 65:115–24. doi: 10.1007/s00251-012-0666-5

30. Boudinot P, Mondot S, Jouneau L, Teyton L, Lefranc MP, Lantz O. Restricting nonclassical MHC genes coevolve with TRAV genes used by innate-like T cells in mammals. *Proc Natl Acad Sci U.S.A.* (2016) 113:E2983–2992. doi: 10.1073/pnas.1600674113

31. Kurioka A, Van Wilgenburg B, Javan RR, Hoyle R, Van Tonder AJ, Harrold CL, et al. Diverse Streptococcus pneumoniae Strains Drive a Mucosal-Associated Invariant T-Cell Response Through Major Histocompatibility Complex class I-Related Molecule-Dependent and Cytokine-Driven Pathways. *J Infect Dis* (2018) 217:988–99. doi: 10.1093/infdis/jix647

32. Georgel P, Radosavljevic M, Macquin C, Bahram S. The non-conventional MHC class I MR1 molecule controls infection by Klebsiella pneumoniae in mice. *Mol Immunol* (2011) 48:769–75. doi: 10.1016/j.molimm.2010.12.002

33. Meierovics A, Yankelevich WJ, Cowley SC. MAIT cells are critical for optimal mucosal immune responses during in *vivo* pulmonary bacterial infection. *Proc Natl Acad Sci U.S.A.* (2013) 110:E3119–3128. doi: 10.1073/pnas.1302799110

34. Wang H, D'souza C, Lim XY, Kostenko L, Pediongco TJ, Eckle SBG, et al. MAIT cells protect against pulmonary Legionella longbeachae infection. *Nat Commun* (2018) 9:3350. doi: 10.1038/s41467-018-05202-8

35. Durand ML, Barshak MB, Sobrin L. Eye infections. *N Engl J Med* (2023) 389:2363–75. doi: 10.1056/NEJMra2216081

36. Ling L, Lin Y, Zheng W, Hong S, Tang X, Zhao P, et al. Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients. *Sci Rep* (2016) 6:20358. doi: 10.1038/srep20358

37. Won EJ, Ju JK, Cho YN, Jin HM, Park KJ, Kim TJ, et al. Clinical relevance of circulating mucosal-associated invariant T cell levels and their anti-cancer activity in patients with mucosal-associated cancer. *Oncotarget* (2016) 7:76274–90. doi: 10.18632/oncotarget.11187

38. Yan J, Allen S, Mcdonald E, Das I, Mak JYW, Liu L, et al. MAIT cells promote tumor initiation, growth, and metastases *via* tumor MR1. *Cancer Discovery* (2020) 10:124–41. doi: 10.1158/2159-8290.CD-19-0569

39. Hinks TSC, Marchi E, Jabeen M, Olshansky M, Kurioka A, Pediongco TJ, et al. Activation and *In Vivo* Evolution of the MAIT Cell Transcriptome in Mice and humans reveals tissue repair functionality. *Cell Rep* (2019) 28:3249–3262.e3245. doi: 10.1016/j.celrep.2019.07.039

40. Zhang Y, Bailey JT, Xu E, Singh K, Lavaert M, Link VM, et al. Mucosal-associated invariant T cells restrict reactive oxidative damage and preserve meningeal barrier integrity and cognitive function. *Nat Immunol* (2022) 23:1714–25. doi: 10.1038/s41590-022-01349-1

41. Preciado-Llanes L, Aulicino A, Canals R, Moynihan PJ, Zhu X, Jambo N, et al. Evasion of MAIT cell recognition by the African Salmonella Typhimurium ST313 pathovar that causes invasive disease. *Proc Natl Acad Sci U.S.A.* (2020) 117:20717–28. doi: 10.1073/pnas.2007472117

42. Shibata K, Shimizu T, Nakahara M, Ito E, Legoux F, Fujii S, et al. The intracellular pathogen Francisella tularensis escapes from adaptive immunity by metabolic adaptation. *Life Sci Alliance* (2022) 5. doi: 10.26508/lsa.202201441

43. Du Halgouet A, Darbois A, Alkobtawi M, Mestdagh M, Alphonse A, Premel V, et al. Role of MR1-driven signals and amphiregulin on the recruitment and repair function of MAIT cells during skin wound healing. *Immunity* (2023) 56:78–92.e76. doi: 10.1016/j.immuni.2022.12.004

44. Godfrey DI, Koay HF, Mccluskey J, Gherardin NA. The biology and functional importance of MAIT cells. *Nat Immunol* (2019) 20:1110–28. doi: 10.1038/s41590-019-0444-8

45. Toubal A, Nel I, Lotersztajn S, Lehuen A. Mucosal-associated invariant T cells and disease. *Nat Rev Immunol* (2019) 19:643–57. doi: 10.1038/s41577-019-0191-y

46. Hinks TSC, Zhang XW. MAIT cell activation and functions. *Front Immunol* (2020) 11:1014. doi: 10.3389/fimmu.2020.01014

47. Mcwilliam HEG, Villadangos JA. MR1 antigen presentation to MAIT cells and other MR1-restricted T cells. *Nat Rev Immunol* (2023). doi: 10.1038/s41577-023-00934-1

48. Gracey E, Qaiyum Z, Almaghlouth I, Lawson D, Karki S, Avvaru N, et al. IL-7 primes IL-17 in mucosal-associated invariant T (MAIT) cells, which contribute to the Th17-axis in ankylosing spondylitis. *Ann Rheum Dis* (2016) 75:2124–32. doi: 10.1136/annrheumdis-2015-208902

49. Hayashi E, Chiba A, Tada K, Haga K, Kitagaichi M, Nakajima S, et al. Involvement of mucosal-associated invariant T cells in ankylosing spondylitis. *J Rheumatol* (2016) 43:1695–703. doi: 10.3899/jrheum.151133

50. Wang JJ, Macardle C, Weedon H, Beroukas D, Banovic T. Mucosal-associated invariant T cells are reduced and functionally immature in the peripheral blood of primary Sjogren's syndrome patients. *Eur J Immunol* (2016) 46:2444–53. doi: 10.1002/eji.201646300

51. Gritz DC, Wong IG. Incidence and prevalence of uveitis in Northern California; the Northern California Epidemiology of Uveitis Study. *Ophthalmology* (2004) 111:491–500;discussion500. doi: 10.1016/j.ophtha.2003.06.014

52. Sonoda KH, Hasegawa E, Namba K, Okada AA, Ohguro N, Goto H, et al. Epidemiology of uveitis in Japan: a 2016 retrospective nationwide survey. *Jpn J Ophthalmol* (2021) 65:184–90. doi: 10.1007/s10384-020-00809-1

53. Mattapallil MJ, Kielczewski JL, Zarate-Blades CR, St Leger AJ, Raychaudhuri K, Silver PB, et al. Interleukin 22 ameliorates neuropathology and protects from central nervous system autoimmunity. *J Autoimmun* (2019) 102:65–76. doi: 10.1016/j.jaut.2019.04.017

54. Moniruzzaman M, Rahman MA, Wang R, Wong KY, Chen AC, Mueller A, et al. Interleukin-22 suppresses major histocompatibility complex II in mucosal epithelial cells. *J Exp Med* (2023) 220. doi: 10.1084/jem.20230106

55. Ye Z, Wu C, Zhang N, Du L, Cao Q, Huang X, et al. Altered gut microbiome composition in patients with Vogt-Koyanagi-Harada disease. *Gut Microbes* (2020) 11:539–55. doi: 10.1080/19490976.2019.1700754

56. Tastan C, Karhan E, Zhou W, Fleming E, Voigt AY, Yao X, et al. Tuning of human MAIT cell activation by commensal bacteria species and MR1-dependent T-cell presentation. *Mucosal Immunol* (2018) 11:1591–605. doi: 10.1038/s41385-018-0072-x

57. Horai R, Zarate-Blades CR, Dillenburg-Pilla P, Chen J, Kielczewski JL, Silver PB, et al. Microbiota-dependent activation of an autoreactive T cell receptor provokes autoimmunity in an immunologically privileged site. *Immunity* (2015) 43:343–53. doi: 10.1016/j.immuni.2015.07.014

58. Menon M, Mohammadi S, Davila-Velderrain J, Goods BA, Cadwell TD, Xing Y, et al. Single-cell transcriptomic atlas of the human retina identifies cell types associated with age-related macular degeneration. *Nat Commun* (2019) 10:4902. doi: 10.1038/s41467-019-12780-8

59. Wang JH, Wong RCB, Liu GS. Retinal aging transcriptome and cellular landscape in association with the progression of age-related macular degeneration. *Invest Ophthalmol Vis Sci* (2023) 64:32. doi: 10.1167/iovs.64.4.32

60. Priya R, Brutkiewicz RR. Brain astrocytes and microglia express functional MR1 molecules that present microbial antigens to mucosal-associated invariant T (MAIT) cells. *J Neuroimmunol* (2020) 349:577428. doi: 10.1016/j.jneuroim.2020.577428

61. Croxford JL, Miyake S, Huang YY, Shimamura M, Yamamura T. Invariant V (alpha)19i T cells regulate autoimmune inflammation. *Nat Immunol* (2006) 7:987–94. doi: 10.1038/ni1370

62. St Leger AJ, Desai JV, Drummond RA, Kugadas A, Almaghrabi F, Silver P, et al. An Ocular Commensal Protects against Corneal Infection by Driving an Interleukin-17 Response from Mucosal gammadelta T Cells. *Immunity* (2017) 47:148–158.e145. doi: 10.1016/j.immuni.2017.06.014

63. Zarzuela JC, Reinoso R, Armentia A, Enriquez-De-Salamanca A, Corell A. Conjunctival intraepithelial lymphocytes, lacrimal cytokines and ocular commensal microbiota: analysis of the three main players in allergic conjunctivitis. *Front Immunol* (2022) 13:911022. doi: 10.3389/fimmu.2022.911022

64. Howson LJ, Napolitani G, Shepherd D, Ghadbane H, Kurupati P, Preciado-Llanes L, et al. MAIT cell clonal expansion and TCR repertoire shaping in human volunteers challenged with Salmonella Paratyphi A. *Nat Commun* (2018) 9:253. doi: 10.1038/s41467-017-02540-x

65. Pan J, Kapur M, Mccallum R. Noninfectious immune-mediated uveitis and ocular inflammation. *Curr Allergy Asthma Rep* (2014) 14:409. doi: 10.1007/s11882-013-0409-1

66. Chang JH, Mccluskey PJ, Wakefield D. Acute anterior uveitis and HLA-B27. *Surv Ophthalmol* (2005) 50:364–88. doi: 10.1016/j.survophthal.2005.04.003

67. Robinson PC, Claushuis TA, Cortes A, Martin TM, Evans DM, Leo P, et al. Genetic dissection of acute anterior uveitis reveals similarities and differences in associations observed with ankylosing spondylitis. *Arthritis Rheumatol* (2015) 67:140–51. doi: 10.1002/art.38873

68. Huang JC, Schleisman M, Choi D, Mitchell C, Watson L, Asquith M, et al. Preliminary report on interleukin-22, GM-CSF, and IL-17F in the pathogenesis of acute anterior uveitis. *Ocul Immunol Inflammation* (2021) 29:558–65. doi: 10.1080/09273948.2019.1686156

69. Georgiou M, Fujinami K, Michaelides M. Inherited retinal diseases: Therapeutics, clinical trials and end points-A review. *Clin Exp Ophthalmol* (2021) 49:270–88. doi: 10.1111/ceo.13917

70. Nagata M, Izumi Y, Ishikawa E, Kiyotake R, Doi R, Iwai S, et al. Intracellular metabolite beta-glucosylceramide is an endogenous Mincle ligand possessing immunostimulatory activity. *Proc Natl Acad Sci U.S.A.* (2017) 114:E3285–94. doi: 10.1073/pnas.1618133114

71. Harriff MJ, Mcmurtrey C, Froyd CA, Jin H, Cansler M, Null M, et al. MR1 displays the microbial metabolome driving selective MR1-restricted T cell receptor usage. *Sci Immunol* (2018) 3. doi: 10.1126/sciimmunol.aao2556

72. Shibata K, Motozono C, Nagae M, Shimizu T, Ishikawa E, Motooka D, et al. Symbiotic bacteria-dependent expansion of MR1-reactive T cells causes autoimmunity in the absence of Bcl11b. *Nat Commun* (2022) 13:6948. doi: 10.1038/s41467-022-34802-8

73. Yvorra T, Steinmetz A, Retailleau P, Lantz O, Schmidt F. Synthesis, biological evaluation and molecular modelling of new potent clickable analogues of 5-OP-RU for their use as chemical probes for the study of MAIT cell biology. *Eur J Med Chem* (2021) 211:113066. doi: 10.1016/j.ejmech.2020.113066

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 13 March 2024
DOI 10.3389/fimmu.2024.1369236

OPEN ACCESS

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RECEIVED 11 January 2024
ACCEPTED 26 February 2024
PUBLISHED 13 March 2024

CITATION
Yigit M, Basoglu OF and Unutmaz D (2024) Mucosal-associated invariant T cells in cancer: dual roles, complex interactions and therapeutic potential.
Front. Immunol. 15:1369236.
doi: 10.3389/fimmu.2024.1369236

Mucosal-associated invariant T cells in cancer: dual roles, complex interactions and therapeutic potential

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Mucosal-associated invariant T (MAIT) cells play diverse roles in cancer, infectious diseases, and immunotherapy. This review explores their intricate involvement in cancer, from early detection to their dual functions in promoting inflammation and mediating anti-tumor responses. Within the solid tumor microenvironment (TME), MAIT cells can acquire an 'exhausted' state and secrete tumor-promoting cytokines. On the other hand, MAIT cells are highly cytotoxic, and there is evidence that they may have an anti-tumor immune response. The frequency of MAIT cells and their subsets has also been shown to have prognostic value in several cancer types. Recent innovative approaches, such as programming MAIT cells with chimeric antigen receptors (CARs), provide a novel and exciting approach to utilizing these cells in cell-based cancer immunotherapy. Because MAIT cells have a restricted T cell receptor (TCR) and recognize a common antigen, this also mitigates potential graft-versus-host disease (GVHD) and opens the possibility of using allogeneic MAIT cells as off-the-shelf cell therapies in cancer. Additionally, we outline the interactions of MAIT cells with the microbiome and their critical role in infectious diseases and how this may impact the tumor responses of these cells. Understanding these complex roles can lead to novel therapeutic strategies harnessing the targeting capabilities of MAIT cells.

KEYWORDS

MAIT cell, CAR-MAIT, immunotherapy, cancer, microbiome, peripheral blood mononuclear cells

Abbreviations: MAIT, Mucosal-Associated Invariant T; PBMC, Peripheral Blood Mononuclear Cells; TCR, T Cell Receptor; MR1, Major Histocompatibility Complex Class I-Related Protein 1; TLR, Toll-Like Receptor; HIV, Human Immunodeficiency Virus; HBV, Hepatitis B Virus; NK, Natural Killer Cells; PD-1, Programmed Cell Death Protein 1; TME, Tumor Microenvironment; CTLA-4, Cytotoxic T-Lymphocyte-Associated Protein 4; TIM-3, T Cell Immunoglobulin and Mucin Domain 3; Treg, Regulatory T Cells; MDSC, Myeloid-Derived Suppressor Cells; aAPC, Artificial Antigen-Presenting Cells; GvHD: Graft-versus-Host Disease; iPSC, Induced Pluripotent Stem Cells; IFN, Interferon; TNF, Tumor Necrosis Factor; IL, Interleukin.

1 Introduction to MAIT cells

Mucosal-associated invariant T (MAIT) cells represent a conserved arm of the immune system, bridging the innate and adaptive responses that play a crucial role in the body's defense against infectious diseases, particularly at mucosal sites (1–4). They are distinguished by their specialized semi-invariant T cell receptors (TCRs), primarily composed of Vα7.2-Jα33 in humans, paired with a limited set of β-chains, which recognize riboflavin (vitamin B2) metabolites, produced by bacteria and fungi, presented by the MR1 molecule on the surface of infected cells (3–7). This unique antigen recognition mechanism allows MAIT cells to respond to a wide range of microbial infections, as these metabolite antigens are conserved across many bacterial and fungal species, not found in human cells (1, 6, 8–12). MAIT cells are also involved in tissue repair and regeneration, privileged interaction between defined members of the microbiota, which sequentially controls both tissue-imprinting and subsequent responses to injury (13, 14).

Upon encountering these microbial metabolites, MAIT cells are quickly activated and initiate a potent immune response through two distinct pathways: TCR-dependent (MR1-dependent) and TCR-independent (MR1-independent) mechanisms (Figure 1A). In the TCR-dependent pathway, they swiftly secrete a variety of pro-inflammatory cytokines, such as IFN-γ, TNF, IL-17, and express cytotoxic molecules like granzyme B and perforin. This enables them to recruit other cells to the site of inflammation such as neutrophils and modulate others such as dendritic cells and macrophages, thus influencing the broader immune response (1, 6, 8, 15–17). Additionally, their activation is augmented by cytokines such as IL-18, IL-23, and IL-1β, as well as TLR agonists and bacterial products (16, 18–21). This multi-signal approach ensures robust MAIT cell activation and proliferation, integral to their role in the immune response.

MAIT cells can also be activated in an TCR-independent (MR1-independent) manner by cytokines released in response to viral infections or by other immune cells. IL-12 and IL-18 are key players in this cytokine-driven pathway, and additional cytokines like IL-7, IL-15, and type-I IFNs also play a role (16, 22–24). The activation mediated by cytokines could offer an additional level of protection against viral infections. This is particularly relevant because these infections do not generate the riboflavin byproducts required for activation through the MR1 pathway (16, 24).

2 MAIT cells during infectious diseases

MAIT cells play a crucial role in the immune response to infections (Figure 1A). They have been shown to directly clear multidrug-resistant bacteria and overcome mechanisms of antimicrobial resistance (25–29). Studies have also supported the protective role of MAIT cells in the antimicrobial immune response, as suggested by mouse models where the deletion of MR1, and hence MAIT cells, rendered mice more susceptible to bacterial infections (10, 30). Moreover, activated MAIT cells produce diverse

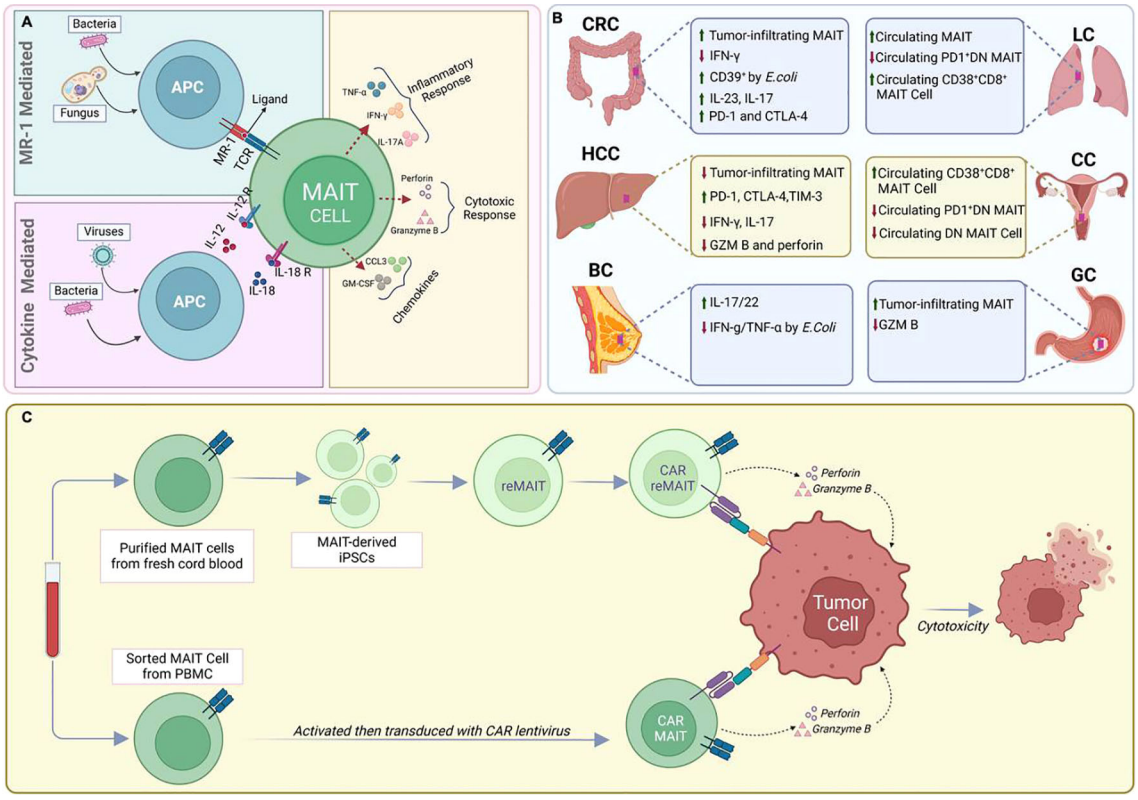

FIGURE 1
MAIT cells in infectious diseases and Cancer. **(A)**. Recognition of viruses or bacteria by MAIT cells **(B)** Functional and phenotypic features of tumor-infiltrating MAIT cells. **(C)** CAR-T cell engineering of natural or IPS-derived human MAIT cells against cancer.

cytokines and cytotoxic effector molecules and accumulate at the site of infection, demonstrating their protective role against various bacterial infections (31–33), including tuberculosis (26, 34), Salmonella (8, 35), Legionella (36, 37), Francisella tularensis (36, 38), klebsiella pneumoniae (39) and clostridium difficile (40). Zhao et al. demonstrated that systemic infection of mice with Francisella tularensis live vaccine strain (LVS) led to substantial expansion of MAIT cells in organs and blood. The study revealed a Th1-like MAIT-1 phenotype, featuring specific transcription factors and cytokine profiles, pivotal for controlling bacterial replication (41). Following infection resolution, Zhao et al. observed a transition of expanded MAIT cells into stable memory-like populations, suggesting a potential foundation for a Francisella tularensis vaccine (41).

Zheng et al. provide a comprehensive review on MAIT cells in gastrointestinal bacterial infections, revealing their intricate roles. They emphasize recent findings, showcasing the dynamic nature of MAIT cell responses, which vary based on etiological agents and anatomical locations (42). MAIT cells can also improve bacterial control during chronic infection and reduce bacterial loads through IL-17A-dependent mechanisms (26, 43).

MAIT cells have also been implicated in the immune response during viral infections such as COVID-19 and HIV infections. In the context of HIV infection, MAIT cells are depleted early on, but they retain functional cytokine expression, suggesting a potential role in controlling microbial translocation in the gut during HIV infection (34, 44). Additionally, MAIT cells have been shown to respond to and suppress HIV-1, suggesting an antiviral protective role *in vivo* (45). In the context of COVID-19, MAIT cells have been found to be highly activated and functionally impaired in COVID-19 patients and that their numbers decline sharply in the circulation (46, 47). Additionally, MAIT cell alterations markedly correlated with disease severity and patient mortality, suggesting a negative role for MAIT cells in severe COVID-19 infection, where their activation and Granzyme B production are at the highest levels (48). MAIT cells play a significant role in hepatitis infections, particularly in chronic liver diseases such as hepatitis B and hepatitis C. Studies have indicated that MAIT cells are involved in chronic hepatitis, with a decrease in their numbers independent of the cause (49). Furthermore, MAIT cells have been found to be severely reduced and exhausted in individuals with chronic hepatitis B virus (HBV) infection, suggesting a potential impact on the immune response in these patients (50)

MAIT cells were also investigated for their role in severe influenza infection. Analysis of patients with avian H7N9 influenza pneumonia revealed higher CD161+Vα7.2+ MAIT cell counts in survivors, suggesting a potential protective function. *In vitro* studies with influenza A virus (IAV)-infected lung epithelial cells and peripheral blood mononuclear cells demonstrated MAIT cell activation, characterized by upregulation of IFNγ and granzyme B. Notably, IL-18, produced by IAV-exposed CD14+ monocytes, was identified as a key factor in MAIT cell cytokine production. This IL-18-dependent activation suggests a protective role for MAIT cells in influenza and potentially in other inflammatory conditions involving IL-18 production (51).

Several authors have previously meticulously compiled comprehensive tables summarizing the multifaceted roles of MAIT cells in various infections (3, 52, 53).

3 Multifaceted impact of MAIT cells in cancer

MAIT cells have also been reported to serve important but diverse roles in human cancer by their dual roles in promoting inflammation and mediating anti-tumor responses, indicating their multifaceted impact on cancer (Figure 1B) (54–58). Associations have been determined between MAIT cell frequency, circulating inflammatory markers, and clinical parameters to elucidate the role of MAIT cells in inflammation-driven cancer, indicating their potential relevance in cancer progression (59, 60).

In multiple cancers, including glioma and others, Kubica et al. found that MR1 is overexpressed, yet its impact on overall survival varies (61). Specifically, in glioma, high MR1 levels correlate with poorer outcomes (61). However, the link between MR1 expression and survival is complex and not universally clear across cancer types since he correlation between MR1 levels and MAIT cell infiltration differs among cancers (52). Beyond solid tumors, MR1 expression is also observed in multiple myeloma cell lines, which can present the vitamin-B derivative ligand to MAIT cells, which in turn can induce cytotoxic activity towards these myeloma cells (62).

The ability of MAIT cells to exert cytotoxic effects and secrete inflammatory cytokines situates them at a crossroads of tumor biology, where they can potentially influence tumor growth, progression, and response to treatment (55, 58, 63, 64). However, the role of MAIT cells in cancer is not straightforward (52). Petley et al. showed that MAIT cells enhance NK cell mediated anti-tumor immunity by inducing an IFN- γ transcriptome in NK cells followed by activation (58). As such, it is worth noting that MAIT cells can play a supplementary rather than a primary role in NK cell-mediated anti-tumor activity. Additionally, Gentles et al. demonstrated that more CD8+CD161+ T cells, including MAIT cells, in tumors are linked to better outcomes in various cancers, suggesting a potential role for these cells in clinical benefits, supporting the theme of anti-tumor effects (65). Additionally, MAIT cells have been shown to influence the tumor microenvironment and could be relevant in cancer immunotherapy, although their exact role remains uncertain (57, 58, 63).

Conversely, other studies have highlighted the tumor-promoting functions of MAIT cells. For example, they can suppress T and NK cells and blocking the MR1 protein may offer a new strategy for cancer immunotherapy (57). In non-small cell lung cancer patients, MAIT cells exhibited an exhausted tumor-promoting phenotype and were found to predict the response to anti-PD-1 immunotherapy (66). Negative impact of IL-17 producing MAIT cells on cancer progression has also been observed, suggesting a potential detrimental role in cancers such as breast cancer (63). MAIT cells can also impair anti-tumor immunity by expressing PD-1 ligands, which can in turn lead to

conventional T cell exhaustion and reduced anti-tumor responses in prostate cancer (67).

The paradoxical behavior of MAIT cells that encompasses both the promotion of tumor growth and the execution of anti-tumor activities is possibly result of their ability to secrete a wide range of cytokines and cytotoxic molecules, influenced by the signals they receive from the tumor microenvironment (TME) (57–59, 68–71). On the one hand, MAIT cells have been observed to produce pro-inflammatory cytokines such as IL-17 and IL-13 within the TME (59, 72, 73). IL-17 is known to be involved in chronic inflammation and has been linked to the promotion of angiogenesis and tumor growth (74, 75). It can enhance the survival and proliferation of cancer cells, and its presence in the TME is often associated with a poor prognosis in several cancers (74–77) Similarly, IL-13 has been implicated in tumor progression and metastasis (73, 78). It contributes to an immunosuppressive TME by inhibiting the activity of cytotoxic cells and promoting the survival and proliferation of tumor cells (73, 78).

MAIT cells are also primarily found in mucosal tissues such as the gut, lungs, and cervix, and are thought to play a crucial role in the immune response in these tissues. As such, they could particularly migrate into tumor areas within these areas, including colorectal and cervical cancers (79). Their prevalence in these tissues positions them as potential early detectors and responders in the development of cancer, serving as the first line of defense against abnormal cellular changes that may lead to cancer (79).

The potential migration of MAIT cells into tumor areas within mucosal tissues has also been supported by the observation that circulating MAIT cells are reduced in mucosa-associated cancer patients, possibly because of migration to these areas (68). MAIT cells were also shown to accumulate in colon adenocarcinomas, indicating their potential to promote local immune responses to tumors, although factors in the tumor microenvironment may act to reduce MAIT cell IFN-γ production (79). Moreover, MAIT cells have been found to display hallmarks of bacterial antigen recognition in colorectal cancer (56). It was also suggested that MAIT cells in colorectal cancer, when activated, can be potential sources of stimulation in the tumor microenvironment (55).

In cervical cancer there also seems to be a link between the number of MAIT cells and myeloid-derived suppressor cells (MDSCs). This connection suggests that MAIT cells, through the release of IL-17 and other cytokines, could be involved in recruiting MDSCs to the tumor site, potentially aiding in tumor growth and progression (54; Lu et al., 2020). Increased CD8+, CD4+, and highly activated CD38+CD8+MAIT cells in cervical cancer patients' peripheral blood compared to healthy donors, along with reduced PD1+ double negative (DN) MAIT cells were also observed (Lu et al., 2020). Importantly, higher levels of circulating PD1+ DN MAIT cells correlated with improved progression-free survival in cervical cancer patients (Lu et al., 2020).

Several studies that have also focused on MAIT cell frequencies as prognostic factors in these cancers. In colorectal cancer (CRC) it was observed that MAIT cell numbers in the periphery decrease, possibly because these cells migrate preferentially to the cancerous areas (59, 70). This pattern of MAIT cell redistribution has also been observed in patients with gastric cancer (GC) and cervical cancer, indicating a similar trend in these types of cancer as well (64, 80). Whereas, increased circulating MAIT cells appear to be correlated with lung cancer progression, further suggesting their potential role in tumor areas within the lungs (71). To provide a concise overview of changes in MAIT cell function across various solid tumors, a summary is presented in Table 1. These clinical observations highlight the potential prognostic potential of MAIT cells in cancer.

Overall, these findings collectively suggest that MAIT cells can display contrasting roles in controlling anti-tumor immune responses depending on their activation status, may have complex and diverse roles in cancer progression and potential as therapeutic targets in cancer treatment (58). However, their exact impact on tumor progression and therapy remains a complex and evolving area of study.

4 Impact of tumor microenvironment on MAIT cell immune responses

As discussed above, the signals received by MAIT cells, such as cytokines, cell-cell interactions, and metabolic cues, can sway them towards either promoting tumor growth or exerting anti-tumor effects (Figure 1B). Understanding these signals and the conditions under which they operate is crucial for harnessing the therapeutic potential of MAIT cells in cancer (25, 56, 67, 68, 70, 73, 87).

Within the TME, MAIT cells can undergo significant phenotypic changes that alter their functionality. One of the most critical transformations is the development of an 'exhausted' phenotype. This state of exhaustion is characterized by a reduced capacity to proliferate, produce cytokines, and mediate cytotoxicity, alongside an increased expression of inhibitory receptors such as PD-1, CTLA-4, and TIM-3. Such phenotypic changes indicate that MAIT cells may contribute to an immunosuppressive tumor microenvironment, potentially aiding in cancer progression (25, 56, 67, 70, 87).

The path to exhaustion typically begins with chronic antigen stimulation and persistent inflammation within the TME (59, 70). As MAIT cells encounter an ongoing barrage of signals - from cancer cell antigens presented by MR1 to inflammatory cytokines and chemokines - they initially respond robustly. However, this sustained activation can lead to a state of overstimulation, may lead to the previously mentioned functional dysregulation (25, 56, 67, 70, 87).

The implications of MAIT cell exhaustion for cancer progression are manifold. Exhausted MAIT cells may contribute to an immunosuppressive TME, allowing cancer cells to evade immune detection and destruction. They may also promote the recruitment and function of regulatory cells, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), which further inhibit the anti-tumor response. In addition, these exhausted MAIT cells can secrete cytokines that support tumor survival, angiogenesis, and metastasis, thus inadvertently aiding cancer progression.

Furthermore, the exhausted phenotype is often associated with a reduced production of IFN-γ, IL-17, granzyme B, and perforin by

TABLE 1 MAIT cells in human cancer.

Human cancer Type	Functionality Changes in response to TME	Reference/ Year Published
Colorectal Cancer (CRC)	↑Tumor-infiltrating MAIT ↑CD39+ MAIT cells (stimulated by E. coli) ↑PD-1 and CTLA-4 ↑Ki-67 ↓IFN-γ	(56)
	↑ IL-23 ↑ IL-17 ↑ IL-6	(81)
	↑Tumor-infiltrating MAIT ↑IL-17 secretion from MAIT	(82)
	↑Tumor-infiltrating MAIT ↓IFN-γ producing MAITs No change in TNF-α or IL-17	(79)
	↑Tumor-infiltrating MAIT ↓IFN-γ ↑IL-17A	(59)
	↑Tumor-infiltrating MAIT ↓IFN-γ	(83)
	↑Tumor-infiltrating DN MAIT ↑PD-1^{high}Tim3^{+}CD39^{+} exhausted phenotype	(70)
Prostate cancer (PC)	↑PD-L1/L2 upregulation on Tumor-infiltrating MAIT (induced by 5-A-RU)	(67)
Gastric cancer (GC)	↑Tumor-infiltrating MAIT ↓ GZM B	(64)
Cervical Cancer (CC)	↓ Circulating MAIT	(80)
	↑Circulating CD38^{+}CD8^{+}MAIT cell ↓Circulating PD1^{+}DN MAIT ↓DN MAIT cell	(84)
Multiple Myeloma (MM)	↓Circulating MAIT ↓CD27 expression on MAIT ↓IFN-γ production from MAIT	(62)
Breast carcinoma	↑IL-17/22 ↓IFN-γ/TNF-alpha by E. Coli	(85)
Colorectal liver metastases (CRLM)	↓Tumor-infiltrating MAIT ↓IFN-γ	(72)
Hepatocellular carcinoma(HCC)	↓Tumor-infiltrating MAIT	(86)
	↓Tumor-infiltrating MAIT ↑PD-1, CTLA-4, TIM-3 ↓IFN-γ, IL-17, GZM B and perforin	(87)
Lung Cancer (LC)	↑Circulating MAIT ↓Circulating PD1^{+}DN MAIT ↑CD38^{+}CD8^{+}MAIT cell	(71)

MAIT cells (88, 89). This not only diminishes the direct anti-tumor activity of MAIT cells but also impacts the broader anti-tumor immune response, as these molecules play crucial roles in orchestrating and executing effective anti-cancer immunity (82, 83, 87–89).

In hepatocellular carcinoma (HCC), tumor tissue localized MAIT cells also exhibited exhausted phenotype with upregulation of inhibitory immune molecules (PD-1, CTLA-4, and TIM-3) and secreted lower quantities of effector molecules (e.g., IFN-g, granzyme B, and perforin) (87). As suggested by this immunosuppressive phenotype, high MAIT cell infiltration into HCC solid tumors was correlated with adverse prognosis (87). Furthermore, these cells often express pro-tumor cytokines like IL-8, possibly contributing to the recruitment of immunosuppressive MDSCs to the tumor site. The presence of such immunosuppressive MAIT cells in HCC has been associated with a negative impact on patient prognosis (87, 90).

Thus, the presence, functional state, and interactions of MAIT cells with other immune cells within the TME can provide valuable insights into disease progression and patient outcomes. However, the dualistic nature of MAIT cells and their varying roles in different cancer types or even within the same cancer type but at different stages or different patients underscore the need for a deeper understanding of their biology. Understanding these mechanisms is crucial because it could lead to novel therapeutic approaches that leverage MAIT cells' natural targeting abilities including genetically engineering MAIT cells to enhance cancer treatment.

5 Role of MAIT cells in cancer treatment

The role of MAIT cells in positive contribution to anti-tumor immune responses is also an area of ongoing investigation (66) especially against solid tumors (65, 91). MAIT cells are capable of exerting anti-tumor effects, predominantly through the action of cytotoxic molecules (92). They can also release granzyme B and perforin, which are essential for the direct killing of target cells (23, 93). The anti-tumor activity of MAIT cells is further enhanced by their ability to produce IFN-γ, a cytokine critical for immune surveillance and the activation of other immune cells, including macrophages and dendritic cells. IFN-γ can enhance the antigen-presenting capabilities of these cells, leading to a more effective anti-tumor immune response (23, 32, 94, 95).

As discussed above, MAIT cells express immune checkpoint receptors and possess cytotoxic functions, making them attractive in tumor immunology research. Innate-like T cell subsets, including MAIT cells, have shown potential in targeting the immunosuppressive TME composed of tumor-associated macrophages (TAMs) and MDSCs (32, 63, 70). Interestingly, Biasi et al. (2021) found that detecting circulating MAIT cells could identify patients' positive responses to anti-PD1 therapy, suggesting their promise as a biomarker for treatment effectiveness (96). Additionally, the frequency of circulating MAIT cells was observed to influence treatment response and survival in melanoma patients undergoing anti-PD1 therapy, emphasizing a potential role for MAIT cells in shaping treatment outcomes (97).

While the current understanding of how MAIT cells are activated within tumors is predominantly focused on their response to microbial metabolites (59) the possibility that they could also be triggered by antigens originating from the tumor itself

has not been ruled out (55, 63). If such tumor-derived antigens that activate MAIT cells are identified, they could serve as precise targets for new immunotherapies, potentially leading to more effective cancer treatments.

Besides solid tumors, the involvement of mucosa-associated invariant T (MAIT) cells in hematological malignancies and transplantation immunity has also been a subject of interest in recent research. MAIT cells may also play multifunction role in hematological malignancies and transplantation immunity, highlighting their potential role as an immunomodulatory factor (98).

However, experimental evidence indicates that MAIT cells can retain their anti-tumor functions even when MR1 is knocked out from the tumor cells, suggesting alternative pathways through which MAIT cells can exert their effects. The nuances of MR1 expression and the conditions that influence the anti-tumor activities of MAIT cells are still under investigation. Discrepancies in MR1 expression across various tumor cell lines and the resulting differences in MAIT cell response underscore the complexity of the immune system's interaction with cancer. Utilization of 3D bioprinted tumor models to study the effect of localization and activation of MAIT cells within the 3D tumor tissue model could help elucidate and these complex interactions (99).

Priya and Brutkiewicz et al. use an artificial antigen-presenting cell (aAPC) comprised of latex beads coated with an MR1 tetramer complex loaded with antigen (Ag) and anti-CD28 antibody, they successfully addressed MAIT cell expansion limitations (100). Furthermore, they demonstrated that aAPC-expanded MAIT cells retained functionality, preserved their original phenotype, and displayed both proinflammatory cytokine secretion and cytotoxicity against GBM cell lines (100). These findings strongly support the potential of aAPC-generated MAIT cells as a promising and efficient option for adoptive immunotherapy against tumors (100).

Recent advancements in immunotherapy have highlighted chimeric antigen receptor (CAR)-based therapies, with CAR-T cells showing promise in targeting hematological malignancies (Figure 1C) (52, 101). However, these therapies have significant limitations, such as severe side effects, and are less effective against solid tumors. Additionally, CAR-T therapies require autologous cells, which are costly and time-consuming to produce (102).

Utilizing MAIT cells engineered with CAR molecules (CAR-MAIT) could be a novel approach that may address some of these challenges. Recently, we developed such CAR-MAIT cells to target lymphomas and breast cancer cells (101). We had found that these engineered cells demonstrate robust cytotoxicity against CD19+ lymphomas and Her2-expressing breast cancer cell lines, while releasing lower levels of inflammatory cytokines, potentially leading to fewer adverse reactions like cytokine release syndrome (101). This approach also avoids the MHC class I restriction, enabling the development of 'off-the-shelf' therapies. Importantly, we were able to expand large numbers of these MAIT cells from PBMCs of healthy donors (typically more than 100-fold). Given that MAIT cell proportions can be as high as 10-20% of CD8 T cells in some donors, we believe that generating abundant cells for off-the-shelf therapeutic applications is likely achievable (101). Furthermore, MAIT cells may continue to expand *in vivo*, as they will still recognize and be activated by their common cognate antigen, thus requiring fewer cell transfers to patients.

Efforts to address the suboptimal ex vivo expansion efficiency of Mucosal-Associated Invariant T (MAIT) cells from healthy donor peripheral blood led to the development of pluripotent stem cell (PSC)-derived MAIT-like cells (Figure 1C) (103). Wakao et al. pioneered the reprogramming of cord blood (CB) MAIT cells into induced pluripotent stem cells (iPSCs) using a Sendai viral vector (103). The resulting MAIT-iPSC clones demonstrated pluripotency through various tests and were differentiated into MAIT-like lymphocytes using a two-step protocol (103). More recently, it was proposed that iPSC-derived Mucosal-Associated Invariant T (MAIT) cells in tumor immune cell therapy or prophylaxis may prevent relapse (104). While phenotypic and functional characteristics of reprogrammed MAIT cells do not fully mirror those of PBMC-derived MAIT cells (103), this innovative approach further aims to address the limitations associated with the ex vivo expansion of MAIT cells, such as the restricted numbers obtained after expansion compared to the ex vivo expansion of all T cells. Together, the ability to efficiently expand MAIT cells ex vivo while maintaining a functional phenotype may facilitate the development of new MAIT cell–based tumor immunotherapies.

One distinguishing characteristic that significantly contributes to their therapeutic appeal is their incapacity to induce graft-versus-host disease (GvHD) (105–107). This is attributed to their restriction by MR1 and their non-recognition of mismatched major histocompatibility complex (MHC) molecules and protein autoantigens (105). Rigorous assessments, including *in vitro* studies and xenogeneic GvHD mouse models, consistently affirm the GvHD-free safety profile of MAIT cells (108). Recent research endeavors have shed light on the multifaceted advantages of MAIT cells, extending beyond their GvHD-free safety profile. Notably, MAIT cells exhibit resistance to xenobiotics, adding an additional layer of appeal for their therapeutic application (2). Studies conducted by Bohineust et al. showcase that these engineered MAIT cells can engraft without eliciting GvHD in preclinical immunodeficient mouse models, presenting a distinct advantage over CD19-targeted CAR-T cells (109).

Furthermore, several studies demonstrated the potential for MAIT cells in solid tumor-targeting immunotherapies due to their natural ability to infiltrate tissue sites of chronic inflammation and their abundance in the tumor microenvironment (99, 101, 109, 110). In this tumor environment, MAIT cells can also act as innate T cells with the potential to bridge innate and adaptive immune immunity, and can rapidly exert their effector functions upon activation without the requirement for clonal expansion, similar to innate immune (111) (112). Upon activation, MAIT cells rapidly secrete cytokines and exert cytotoxic functions, making them highly relevant in tumor immunity (70). Indeed, such feature would be significant in that even in the case where tumors escape the attack from CAR-MAIT cells, they can still exert anti-tumor activity.

Overall, we think CAR-MAIT cells represent a significant advancement in our arsenal of immunotherapy. Their development could overcome the limitations of current CAR-T therapies and harness the immune system's power more safely and effectively.

Future studies, especially *in vivo* experiments will be needed to fully understand the therapeutic potential of CAR-MAIT cells.

6 MAIT cell and microbiome interplay in cancer

MAIT cells also play a crucial role in interacting with the microbiota and its products, protecting body barriers, and maintaining tissue homeostasis (113). MAIT cells have been found to be enriched in the human liver, which is constantly exposed to bacterial products from the intestine, indicating their role in the gut microbiota-liver axis (114, 115). Furthermore, MAIT cells have been shown to control barrier function and attenuate pathogenic T cell responses in the colon, suggesting their importance in maintaining gut integrity (116). The gut microbiome is crucial for the development and function of MAIT cells, and their association with the tumor microbiome supports the interest in manipulating the gut microbiome to reshape their response (56, 117).

Additionally, MAIT cells are thought to play a role in bacterial immunity, as they interact with metabolites synthesized by various yeast and bacteria (118). Moreover, MAIT cells have been proposed to play an important role in protecting mucosal surfaces against multiple bacterial pathogens (119). In contrast, studies have also revealed a deleterious role of MAIT cells in promoting inflammation, gut mucosa dysfunction, and gut microbiota alteration in obesity, indicating a complex role of MAIT cells in different contexts (3). Furthermore, the reduction and functional immaturity of MAIT cells in the peripheral blood of patients with primary Sjögren's syndrome suggest a potential link between MAIT cells and autoimmune conditions (119).

The potential role of MAIT cells in the regulation of metabolic pathways and their crosstalk with the microbiota represents an exciting new line of research (120). In our studies focusing on activation of MAIT cells differing in their TCR Vbeta regions, we found that MAIT cells responded selectively to microorganisms that produce riboflavin metabolites, which are common across a variety of bacterial species within the microbiota (11). This suggests that MAIT cells play a role in fine-tuning the immune response to a broad spectrum of microbial inhabitants in the human body, including both commensal and pathogenic species. This ability to discriminate between different bacteria can be crucial for maintaining a balanced microbiome. The insights into how MAIT cells interact with the microbiome could have implications for understanding and treating diseases that are influenced by microbiota imbalances, such as inflammatory bowel disease, obesity, and certain types of cancer or immunotherapy as discussed below.

Growing evidence highlights a relationship between the human microbiome and cancer, particularly in the context of solid tumors. The composition of the microbiome has been found to offer prognostic insights and is implicated in the initiation, growth, and spread of tumors. Additionally, certain microbial signatures have been identified that may either contribute to tumor development or enhance the efficacy of immunotherapies (121–123).

MAIT cells, which have an affinity for microbe-rich environments and respond to microbial metabolites such as those from riboflavin metabolism, are poised to play a crucial role in the dialogue between the microbiome and the tumor microenvironment (52). They possess the unique ability to detect these metabolites presented by the MR1 protein on the surface of various cells, including tumor cells (6). Notably, Liu and Brutkiewicz et al. investigated the role of TLR9 activation in MR1-mediated bacterial antigen presentation and concluded that the activation of Toll-like Receptor 9 (TLR9) is crucial for Major Histocompatibility Complex Class I-Related gene (MR1)-mediated bacterial antigen presentation (124). Interestingly, studies have shown that melanoma cells can increase their expression of MR1 when exposed to the microbial metabolite 5-OP-RU, highlighting the potential for microbial components to modulate immune recognition (62).

Changes in the microbiota, termed dysbiosis, have been linked to tumor development and progression. Dysbiosis can lead to altered production of microbial metabolites, affecting the activation and function of MAIT cells. For example, a microbiota that favors inflammation may promote the secretion of pro-tumorigenic cytokines by MAIT cells, such as IL-17 and IL-13, contributing to tumor growth and progression (123). Conversely, certain microbial populations might induce anti-tumor responses by MAIT cells, enhancing their cytotoxic functions and the production of IFN-γ, which could suppress tumor growth. For instance, some studies suggest that specific bacterial strains can boost the efficacy of cancer immunotherapies, potentially through the activation of MAIT cells (125).

Moreover, the TME itself can alter the local microbiota, creating a feedback loop that further influences MAIT cell function (14, 126, 127). Tumor-induced changes in the local environment, such as hypoxia or altered nutrient availability, can select for microbial populations that either exacerbate or mitigate tumor progression (127).

The microbiota also plays a role in the modulation of the immune checkpoint molecules on MAIT cells. For example, certain bacteria might influence the expression of PD-1 on MAIT cells, affecting their state of exhaustion and ability to fight cancer cells (70, 128). Thus, given the central role of the microbiota in regulating immune responses, therapeutic strategies are being considered that could modulate the microbiota to enhance the anti-tumor activity of MAIT cells. These include the use of prebiotics, probiotics, or fecal microbiota transplants to reshape the microbial ecosystem in favor of a more robust anti-tumor immune response.

MAIT cells' interactions with both cancer cells and the microbiome are integral to their dual role in tumor biology. Their capacity to respond to early cancerous changes, influenced by shifts in the microbiome, along with the ongoing research into their tumor recognition mechanisms, underscores the potential of MAIT cells as targets for innovative cancer therapies (56, 63).

7 Conclusions and future directions

In conclusion, current advances in MAIT cell immunobiology underscores the necessity to unravel the involvement and therapeutic

potential of MAIT cells in in cancer biology, as well as to exploit the microbiome-MAIT cell interaction for the development of new cancer immunotherapies. The abundance of MAIT cells, ability to expand them *in vitro* with their antigen and possibility of generating these cells from iPSCs in humans presents an exciting avenue for future studies to develop them into CAR-MAIT cells. Other diverse avenues for future studies may include the exploration and development of immunotherapeutic strategies through utilization of MAIT cell anti-tumor recognition and functions and the potential use of small molecule or microbiome-derived therapeutics to stimulate their responses. These approaches highlight the promising prospects for advancing cancer treatment through understanding and developing MAIT cell-based therapeutics.

Author contributions

DU: Conceptualization, Writing – original draft, Writing – review & editing. MY: Writing – original draft, Writing – review & editing. OB: Visualization, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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

References

1. Martin E, Treiner E, Duban L, Guerri L, Laude H, Toly C, et al. Stepwise development of MAIT cells in mouse and human. *PloS Biol.* (2009) 7:e54. doi: 10.1371/journal.pbio.1000054

2. Dusseaux M, Martin E, Serriari N, Peguillet I, Premel V, Louis D, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood.* (2011) 117:1250–9. doi: 10.1182/blood-2010-08-303339

3. Toubal A, Nel I, Lotersztajn S, Lehuen A. Mucosal-associated invariant T cells and disease. *Nat Rev Immunol.* (2019) 19:643–57. doi: 10.1038/s41577-019-0191-y

4. Ioannidis M, Cerundolo V, Salio M. The immune modulating properties of mucosal-associated invariant T cells. *Front Immunol.* (2020) 11:1556. doi: 10.3389/fimmu.2020.01556

5. Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature.* (2003) 422:164–9. doi: 10.1038/nature01433

6. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature.* (2012) 491:717–23. doi: 10.1038/nature11605

7. Eckle SB, Corbett AJ, Keller AN, Chen Z, Godfrey DI, Liu L, et al. Recognition of vitamin B precursors and byproducts by mucosal associated invariant T cells. *J Biol Chem.* (2015) 290:30204–11. doi: 10.1074/jbc.R115.685990

8. Le Bourhis L, Martin E, Peguillet I, Guihot A, Froux N, Core M, et al. Antimicrobial activity of mucosal-associated invariant T cells. *Nat Immunol.* (2010) 11:701–8. doi: 10.1038/ni.1890

9. Lepore M, Kalinichenko A, Colone A, Paleja B, Singhal A, Tschumi A, et al. Parallel T-cell cloning and deep sequencing of human MAIT cells reveal stable oligoclonal TCRbeta repertoire. *Nat Commun.* (2014) 5:3866. doi: 10.1038/ncomms4866

10. Meermeier EW, Harriff MJ, Karamooz E, Lewinsohn DM. MAIT cells and microbial immunity. *Immunol Cell Biol.* (2018) 96:607–17. doi: 10.1111/imcb.12022

11. Tastan C, Karhan E, Zhou W, Fleming E, Voigt AY, Yao X, et al. Tuning of human MAIT cell activation by commensal bacteria species and MR1-dependent T-cell presentation. *Mucosal Immunol.* (2018) 11:1591–605. doi: 10.1038/s41385-018-0072-x

12. Garner LC, Amini A, FitzPatrick MEB, Lett MJ, Hess GF, Filipowicz Sinnreich M, et al. Single-cell analysis of human MAIT cell transcriptional, functional and clonal diversity. *Nat Immunol.* (2023) 24:1565–78. doi: 10.1038/s41590-023-01575-1

13. Constantinides MG, Link VM, Tamoutounour S, Wong AC, Perez-Chaparro PJ, Han SJ, et al. MAIT cells are imprinted by the microbiota in early life and promote tissue repair. *Science.* (2019) 366. doi: 10.1126/science.aax6624

14. Jabeen M, Hinks T. MAIT cells and the microbiome. *Front Immunol.* (2023) 14. doi: 10.3389/fimmu.2023.1127588

15. Meierovics AI, Cowley SC. MAIT cells promote inflammatory monocyte differentiation into dendritic cells during pulmonary intracellular infection. *J Exp Med.* (2016) 213:2793–809. doi: 10.1084/jem.20160637

16. Hinks TSC, Zhang XW. MAIT cell activation and functions. *Front Immunol.* (2020) 11:1014. doi: 10.3389/fimmu.2020.01014

17. Fan Q, Nan H, Li Z, Li BT, Zhang FZ, Bi LQ. New insights into MAIT cells in autoimmune diseases. *Biomedicine Pharmacotherapy.* (2023) 159. doi: 10.1016/j.biopha.2023.114250

18. Turtle CJ, Delrow JJ, Joslyn RC, Swanson HM, Basom R, Tabellini L, et al. Innate signals overcome acquired TCR signaling pathway regulation and govern the fate of human CD161hi CD8α+ Semi-invariant T cells. *Blood.* (2011) 118(10):2752–62. doi: 10.1182/blood-2011-02-334698

19. Hagel JP, Garner LC, Bilton M, Mehta H, Leng TQ, Hackstein CP, et al. Human MAIT cell activation *in vitro*. *Mait Cells: Methods Protoc.* (2020) 2098:97–124. doi: 10.1007/978-1-0716-0207-2_7

20. Tao HS, Pan Y, Chu S, Li L, Xie JH, Wang P, et al. Differential controls of MAIT cell effector polarization by mTORC1/mTORC2 via integrating cytokine and costimulatory signals. *Nat Commun.* (2021) 12. doi: 10.1038/s41467-021-22162-8

21. Li YR, Zhou K, Wilson M, Kramer A, Zhu Y, Dawson N, et al. Mucosal-associated invariant T cells for cancer immunotherapy. *Mol Ther.* (2023) 31:631–46. doi: 10.1016/j.ymthe.2022.11.019

22. Ussher JE, Bilton M, Attwod E, Shadwell J, Richardson R, de Lara C, et al. CD161 ++ CD8+ T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner. *Eur J Immunol.* (2014) 44:195–203. doi: 10.1002/eji.201343509

23. Kurioka A, Ussher JE, Cosgrove C, Clough C, Fergusson JR, Smith K, et al. MAIT cells are licensed through granzyme exchange to kill bacterially sensitized targets. *Mucosal Immunol.* (2015) 8(2):429–40. doi: 10.1038/mi.2014.81

24. van Wilgenburg B, Scherwitzl I, Hutchinson EC, Leng T, Kurioka A, Kulicke C, et al. MAIT cells are activated during human viral infections. *Nat Commun.* (2016) 7:11653. doi: 10.1038/ncomms11653

25. Leeansyah E, Ganesh A, Quigley MF, Sonnerborg A, Andersson J, Hunt PW, et al. Activation, exhaustion, and persistent decline of the antimicrobial MR1-restricted MAIT-cell population in chronic HIV-1 infection. *Blood.* (2013) 121:1124–35. doi: 10.1182/blood-2012-07-445429

26. Sakai S, Kauffman KD, Oh S, Nelson CE, Barry CE 3rd, Barber DL. MAIT cell-directed therapy of Mycobacterium tuberculosis infection. *Mucosal Immunol.* (2021) 14:199–208. doi: 10.1038/s41385-020-0332-4

27. Chua WJ, Truscott SM, Eickhoff CS, Blazevic A, Hoft DF, Hansen TH. Polyclonal mucosa-associated invariant T cells have unique innate functions in bacterial infection. *Infect Immun.* (2012) 80:3256–67. doi: 10.1128/IAI.00279-12

28. Boulouis C, Sia WR, Gulam MY, Teo JQM, Png YT, Phan TK, et al. Human MAIT cell cytolytic effector proteins synergize to overcome carbapenem resistance in. *PloS Biol*. (2020) 18. doi: 10.1101/2020.01.16.908806

29. Wu SH, Yang X, Lou YL, Xiao XX. MAIT cells in bacterial infectious diseases: heroes, villains, or both?. *Clin Exp Immunol*. (2023). doi: 10.1093/cei/uxad102

30. van Wilgenburg B, Loh L, Chen ZJ, Pediongco TJ, Wang HM, Shi M, et al. MAIT cells contribute to protection against lethal influenza infection in *vivo*. *Nat Commun*. (2018) 9. doi: 10.1038/s41467-018-07207-9

31. Hartmann N, Harriff MJ, McMurtrey CP, Hildebrand WH, Lewinsohn DM, Kronenberg M. Role of MAIT cells in pulmonary bacterial infection. *Mol Immunol*. (2018) 101:155–59. doi: 10.1016/j.molimm.2018.06.270

32. Rudak PT, Choi JJ, Haeryfar SMM. MAIT cell-mediated cytotoxicity: roles in host defense and therapeutic potentials in infectious diseases and cancer. *J Leukocyte Biol*. (2018) 104(3):473–86. doi: 10.1002/JLB.4RI0118-023R

33. Nel I, Bertrand L, Toubal A, Lehuen A. MAIT cells, guardians of skin and mucosa?. *Mucosal Immunol*. (2021) 14:803–14. doi: 10.1038/s41385-021-00391-w

34. Fernandez CS, Amarasena T, Kelleher AD, Rossjohn J, McCluskey J, Godfrey DI, et al. MAIT cells are depleted early but retain functional cytokine expression in HIV infection. *Immunol Cell Biol*. (2015) 93:177–88. doi: 10.1038/icb.2014.91

35. Chen Z, Wang H, D'Souza C, Sun S, Kostenko L, Eckle SB, et al. Mucosal-associated invariant T-cell activation and accumulation after in *vivo* infection depends on microbial riboflavin synthesis and co-stimulatory signals. *Mucosal Immunol*. (2017) 10:58–68. doi: 10.1038/mi.2016.39

36. Meierovics A, Yankelevich WJ, Cowley SC. MAIT cells are critical for optimal mucosal immune responses during in *vivo* pulmonary bacterial infection. *Proc Natl Acad Sci U.S.A*. (2013) 110:E3119–28. doi: 10.1073/pnas.1302799110

37. Wang H, D'Souza C, Lim XY, Kostenko L, Pediongco TJ, Eckle SBG, et al. MAIT cells protect against pulmonary Legionella longbeachae infection. *Nat Commun*. (2018) 9:3350. doi: 10.1038/s41467-018-05202-8

38. Shibata K, Shimizu T, Nakahara M, Ito E, Legoux F, Fujii S, et al. The intracellular pathogen Francisella tularensis escapes from adaptive immunity by metabolic adaptation. *Life Sci Alliance*. (2022) 5. doi: 10.26508/lsa.202201441

39. Georgel P, Radosavljevic M, Macquin C, Bahram S. The non-conventional MHC class I MR1 molecule controls infection by Klebsiella pneumoniae in mice. *Mol Immunol*. (2011) 48:769–75. doi: 10.1016/j.molimm.2010.12.002

40. Smith AD, Foss ED, Zhang I, Hastie JL, Giordano NP, Gasparyan L, et al. Microbiota of MR1 deficient mice confer resistance against Clostridium difficile infection. *PloS One*. (2019) 14:e0223025. doi: 10.1371/journal.pone.0223025

41. Zhao Z, Wang H, Shi M, Zhu T, Pediongco T, Lim XY, et al. Francisella tularensis induces Th1 like MAIT cells conferring protection against systemic and local infection. *Nat Commun*. (2021) 12:4355. doi: 10.1038/s41467-021-24570-2

42. Zheng Y, Han F, Ho A, Xue Y, Wu Z, Chen X, et al. Role of MAIT cells in gastrointestinal tract bacterial infections in humans: More than a gut feeling. *Mucosal Immunol*. (2023) 16:740–52. doi: 10.1016/j.mucimm.2023.06.005

43. Sakai S, Lora NE, Kauffman KD, Dorosky DE, Oh S, Namasivayam S, et al. Functional inactivation of pulmonary MAIT cells following 5-OP-RU treatment of non-human primates. *Mucosal Immunol*. (2021) 14:1055–66. doi: 10.1038/s41385-021-00425-3

44. Barber-Axthelm IM, Kent SJ, Juno JA. Understanding the role of mucosal-associated invariant T-cells in non-human primate models of HIV infection. *Front Immunol*. (2020) 11:2038. doi: 10.3389/fimmu.2020.02038

45. Phetsouphanh C, Phalora P, Hackstein CP, Thornhill J, Munier CML, Meyerowitz J, et al. Human MAIT cells respond to and suppress HIV-1. *Elife*. (2021) 10. doi: 10.7554/eLife.50324

46. Parrot T, Gorin JB, Ponzetta A, Maleki KT, Kammann T, Emgard J, et al. MAIT cell activation and dynamics associated with COVID-19 disease severity. *Sci Immunol*. (2020) 5. doi: 10.1101/2020.08.27.20182550

47. Yang Q, Wen Y, Qi F, Gao X, Chen W, Xu G, et al. Suppressive monocytes impair MAIT cells response via IL-10 in patients with severe COVID-19. *J Immunol*. (2021) 207:1848–56. doi: 10.4049/jimmunol.2100228

48. Flament H, Rouland M, Beaudoin L, Toubal A, Bertrand L, Lebourgeois S, et al. Outcome of SARS-CoV-2 infection is linked to MAIT cell activation and cytotoxicity. *Nat Immunol*. (2021) 22:322–35. doi: 10.1038/s41590-021-00870-z

49. Tan HY, Yong YK, Ng CS, Vimali J, Mohamed R, Murugesan A, et al. Mucosal-associated invariant T cells: diplomatic front-runners in the fight against hepatitis B virus infection. *Crit Rev Immunol*. (2021) 41:1–16. doi: 10.1615/CritRevImmunol.v41.i5

50. Huang W, He W, Shi X, Ye Q, He X, Dou L, et al. Mucosal-associated invariant T-cells are severely reduced and exhausted in humans with chronic HBV infection. *J Viral Hepat*. (2020) 27:1096–107. doi: 10.1111/jvh.13341

51. Loh L, Wang Z, Sant S, Koutsakos M, Jegaskanda S, Corbett AJ, et al. Human mucosal-associated invariant T cells contribute to antiviral influenza immunity via IL-18-dependent activation. *Proc Natl Acad Sci U.S.A*. (2016) 113:10133–8. doi: 10.1073/pnas.1610750113

52. Godfrey DI, Koay HF, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. *Nat Immunol*. (2019) 20:1110–28. doi: 10.1038/s41590-019-0444-8

53. Joyce S, Okoye GD, Driver JP. Die Kampfe und schlachten-the struggles and battles of innate-like effector T lymphocytes with microbes. *Front Immunol*. (2023) 14:1117825. doi: 10.3389/fimmu.2023.1117825

54. Haeryfar SMM, Shaler CR, Rudak PT. Mucosa-associated invariant T cells in malignancies: a faithful friend or formidable foe? *Cancer Immunol Immunother*. 67 (12):1885–96. doi: 10.1007/s00262-018-2132-1

55. Berzins SP, Wallace ME, Kannourakis G, Kelly J. A role for MAIT cells in colorectal cancer. *Front Immunol*. (2020) 11:949. doi: 10.3389/fimmu.2020.00949

56. Li S, Simoni Y, Becht E, Loh CY, Li N, Lachance D, et al. Human tumor-infiltrating MAIT cells display hallmarks of bacterial antigen recognition in colorectal cancer. *Cell Rep Med*. (2020) 1:100039. doi: 10.1016/j.xcrm.2020.100039

57. Yan J, Allen S, McDonald E, Das I, Mak JYW, Liu L, et al. MAIT cells promote tumor initiation, growth, and metastases via tumor MR1. *Cancer Discov*. (2020) 10:124–41. doi: 10.1158/2159-8290.CD-19-0569

58. Petley EV, Koay HF, Henderson MA, Sek K, Todd KL, Keam SP, et al. MAIT cells regulate NK cell-mediated tumor immunity. *Nat Commun*. (2021) 12:4746 doi: 10.1038/s41467-021-25009-4

59. Ling L, Lin Y, Zheng W, Hong S, Tang X, Zhao P, et al. Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients. *Sci Rep*. (2016) 6:20358. doi: 10.1038/srep20358

60. Melo AM, O'Brien AM, Phelan JJ, Kennedy SA, Wood NAW, Veerapen N, et al. Mucosal-associated invariant T cells display diminished effector capacity in oesophageal adenocarcinoma. *Front Immunol*. (2019) 10:1580. doi: 10.3389/fimmu.2019.01580

61. Kubica P, Lara-Velazquez M, Bam M, Siraj S, Ong I, Liu P, et al. MR1 overexpression correlates with poor clinical prognosis in glioma patients. *Neurooncol Adv*. (2021) 3:vdab034. doi: 10.1093/noajnl/vdab034

62. Gherardin NA, Loh L, Admojo L, Davenport AJ, Richardson K, Rogers A, et al. Enumeration, functional responses and cytotoxic capacity of MAIT cells in newly diagnosed and relapsed multiple myeloma. *Sci Rep*. (2018) 8:4159. doi: 10.1038/s41598-018-22130-1

63. Cogswell DT, Gapin L, Tobin HM, McCarter MD, Tobin RP. MAIT cells: partners or enemies in cancer immunotherapy?. *Cancers (Basel)*. (2021) 13. doi: 10.3390/cancers13071502

64. Shao C, Zhu C, Zhu Y, Hao J, Li Y, Hu H, et al. Decrease of peripheral blood mucosal-associated invariant T cells and impaired serum Granzyme-B production in patients with gastric cancer. *Cell Biosci*. (2021) 11:12. doi: 10.1186/s13578-020-00518-9

65. Gentles AJ, Newman AM, Liu CL, Bratman SV, Feng W, Kim D, et al. The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med*. (2015) 21:938–45. doi: 10.1038/nm.3909

66. Shi L, Lu J, Zhong D, Song M, Liu J, You W, et al. Clinicopathological and predictive value of MAIT cells in non-small cell lung cancer for immunotherapy. *J Immunother Cancer*. (2023) 11. doi: 10.1136/jitc-2022-005902

67. Jarvis EM, Collings S, Authier-Hall A, Dasyam N, Luey B, Nacey J, et al. Mucosal-associated invariant T (MAIT) cell dysfunction and PD-1 expression in prostate cancer: implications for immunotherapy. *Front Immunol*. (2021) 12:748741. doi: 10.3389/fimmu.2021.748741

68. Won EJ, Ju JK, Cho Y-N, Jin H-M, Park K-J, Kim T-J, et al. Clinical relevance of circulating mucosal-associated invariant T cell levels and their anti-cancer activity in patients with mucosal-associated cancer. *Oncotarget*. (2016). doi: 10.18632/oncotarget.v7i46

69. Mo J, Li Z, Gao Z, Wu J, Bao Y. The study of mucosal-associated invariant T cells in colon cancer and roles in immune activities. *Oncotargets Ther*. (2021) 2021 (14):5263–73. doi: 10.2147/OTT.S332822

70. Rodin W, Sundstrom P, Ahlmanner F, Szeponik L, Zajt KK, Wettergren Y, et al. Exhaustion in tumor-infiltrating Mucosal-Associated Invariant T (MAIT) cells from colon cancer patients. *Cancer Immunol Immunother*. (2021) 70:3461–75. doi: 10.1007/s00262-021-02939-y

71. Zhang Q, Li P, Zhou W, Fang S, Wang J. Participation of increased circulating MAIT cells in lung cancer: a pilot study. *J Cancer*. (2022) 13:1623–29. doi: 10.7150/jca.69415

72. Shaler CR, Tun-Abraham ME, Skaro AI, Khazaie K, Corbett AJ, Mele T, et al. Mucosa-associated invariant T cells infiltrate hepatic metastases in patients with colorectal carcinoma but are rendered dysfunctional within and adjacent to tumor microenvironment. *Cancer Immunol Immunother*. (2017) 66:1563–75. doi: 10.1007/s00262-017-2050-7

73. Kelly J, Minoda Y, Meredith T, Cameron G, Philipp MS, Pellicci DG, et al. Chronically stimulated human MAIT cells are unexpectedly potent IL-13 producers. *Immunol Cell Biol*. (2019) 97:689–99. doi: 10.1111/imcb.12281

74. Qian X, Chen H, Wu X, Hu L, Huang Q, Jin Y. Interleukin-17 acts as double-edged sword in anti-tumor immunity and tumorigenesis. *Cytokine*. (2017) 89:34–44. doi: 10.1016/j.cyto.2015.09.011

75. Zhao J, Chen X, Herjan T, Li X. The role of interleukin-17 in tumor development and progression. *J Exp Med*. (2020) 217. doi: 10.1084/jem.20190297

76. Wu F, Xu J, Huang Q, Han J, Duan L, Fan J, et al. The role of interleukin-17 in lung cancer. *Mediators Inflammation*. (2016) 2016:8494079. doi: 10.1155/2016/8494079

77. Hurtado CG, Wan F, Housseau F, Sears CL. Roles for interleukin 17 and adaptive immunity in pathogenesis of colorectal cancer. *Gastroenterology*. (2018) 155:1706–15. doi: 10.1053/j.gastro.2018.08.056

78. Li X, Liu MK, Shi Q, Fang Y, Fu D, Shen ZX, et al. Elevated serum IL-13 level is associated with increased Treg cells in tumor microenvironment and disease

progression of diffuse large B-cell lymphoma. *Hematological Oncol*. (2023) 41:230–38. doi: 10.1002/hon.2993

79. Sundström P, Ahlmanner F, Akéus P, Sundquist M, Alsén S, Yrlid U, et al. Human mucosa-associated invariant T cells accumulate in colon adenocarcinomas but produce reduced amounts of IFN-γ. *J Immunol*. (2015) 195(7):3472–81. doi: 10.4049/jimmunol.1500258

80. Huang WC, Hsiao YC, Wu CC, Hsu YT, Chang CL. Less circulating mucosal-associated invariant T cells in patients with cervical cancer. *Taiwan J Obstet Gynecol*. (2019) 58:117–21. doi: 10.1016/j.tjog.2018.11.022

81. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, et al. Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature*. (2012) 491:254–8. doi: 10.1038/nature11465

82. Zabijak L, Attencourt C, Guignant C, Chatelain D, Marcelo P, Marolleau J-P, et al. Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients. *Cancer Immunol Immunotherapy*. (2015) 64:1601–8. doi: 10.1007/s00262-015-1764-7

83. Sundstrom P, Ahlmanner F, Akeus P, Sundquist M, Alsen S, Yrlid U, et al. Human mucosa-associated invariant T cells accumulate in colon adenocarcinomas but produce reduced amounts of IFN-γ. *J Immunol*. (2015) 195:3472–81. doi: 10.4049/jimmunol.1500258

84. Lu Z, Zhu M, Marley JL, Bi K, Wang K, Zhai M, et al. The combined action of monocytic myeloid-derived suppressor cells and mucosal-associated invariant T cells promotes the progression of cervical cancer. *Int J Cancer*. 148(6):1499–507. doi: 10.1002/ijc.33411

85. Zumwalde NA, Haag JD, Gould MN, Gumperz JE. Mucosal associated invariant T cells from human breast ducts mediate a Th17-skewed response to bacterially exposed breast carcinoma cells. *Breast Cancer Res*. (2018) 20:111. doi: 10.1186/s13058-018-1036-5

86. Zheng C, Zheng L, Yoo JK, Guo H, Zhang Y, Guo X, et al. Landscape of infiltrating T cells in liver cancer revealed by single-cell sequencing. *Cell*. (2017) 169:1342–56.e16. doi: 10.1016/j.cell.2017.05.035

87. Duan M, Goswami S, Shi JY, Wu LJ, Wang XY, Ma JQ, et al. Activated and exhausted MAIT cells foster disease progression and indicate poor outcome in hepatocellular carcinoma. *Clin Cancer Res*. (2019) 25:3304–16. doi: 10.1158/1078-0432.CCR-18-3040

88. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nat Rev Immunol*. (2015) 15:486–99. doi: 10.1038/nri3862

89. McLane LM, Abdel-Hakeem MS, Wherry EJ. CD8 T cell exhaustion during chronic viral infection and cancer. *Annu Rev Immunol*. (2019) 37:457–495. doi: 10.1146/annurev-immunol-041015-055318

90. Tobin RP, Jordan KR, Kapoor P, Spongberg E, Davis D, Vorwald VM, et al. IL-6 and IL-8 are linked with myeloid-derived suppressor cell accumulation and correlate with poor clinical outcomes in melanoma patients. *Front Oncol*. (2019) 9:1223. doi: 10.3389/fonc.2019.01223

91. Kurioka A, Cosgrove C, Simoni Y, Wilgenburg Bv, Geremia A, Björkander S, et al. CD161 defines a functionally distinct subset of pro-inflammatory natural killer cells. *Front Immunol*. (2018) 9. doi: 10.3389/fimmu.2018.00486

92. Väyrynen JP, Kantola T, Klintrup K, Bloigu R, Karhu T, Mäkelä J, et al. The relationships between serum cytokine levels and tumor infiltrating immune cells and their clinical significance in colorectal cancer. *Int J Cancer*. (2016). 139(1):112–21. doi: 10.1002/ijc.30040

93. Lamichhane R, Schneider M, Sara M, de la Harpe SM, Harrop TWR, Hannaway RF, et al. TCR- or cytokine-activated CD8+ Mucosal-associated invariant T cells are rapid polyfunctional effectors that can coordinate immune responses. *Cell Rep*. (2019). doi: 10.1101/600189

94. Mori L, Lepore M, De Libero G. The immunology of CD1- and MR1-restricted T cells. *Annu Rev Immunol*. (2016) 34:479–510. doi: 10.1146/annurev-immunol-032414-112008

95. Salio M, Gasser O, Lopez CG, Martens AWJ, Veerapen N, Gileadi U, et al. Activation of human mucosal-associated invariant T cells induces CD40L-dependent maturation of monocyte-derived and primary dendritic cells. *J Immunol*. (2017) 199 (8):2631–8. doi: 10.4049/jimmunol.1700615

96. De Biasi S, Gibellini L, Lo Tartaro D, Puccio S, Rabacchi C, Mazza EMC, et al. Circulating mucosal-associated invariant T cells identify patients responding to anti-PD-1 therapy. *Nat Commun*. 12(1):1669. doi: 10.1038/s41467-021-21928-4

97. Vorwald VM, Davis DM, Van Gulick RJ, Torphy RJ, Borgers JS, Klarquist J, et al. Circulating CD8(+) mucosal-associated invariant T cells correlate with improved treatment responses and overall survival in anti-PD-1-treated melanoma patients. *Clin Transl Immunol*. (2022) 11:e1367. doi: 10.1002/cti2.1367

98. Gao MG, Zhao XS. Mining the multifunction of mucosal-associated invariant T cells in hematological Malignancies and transplantation immunity: A promising hexagon soldier in immunomodulatory. *Front Immunol*. (2022) 13:931764. doi: 10.3389/fimmu.2022.931764

99. Dey M, Kim MH, Nagamine M, Karhan E, Kozhaya L, Dogan M, et al. Biofabrication of 3D breast cancer models for dissecting the cytotoxic response of human T cells expressing engineered MAIT cell receptors. *Biofabrication*. (2022) 14. doi: 10.1101/2022.01.29.478309

100. Priya R, Brutkiewicz RR. MR1 tetramer-based artificial APCs expand MAIT cells from human peripheral blood that effectively kill glioblastoma cells. *Immunohorizons*. (2021) 5:500–11. doi: 10.4049/immunohorizons.2100003

101. Dogan M, Karhan E, Kozhaya L, Placek L, Chen X, Yigit M, et al. Engineering human MAIT cells with chimeric antigen receptors for cancer immunotherapy. *J Immunol*. (2022) 209(8). doi: 10.4049/jimmunol.2100856

102. Sterner RC, Sterner RM. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J*. (2021) 11:69. doi: 10.1038/s41408-021-00459-7

103. Wakao H, Yoshikiyo K, Koshimizu U, Furukawa T, Enomoto K, Matsunaga T, et al. Expansion of functional human mucosal-associated invariant T cells via reprogramming to pluripotency and redifferentiation. *Cell Stem Cell*. (2013) 12:546–58. doi: 10.1016/j.stem.2013.03.001

104. Sugimoto C, Fujita H, Wakao H. Harnessing the power of mucosal-associated invariant T (MAIT) cells in cancer cell therapy. *Biomedicines*. (2022) 10. doi: 10.3390/biomedicines10123160

105. Kurioka A, Jahun AS, Hannaway RF, Walker LJ, Fergusson JR, Sverremark-Ekstrom E, et al. Shared and distinct phenotypes and functions of human CD161++ Valpha7.2+ T cell subsets. *Front Immunol*. (2017) 8:1031. doi: 10.3389/fimmu.2017.01031

106. Li YR, Brown J, Yu Y, Lee D, Zhou K, Dunn ZS, et al. Targeting immunosuppressive tumor-associated macrophages using innate T cells for enhanced antitumor reactivity. *Cancers (Basel)*. (2022) 14. doi: 10.3390/cancers14112749

107. Wang Z, Zhang S, Zhang X, Liu L, Zhou L, Shen Y, et al. Mucosal-associated invariant T cells predict increased acute graft-versus-host-disease incidence in patients receiving allogeneic hematopoietic stem cell transplantation. *Cancer Cell Int*. (2022) 22:297. doi: 10.1186/s12935-022-02703-x

108. Tourret M, Talvard-Balland N, Lambert M, Ben Youssef G, Chevalier MF, Bohineust A, et al. Human MAIT cells are devoid of alloreactive potential: prompting their use as universal cells for adoptive immune therapy. *J Immunother Cancer*. (2021) 9. doi: 10.1101/2021.04.29.21256184

109. Bohineust A, Tourret M, Derivry L, Caillat-Zucman S. Mucosal-associated invariant T (MAIT) cells, a new source of universal immune cells for chimeric antigen receptor (CAR)-cell therapy. *Bull Cancer*. (2021) 108:S92–5. doi: 10.1016/j.bulcan.2021.07.003

110. Labuz D, Cacioppo J, Li K, Aube J, Leung DT. Enhancing mucosal-associated invariant T cell function and expansion with human selective serum. *Immunohorizons*. (2023) 7:116–24. doi: 10.4049/immunohorizons.2200082

111. Wakao H, Sugimoto C, Kimura S, Wakao R. Mucosal-associated invariant T cells in regenerative medicine. *Front Immunol*. (2017) 8:1711. doi: 10.3389/fimmu.2017.01711

112. Garner LC, Klenerman P, Provine NM. Insights into mucosal-associated invariant T cell biology from studies of invariant natural killer T cells. *Front Immunol*. (2018) 9:1478. doi: 10.3389/fimmu.2018.01478

113. Mechelli R, Romano S, Romano C, Morena E, Buscarinu MC, Bigi R, et al. MAIT cells and microbiota in multiple sclerosis and other autoimmune diseases. *Microorganisms*. (2021) 9. doi: 10.3390/microorganisms9061132

114. Tang XZ, Jo J, Tan AT, Sandalova E, Chia A, Tan KC, et al. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J Immunol*. (2013) 190:3142–52. doi: 10.4049/jimmunol.1203218

115. Gebru YA, Choi MR, Raja G, Gupta H, Sharma SP, Choi YR, et al. Pathophysiological roles of mucosal-associated invariant T cells in the context of gut microbiota-liver axis. *Microorganisms*. (2021) 9. doi: 10.3390/microorganisms9020296

116. Varelias A, Gartlan KH, Wilkinson AN, Olver SD, Samson LD, Tey SK, et al. Expansion of IL-17A-secreting CD8(+) mucosa-associated invariant T cells in peripheral blood following stem cell mobilization. *Blood Adv*. (2019) 3:718–23. doi: 10.1182/bloodadvances.2018025601

117. Gibbs A, Healy K, Kaldhusdal V, Sundling C, Franzen-Boger M, Edfeldt G, et al. Preserved mucosal-associated invariant T cells in the cervical mucosa of HIV-infected women with dominant expression of the TRAV1-2-TRAJ20 T cell receptor alpha-chain. *J Infect Dis*. (2022) 226:1428–40. doi: 10.1093/infdis/jiac171

118. Koppejan H, Jansen DTSL, Hameetman M, Thomas R, Toes REM, van Gaalen FA. Altered composition and phenotype of mucosal-associated invariant T cells in early untreated rheumatoid arthritis. *Arthritis Res Ther*. (2019) 21:3. doi: 10.1186/s13075-018-1799-1

119. Wang H, Hogquist KA. How MAIT cells get their start. *Nat Immunol*. (2016) 17:1238–40. doi: 10.1038/ni.3584

120. Magalhaes I, Pingris K, Poitou C, Bessoles S, Venteclef N, Kiaf B, et al. Mucosal-associated invariant T cell alterations in obese and type 2 diabetic patients. *J Clin Invest*. (2015) 125:1752–62. doi: 10.1172/JCI78941

121. Elinav E, Garrett WS, Trinchieri G, Wargo J. The cancer microbiome. *Nat Rev Cancer*. (2019) 19:371–76. doi: 10.1038/s41568-019-0155-3

122. Doocey CM, Finn K, Murphy C, Guinane CM. The impact of the human microbiome in tumorigenesis, cancer progression, and biotherapeutic development. *BMC Microbiol*. (2022) 22:53. doi: 10.1186/s12866-022-02465-6

123. Sadrekarimi H, Gardanova ZR, Bakhshesh M, Ebrahimzadeh F, Yaseri AF, Thangavelu L, et al. Emerging role of human microbiome in cancer development and

response to therapy: special focus on intestinal microflora. *J Transl Med*. (2022) 20:301. doi: 10.1186/s12967-022-03492-7

124. Liu J, Brutkiewicz RR. The Toll-like receptor 9 signalling pathway regulates MR1-mediated bacterial antigen presentation in B cells. *Immunology*. (2017) 152:232–42. doi: 10.1111/imm.12759

125. Yang W, Cong Y. Gut microbiota-derived metabolites in the regulation of host immune responses and immune-related inflammatory diseases. *Cell Mol Immunol*. (2021) 18:866–77. doi: 10.1038/s41423-021-00661-4

126. Qiu Q, Lin Y, Ma Y, Li X, Liang J, Chen Z, et al. Exploring the emerging role of the gut microbiota and tumor microenvironment in cancer immunotherapy. *Front Immunol*. (2020) 11:612202. doi: 10.3389/fimmu.2020.612202

127. Park EM, Chelvanambi M, Bhutiani N, Kroemer G, Zitvogel L, Wargo JA. Targeting the gut and tumor microbiota in cancer. *Nat Med*. (2022) 28:690–703. doi: 10.1038/s41591-022-01779-2

128. Blake SJ, Wolf Y, Boursi B, Lynn DJ. Role of the microbiota in response to and recovery from cancer therapy. *Nat Rev Immunol*. (2023). doi: 10.1038/s41577-023-00951-0

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 28 May 2024
DOI 10.3389/fimmu.2024.1402412

OPEN ACCESS

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RECEIVED 17 March 2024
ACCEPTED 14 May 2024
PUBLISHED 28 May 2024

CITATION
Hayashizaki K, Kamii Y and Kinjo Y (2024) Glycolipid antigen recognition by invariant natural killer T cells and its role in homeostasis and antimicrobial responses. *Front. Immunol.* 15:1402412. doi: 10.3389/fimmu.2024.1402412

Glycolipid antigen recognition by invariant natural killer T cells and its role in homeostasis and antimicrobial responses

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Due to the COVID-19 pandemic, the importance of developing effective vaccines has received more attention than ever before. To maximize the effects of vaccines, it is important to select adjuvants that induce strong and rapid innate and acquired immune responses. Invariant natural killer T (iNKT) cells, which constitute a small population among lymphocytes, bypass the innate and acquired immune systems through the rapid production of cytokines after glycolipid recognition; hence, their activation could be used as a vaccine strategy against emerging infectious diseases. Additionally, the diverse functions of iNKT cells, including enhancing antibody production, are becoming more understood in recent years. In this review, we briefly describe the functional subset of iNKT cells and introduce the glycolipid antigens recognized by them. Furthermore, we also introduce novel vaccine development taking advantages of iNKT cell activation against infectious diseases.

KEYWORDS

iNKT, glycolipid, infection, follicular helper, vaccine

Introduction

Invariant natural killer T (iNKT) cells, which express an invariant T cell receptor (TCR) α chain, are a subpopulation of T cells that possess both T cell and NK cell phenotypes (1–3). Conventional T cells recognize peptides presented on MHC class I or II molecules, whereas iNKT cells recognize endogenous or exogenous glycolipids presented by CD1d (4–6). Additionally, iNKT cells have already acquired effector functions during thymic development, and similar to memory T cells, they rapidly produce large amounts of cytokines, such as IFNγ and IL-4, after activation. Thus, iNKT cells play a role in bridging innate and acquired immune responses. Since the discovery of iNKT cells, specific ligands recognized by iNKT cells have been explored and discovered (7, 8). iNKT cells recognize several glycolipid antigens with similar structures through an invariant TCR, a unique

feature of these cells. In this article, we review the features of iNKT cells especially in response to microbes.

Effector subsets of iNKT cells

The invariant TCR of iNKT cells consists of Vα14-Jα18 chains and a restricted repertoire of Vβ chains (Vβ8, 7, 2) in mice, and Vα24-Jα18 chains and Vβ11 in humans (1–3). Upon TCR stimulation, iNKT cells rapidly produce various cytokines, including IFNγ, IL-2, IL-4, IL-13, and IL-17A, and stimulate other immune cells. iNKT cells are classified into several effector subsets similar to conventional T cells based on cytokine production and regulatory transcription factors (9–11). NKT1 cells predominantly produce IFNγ as T helper (Th) 1 cells, NKT2 cells produce IL-4 and IL-13 as Th2 cells, and NKT17 cells have functions similar to those of Th17 cells. These effector subsets are already mature in the thymus and are distributed to the tissues via their specific chemokine receptors and adhesion molecules. However, depending on the intensity of TCR stimulation and the environment, iNKT cells may produce cytokines such as IL-4 from NKT1 cells and IFNγ from NKT2 cells. This suggests that iNKT cells function as "tuning players" in immune responses. iNKT subsets localize differently among tissues (12). NKT1 cells are mostly found in the liver, while NKT2 cells are found in lung and mesenteric lymph nodes. In contrast, NKT17 cells are found more abundantly in lymph nodes throughout the body. This distribution bias may be caused by differences in the cytokines required for homeostasis in each organ (13). Other functional subsets of iNKT cells include NKT10 cells (14), which is an immunosuppressive NKT subset that produces IL-10, and follicular helper NKT (NKT_{FH}) cells (15, 16), which are phenotypically similar to follicular helper T (T_{FH}) cells and stimulate B cells with IL-21 and costimulatory molecules. NKT10 and NKT_{FH} cells localized in peripheral tissues, such as adipose and lymphoid tissue respectively. Although the detailed mechanisms of differentiation into NKT10 cells remain unknown, the quality and intensity of TCR stimulation may likely be involved (17, 18).

In contrast to the above effector subset classification, functional NKT cells may be classified based on the differential surface expression of CD244 and CXCR6 (19). $CD244^+$ $CXCR6^+$ iNKT cells (C2 NKT cells) are systemically circulating cells and produce more IFNγ and granzymes than $CD244^-$ $CXCR6^+$ tissue-resident iNKT cells (C1 NKT cells). Hence, C2 NKT cells participate in antitumor and antimicrobial responses. As iNKT cells in human blood can be identified by similar surface molecules, this classification would be useful for the analysis of iNKT cells in humans.

Glycolipid mediated iNKT cell activation

The prototype antigen for iNKT cells is α-galactosylceramide (αGalCer) that is synthesized based on Agelasphin, which was isolated from a marine sponge and has antitumor activity (5, 20). Even a minimal amount of αGalCer induces IFNγ and IL-4 production by iNKT cells. αGalCer has also been used to study iNKT cell function and vaccines based on their activation, because this glycolipid induces more robust TCR stimulation in iNKT cells than known endogenous and pathogen-derived glycolipid antigens (21). αGalCer-activated iNKT cells reportedly become anergic after transient activation and proliferation (22). Additionally, when activated with αGalCer, NKT1 cells produce large amounts of IFNγ and highly express IL-4, which is produced mainly by NKT2 cells in steady state and is considered a characteristic of NKT2 cells (23). Therefore, while understanding the function of iNKT cells under physiological conditions, αGalCer results should be carefully interpreted. However, αGalCer has become a promising ligand in vaccine studies utilizing the effects of iNKT cell activation (24, 25). Moreover, iNKT cells have the potential for replacing conventional adjuvants. Vaccine studies using αGalCer will be discussed later in this article.

Physiological glycolipid antigens of iNKT cells

The physiological glycolipid antigen that most iNKT cells recognize was identified with the intestinal symbiont *Sphingomonas* sp. The α-linked glycosphingolipids (GSLs; containing a galacturonic acid or glucuronic acid moiety) of *Sphingomonas* sp. induce iNKT cells to produce IFNγ and IL-4 in a CD1d-dependent manner (26, 27). *Bacteroides*, a gram-negative bacterium that comprises 50% of human intestinal bacteria, produces sphingolipids similar to αGalCer (28–30). *Bacteroides fragilis* produces sphingolipids to regulate both activation and inhibition of iNKT cells. Among these sphingolipids, GSL-Bf717 inhibits proliferation and IFNγ and IL-4 production in iNKT cells; this effect is important to regulate the number and function of iNKT cells in intestinal tissues (29). In that study, it was shown that the regulation of iNKT cells by GSL-Bf717 in the neonatal stages influences the sensitivity of iNKT cell-mediated colitis in adult mice. Another study showed that *B. fragilis* regulates intestinal iNKT cell function via sphingolipid synthesis and is dependent on branched amino acids ingested by the host (31). Antibiotic-associated dysbiosis reportedly affects the number and function of iNKT cells, resulting in pathological conditions (32), although gut microbiota composition was not changed in iNKT cell-deficient or transgenic mice (33). This suggests that indigenous bacteria contribute to intestinal homeostasis by regulating iNKT cells.

From the perspective of host protection, exogenous glycolipid antigens recognized by iNKT cells were identified in several pathogens. *Borrelia burgdorferi*—the pathogenic bacterium that causes Lyme disease—produces a diacylglycerol (DAG)-based glycolipid containing α-linked galactose (αGal-DAG). iNKT cells recognize *B. burgdorferi* αGal-DAG via TCR and produce IFNγ and IL-4 (34–36). *B. burgdorferi* infections in iNKT cell-deficient mice results in more severe arthritis and carditis compared to non-iNKT cell-deficient control mice. Additionally, numerous spirochetes

accumulate in the lesions, suggesting that antigen recognition by iNKT cells is important for antibacterial immunity. *Streptococcus pneumoniae* causes pneumonia, which can result in bacteremia and meningitis, especially in children and the elderly, and produces an α-linked glucose (Glc) containing DAG (αGlc-DAG). *S. pneumoniae* αGlc-DAG was recognized by TCR on iNKT cells (37). Neutrophil accumulation in the lungs via IFNγ and cytokines and chemokines, including IL-17 and GM-CSF produced by iNKT cells, is reportedly important during pneumococcal infections (38–40). Dendritic cells (DCs) play an important role in the stimulation of iNKT cells. Maturation of DCs through Toll like receptors upregulates CD1d and introduces TCR stimulation into surrounding iNKT cells together with IL-12. Activated iNKT cells then produce IFNγ and express CD40L, enhancing IL-12 production from DCs that further augments the immune response. More importantly, these glycolipids can activate not only mouse but also human iNKT cells.

Not all pathogens have glycolipid antigens that are recognized by iNKT cells. The filamentous fungus *Aspergillus fumigatus* and the gram-negative bacterium *Salmonella typhimurium* reportedly activate iNKT cells despite not having microbial glycolipid antigens (41, 42), and that this effect is CD1d dependent. This suggests that iNKT cells are activated by endogenous antigen/CD1d-mediated TCR stimulation as well as indirect stimulation by cytokines from antigen-presenting cells that were activated via pattern recognition receptors.

How are iNKT cells able to recognize a wide variety of glycolipids? Previous structural analysis of the iNKT cell TCR-glycolipid-CD1d complex has demonstrated that the TCR of iNKT cells changes the conformation of the CD1d-glycolipid antigen complex (43, 44), which may allow iNKT cells to recognize different but structurally similar glycolipid antigens. Contextually, iNKT cells reportedly produce IFNγ when exposed to IL-12 from antigen-presenting cells stimulated by LPS (21, 45). However, during TCR-independent activation, iNKT cells produced IFNγ but not IL-4 (21). Thus, although iNKT cells can be stimulated in the absence of TCR stimulation similarly as NK cells, CD1d-dependent TCR stimulation is important for IL-4 production.

Vaccine and NKT_{FH} cells

There have been attempts to employ glycolipids as therapeutics for various infectious diseases as well as in vaccines. iNKT cell-mediated vaccines augment cellular and humoral immunity. For cellular immunity, mice immunized with malarial antigen plus αGalCer were more protected against malaria than those immunized with the antigen alone (46). Additionally, mice immunized with the *Mycobacterium tuberculosis* antigen plus αGalCer were also protected from bacterial infection (47). Following administration, αGalCer stimulates iNKT cells, thereby increasing the number of antigen-specific CD8 T cells in an IFNγ-dependent manner. Other synthetic glycolipids were used in several studies. Although αGalCer induces IFNγ and IL-4 production in iNKT cells, α-C-GalCer (48) and OCH (49) induce relatively biased cytokine production toward IFNγ and IL-4, respectively, in these cells.

iNKT cells reportedly enhance B cell responses during influenza infections under physiological conditions (50). iNKT cells play an essential role in the initial formation of germinal centers (GC), which is microstructure that regulates selection and proliferation of antigen-specific B cells and is essential for antibody production. During infection with the influenza virus, iNKT cells become a source of IL-4 that is important not only for the induction of GC but also for class switching and production of IgG1. Also, vaccines containing a glycolipid as an adjuvant efficiently induce antibody-producing responses mediated by B cells through the activation of iNKT cells. Intranasal influenza hemagglutinin (HA) and αGalCer vaccines more strongly induce HA-specific IgG in serum and HA-specific IgA in mucosa than HA vaccine alone as well as exert a potent protective effect even against lethal doses of influenza virus infection (51–53). Although follicular helper T (T_{FH}) cells play an important role in the antibody-producing response *in vivo* (54), follicular helper NKT (NKT_{FH}) cells play a more central role in the antibody-producing response induced by αGalCer-contained vaccines (15, 16).

A vaccine containing liposome-encapsulated PBS57, an αGalCer analog and pneumococcal capsular polysaccharide (CPS) induces NKT_{FH} cells (55). CPS-specific IgG1 induction by this vaccine is dependent on CD1d expression on B cells and DCs, indicate that the interaction of CPS-specific B cells and NKT_{FH} cells is important for specific antibody production. NKT_{FH} cells contribute to antigen-specific B cell responses via stimulatory molecules, including as IL-21 and ICOS, and their follicular differentiation is controlled by transcription factor Bcl6 (15). However, the detailed differentiation mechanisms have not been elucidated. Normally, NKT_{FH} cells are not found in the thymus and appear in the spleen and lymph nodes only upon the activation of iNKT cells with αGalCer. This indicates that potent TCR stimulation by αGalCer is important for NKT_{FH} cell differentiation. However, αGalCer stimulation alone does not induce NKT_{FH} cell differentiation *in vitro*, suggesting that environmental factors are essential for acquiring follicular phenotypes. Moreover, our recent study clarified that Gr-1^+ cells promote NKT_{FH} cell differentiation by producing interleukin-27 (IL-27) post-αGalCer administration (56). IL-27 modulates mitochondrial metabolism in activated iNKT cells and optimizes the energy demand required for NKT_{FH} cell differentiation. Gr-1^+ cell-derived IL-27 is induced by iNKT cells via IFNγ production.

Recently, it was shown that administration of nanoparticles embedded with αGalCer activates iNKT cells *in vivo* more efficiently than soluble αGalCer. Additionally, delivery by nanoparticles enables activation of iNKT cells at doses 1,000× lower than those used for *in vivo* studies and does not induce iNKT cell anergy. Although nanoparticle vaccines inhibit T-independent reactions by tolerizing or eliminating polysaccharide-specific B cells, T-dependent reactions following vaccination efficiently induces antigen-specific antibodies and protects mice from lethal *S. pneumoniae* infection (57).

The administration of NKT-specific glycolipid alone induces protection against microbial infections. 7DW8-5, the primary compound of αGalCer, activates human and mouse iNKT cells more potently than αGalCer (58). Intranasal administration of

TABLE 1 Glycolipid antigens for invariant natural killer T (iNKT) cells .

	Antigens	Sources (Microorganisms etc)	Cytokines*	References
Bacterial	GSLs	*Sphingomonas sp.*	IFNγ, IL-4	(26, 27)
	GSL-Bf717, $\alpha GalCer_{Bf}$	*Bacteroides fragilis*	IFNγ, IL-4	(28–31)
	αGal-DAG	*Borrelia burgdorferi*	IFNγ, IL-4	(34–36)
	αGlc-DAG	*Streptococcus pneumoniae*	IFNγ, IL-4	(37)
Synthetic	αGalCer	Synthesized based on the structure of agelasphins**	IFNγ, IL-4 etc.	(5, 20, 21)
	OCH	αGalCer analog	IFNγ < IL-4	(49)
	α-C-GalCer (7DW8-5)	αGalCer analog	IFNγ > IL-4	(48, 58)

** Agelasphins are marine sponge glycolipids. *Cytokines produced by iNKT cells.

7DW8-5 provided protection against respiratory pathogens, including SARS-COV2, RSV, and influenza viruses. However, as administrating 7DW8-5 to mice postinfection was ineffective, glycolipids may be more suitable for vaccine-like therapy. Herein, the authors underscore that αGalCer and 7DW8-5 may potentially be used in clinical applications as long as their safety is considered. More importantly, their simplicity and affordability will assist in the development of next-generation vaccines. αGalCer has already been used to treat patients with tumors, and that no significant toxicity was reportedly observed (59). Additionally, toxicity was not observed in monkeys treated with excessive amounts of 7DW8-5 as a vaccine adjuvant (60). Although additional safety studies are warranted, αGalCer and 7DW8-5 are expected to be used in clinical studies.

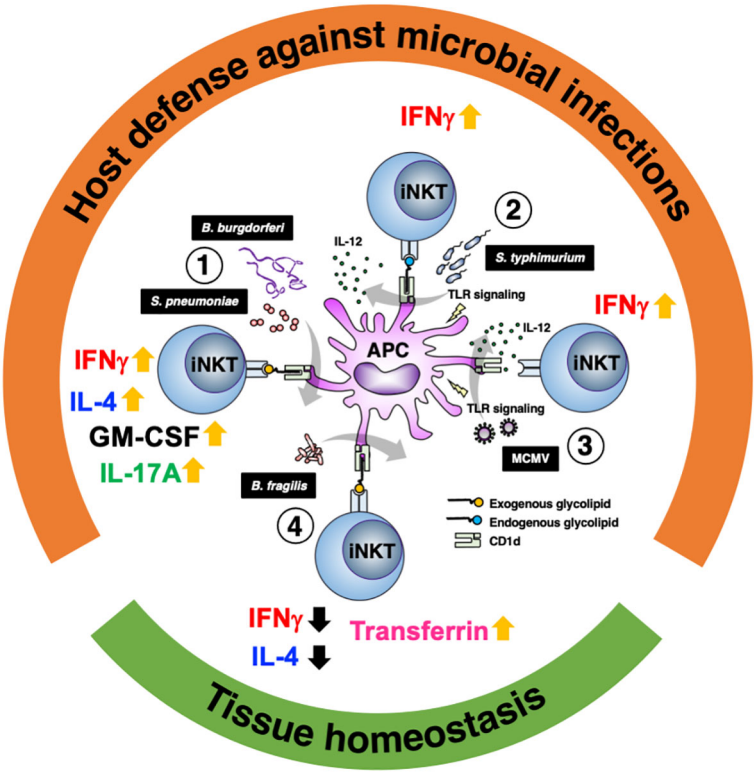

FIGURE 1
Glycolipid-dependent or -independent reaction of iNKT cells direct multiple outcomes. 1) Several microbial lipid antigens, which are derived *Streptococcus pneumoniae* and *Borrelia burgdorferi*, activate iNKT cells through CD1d presentation on antigen-presenting cells (APCs). Activated iNKT cells rapidly produce IFNγ and augment innate and acquired immune responses, which are essential for protection against acute bacterial infections. Not only IFNγ but IL-4, IL-17 and GM-CSF are also involved in iNKT cell-mediated protective responses. 2) Infection with *Salmonella typhimurium* stimulates APCs by LPS and induces IL-12 production (probably also IL-18 production). iNKT cell activation and IFNγ production are dependent not only on IL-12 but also partially on TCR stimulation by endogenous ligands presented by CD1d. 3) During viral infections involving murine cytomegalovirus (MCMV), iNKT cells produce IFNγ even in the absence of glycolipid antigens and is dependent on IL-12 and Toll like receptor signaling. 4) In intestinal tissues, among symbionts, *Bacteroides fragilis* affect the number of iNKT cells in the young and regulate homeostasis throughout life via multiple α-galactosylceramide (BfαGCs), which has immunomodulatory signaling and actions. In the skin, iNKT cells also support tissue homeostasis by regulating local iron metabolism with transferrin production, which depends on CD1d.

Concluding remarks

iNKT cells play an important role in microbial infections. Their importance is evidenced by the fact that in some infections, pathogens adopt strategies to reduce CD1d expression (61, 62). Although iNKT cells constitute a small population of T cells with a poor diversity of TCR, they enhance as well as regulate immune responses through the recognition of various glycolipid ligands (Table 1). iNKT cells are more prominent in subtle responses to environment as compared with conventional T cells, i.e., as a "tuning player" in immune reactions, which may be important for induction of proper immune reactions. These functions would be why iNKT cells have persisted despite evolution. Studies have also shown the functional significance of iNKT cells not only for protection against pathogens but also in the establishment and maintenance of tissue homeostasis (Figure 1). Tissue accumulation of iNKT cells during fetal life is necessary to maintain intestinal tract and skin homeostasis (63, 64), and iNKT cells are also involved in regulating peripheral serotonin release. These show that iNKT cells contribute not only to the immune system but also to systemic homeostasis (65).

Although iNKT cells rapidly respond during microbial infections, their functions are diverse, and their importance in various tissues need to be elucidated. iNKT cell-mediated vaccines are potent and are expected to be components of next-generation vaccines. However, due to their diverse functions, the route of administration, timing, and duration warrants further investigation.

Author contributions

KH: Writing – original draft, Writing – review & editing. YaK: Writing – original draft. YuK: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan [Grants-in Aid for Scientific Research (B) #19H03705, #22H03123, #23K24382 (C) #21K07085, Grants-in Aid for Early-Career Scientists #19K16704], The Uehara Memorial Foundation, Yakult Bio-Science Foundation, Takeda Science Foundation, Senshin Medical Research Foundation, Daiichi Sankyo Foundation of Life Science, Astellas Foundation for Research on Metabolic Disorders, and The Jikei University Graduate Research Fund.

Acknowledgments

We are grateful to the NIH Tetramer Core facility for kindly providing PBS57 loaded CD1d tetramers.

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

References

1. Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu Rev Immunol.* (2005) 23:877–900. doi: 10.1146/annurev.immunol.23.021704.115742

2. Godfrey DI, Pellicci DG, Patel O, Kjer-Nielsen L, McCluskey J, Rossjohn J. Antigen recognition by CD1d-restricted NKT T cell receptors. *Semin Immunol.* (2010) 22:61–7. doi: 10.1016/j.smim.2009.10.004

3. Gapin L. Development of invariant natural killer T cells. *Curr Opin Immunol.* (2016) 39:68–74. doi: 10.1016/j.coi.2016.01.001

4. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1+ T lymphocytes. *Science.* (1995) 268:863–5. doi: 10.1126/science.7538697

5. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science.* (1997) 278:1626–9. doi: 10.1126/science.278.5343.1626

6. McEwen-Smith RM, Salio M, Cerundolo V. CD1d-dependent endogenous and exogenous lipid antigen presentation. *Curr Opin Immunol.* (2015) 34:116–25. doi: 10.1016/j.coi.2015.03.004

7. Tupin E, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol.* (2007) 5:405–17. doi: 10.1038/nrmicro1657

8. Kinjo Y, Ueno K. iNKT cells in microbial immunity: recognition of microbial glycolipids. *Microbiol Immunol.* (2011) 55:472–82. doi: 10.1111/mim.2011.55.issue-7

9. Georgiev H, Ravens I, Benarafa C, Forster R, Bernhardt G. Distinct gene expression patterns correlate with developmental and functional traits of iNKT subsets. *Nat Commun.* (2016) 7:13116. doi: 10.1038/ncomms13116

10. Lee YJ, Starrett GJ, Lee ST, Yang R, Henzler CM, Jameson SC, et al. Lineage-Specific Effector Signatures of Invariant NKT Cells Are Shared amongst gammadelta T, Innate Lymphoid, and Th Cells. *J Immunol.* (2016) 197:1460–70. doi: 10.4049/jimmunol.1600643

11. Engel I, Seumois G, Chavez L, Samaniego-Castruita D, White B, Chawla A, et al. Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat Immunol.* (2016) 17:728–39. doi: 10.1038/ni.3437

12. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-specific distribution of iNKT cells impacts their cytokine response. *Immunity.* (2015) 43:566–78. doi: 10.1016/j.immuni.2015.06.025

13. Crosby CM, Kronenberg M. Tissue-specific functions of invariant natural killer T cells. *Nat Rev Immunol.* (2018) 18:559–74. doi: 10.1038/s41577-018-0034-2

14. Lynch L, Michelet X, Zhang S, Brennan PJ, Moseman A, Lester C, et al. Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T(reg) cells and macrophages in adipose tissue. *Nat Immunol.* (2015) 16:85–95. doi: 10.1038/ni.3047

15. Chang PP, Barral P, Fitch J, Pratama A, Ma CS, Kallies A, et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol.* (2011) 13:35–43. doi: 10.1038/ni.2166

16. King IL, Fortier A, Tighe M, Dibble J, Watts GF, Veerapen N, et al. Invariant natural killer T cells direct B cell responses to cognate lipid antigen in an IL-21-dependent manner. *Nat Immunol*. (2011) 13:44–50. doi: 10.1038/ni.2172

17. Lang ML. The influence of invariant natural killer T cells on humoral immunity to T-dependent and -independent antigens. *Front Immunol*. (2018) 9:305. doi: 10.3389/fimmu.2018.00305

18. Vieth JA, Das J, Ranaivoson FM, Comoletti D, Denzin LK, Sant'Angelo DB. TCRalpha-TCRbeta pairing controls recognition of CD1d and directs the development of adipose NKT cells. *Nat Immunol*. (2017) 18:36–44. doi: 10.1038/ni.3622

19. Cui G, Shimba A, Jin J, Ogawa T, Muramoto Y, Miyachi H, et al. A circulating subset of iNKT cells mediates antitumor and antiviral immunity. *Sci Immunol*. (2022) 7:eabj8760. doi: 10.1126/sciimmunol.abj8760

20. Yamaguchi Y, Motoki K, Ueno H, Maeda K, Kobayashi E, Inoue H, et al. Enhancing effects of (2S,3S,4R)-1-O-(alpha-D-galactopyranosyl)-2-(N-hexacosanoylamino) -1,3,4-octadecanetriol (KRN7000) on antigen-presenting function of antigen-presenting cells and antimetastatic activity of KRN7000-pretreated antigen-presenting cells. *Oncol Res*. (1996) 8:399–407.

21. Holzapfel KL, Tyznik AJ, Kronenberg M, Hogquist KA. Antigen-dependent versus -independent activation of invariant NKT cells during infection. *J Immunol*. (2014) 192:5490–8. doi: 10.4049/jimmunol.1400722

22. Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR, et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest*. (2005) 115:2572–83. doi: 10.1172/JCI24762

23. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol*. (2013) 14:1146–54. doi: 10.1038/ni.2731

24. Fujii SI, Yamasaki S, Sato Y, Shimizu K. Vaccine designs utilizing invariant NKT-licensed antigen-presenting cells provide NKT or T cell help for B cell responses. *Front Immunol*. (2018) 9:1267. doi: 10.3389/fimmu.2018.01267

25. Kinjo Y, Takatsuka S, Kitano N, Kawakubo S, Abe M, Ueno K, et al. Functions of CD1d-restricted invariant natural killer T cells in antimicrobial immunity and potential applications for infection control. *Front Immunol*. (2018) 9:1266. doi: 10.3389/fimmu.2018.01266

26. Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD, et al. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature*. (2005) 434:520–5. doi: 10.1038/nature03407

27. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C3rd, Zhou D, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature*. (2005) 434:525–9. doi: 10.1038/nature03408

28. Wieland Brown LC, Penaranda C, Kashyap PC, Williams BB, Clardy J, Kronenberg M, et al. Production of alpha-galactosylceramide by a prominent member of the human gut microbiota. *PloS Biol*. (2013) 11:e1001610. doi: 10.1371/journal.pbio.1001610

29. An D, Oh SF, Olszak T, Neves JF, Avci FY, Erturk-Hasdemir D, et al. Sphingolipids from a symbiotic microbe regulate homeostasis of host intestinal natural killer T cells. *Cell*. (2014) 156:123–33. doi: 10.1016/j.cell.2013.11.042

30. Cameron G, Nguyen T, Ciula M, Williams SJ, Godfrey DI. Glycolipids from the gut symbiont *Bacteroides fragilis* are agonists for natural killer T cells and induce their regulatory differentiation. *Chem Sci*. (2023) 14:7887–96. doi: 10.1039/D3SC02124F

31. Oh SF, Praveena T, Song H, Yoo JS, Jung DJ, Erturk-Hasdemir D, et al. Host immunomodulatory lipids created by symbionts from dietary amino acids. *Nature*. (2021) 600:302–7. doi: 10.1038/s41586-021-04083-0

32. Strati F, Pujolassos M, Burrello C, Giuffre MR, Lattanzi G, Caprioli F, et al. Antibiotic-associated dysbiosis affects the ability of the gut microbiota to control intestinal inflammation upon fecal microbiota transplantation in experimental colitis models. *Microbiome*. (2021) 9:39. doi: 10.1186/s40168-020-00991-x

33. Lin Q, Kuypers M, Liu Z, Copeland JK, Chan D, Robertson SJ, et al. Invariant natural killer T cells minimally influence gut microbiota composition in mice. *Gut Microbes*. (2022) 14:2104087. doi: 10.1080/19490976.2022.2104087

34. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MR, et al. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol*. (2006) 7:978–86. doi: 10.1038/ni1380

35. Wang J, Li Y, Kinjo Y, Mac TT, Gibson D, Painter GF, et al. Lipid binding orientation within CD1d affects recognition of *Borrelia burgdorferi* antigens by NKT cells. *Proc Natl Acad Sci U S A*. (2010) 107:1535–40. doi: 10.1073/pnas.0909479107

36. Lee WY, Moriarty TJ, Wong CH, Zhou H, Strieter RM, van Rooijen N, et al. An intravascular immune response to *Borrelia burgdorferi* involves Kupffer cells and iNKT cells. *Nat Immunol*. (2010) 11:295–302. doi: 10.1038/ni.1855

37. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li X, et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat Immunol*. (2011) 12:966–74. doi: 10.1038/ni.2096

38. Kawakami K, Yamamoto N, Kinjo Y, Miyagi K, Nakasone C, Uezu K, et al. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against *Streptococcus pneumoniae* infection. *Eur J Immunol*. (2003) 33:3322–30. doi: 10.1002/eji.200324254

39. Nakamatsu M, Yamamoto N, Hatta M, Nakasone C, Kinjo T, Miyagi K, et al. Role of interferon-gamma in Valpha14+ natural killer T cell-mediated host defense against *Streptococcus pneumoniae* infection in murine lungs. *Microbes Infect*. (2007) 9:364–74. doi: 10.1016/j.micinf.2006.12.003

40. Murray MP, Crosby CM, Marcovecchio P, Hartmann N, Chandra S, Zhao M, et al. Stimulation of a subset of natural killer T cells by CD103(+) DC is required for GM-CSF and protection from pneumococcal infection. *Cell Rep*. (2022) 38:110209. doi: 10.1016/j.celrep.2021.110209

41. Cohen NR, Tatituri RV, Rivera A, Watts GF, Kim EY, Chiba A, et al. Innate recognition of cell wall beta-glucans drives invariant natural killer T cell responses against fungi. *Cell Host Microbe*. (2011) 10:437–50. doi: 10.1016/j.chom.2011.09.011

42. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol*. (2003) 4:1230–7. doi: 10.1038/ni1002

43. Girardi E, Yu ED, Li Y, Tarumoto N, Pei B, Wang J, et al. Unique interplay between sugar and lipid in determining the antigenic potency of bacterial antigens for NKT cells. *PloS Biol*. (2011) 9:e1001189. doi: 10.1371/journal.pbio.1001189

44. Zajonc DM, Girardi E. Recognition of microbial glycolipids by natural killer T cells. *Front Immunol*. (2015) 6:400. doi: 10.3389/fimmu.2015.00400

45. Velazquez P, Cameron TO, Kinjo Y, Nagarajan N, Kronenberg M, Dustin ML. Cutting edge: activation by innate cytokines or microbial antigens can cause arrest of natural killer T cell patrolling of liver sinusoids. *J Immunol*. (2008) 180:2024–8. doi: 10.4049/jimmunol.180.4.2024

46. Gonzalez-Aseguinolaza G, Van Kaer L, Bergmann CC, Wilson JM, Schmieg J, Kronenberg M, et al. Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines. *J Exp Med*. (2002) 195:617–24. doi: 10.1084/jem.20011889

47. Khan A, Singh S, Galvan G, Jagannath C, Sastry KJ. Prophylactic sublingual immunization with mycobacterium tuberculosis subunit vaccine incorporating the natural killer T cell agonist alpha-galactosylceramide enhances protective immunity to limit pulmonary and extra-pulmonary bacterial burden in mice. *Vaccines (Basel)*. (2017) 5(4):47. doi: 10.3390/vaccines5040047

48. Schmieg J, Yang G, Franck RW, Tsuji M. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. *J Exp Med*. (2003) 198:1631–41. doi: 10.1084/jem.20031192

49. Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature*. (2001) 413:531–4. doi: 10.1038/35097097

50. Gaya M, Barral P, Burbage M, Aggarwal S, Montaner B, Warren Navia A, et al. Initiation of antiviral B cell immunity relies on innate signals from spatially positioned NKT cells. *Cell*. (2018) 172:517–33 e20. doi: 10.1016/j.cell.2017.11.036

51. Ko SY, Ko HJ, Chang WS, Park SH, Kweon MN, Kang CY. alpha-Galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor. *J Immunol*. (2005) 175:3309–17. doi: 10.4049/jimmunol.175.5.3309

52. Youn HJ, Ko SY, Lee KA, Ko HJ, Lee YS, Fujihashi K, et al. A single intranasal immunization with inactivated influenza virus and alpha-galactosylceramide induces long-term protective immunity without redirecting antigen to the central nervous system. *Vaccine*. (2007) 25:5189–98. doi: 10.1016/j.vaccine.2007.04.081

53. Kamijuku H, Nagata Y, Jiang X, Ichinohe T, Tashiro T, Mori K, et al. Mechanism of NKT cell activation by intranasal coadministration of alpha-galactosylceramide, which can induce cross-protection against influenza viruses. *Mucosal Immunol*. (2008) 1:208–18. doi: 10.1038/mi.2008.2

54. Crotty S. T follicular helper cell biology: A decade of discovery and diseases. *Immunity*. (2019) 50:1132–48. doi: 10.1016/j.immuni.2019.04.011

55. Bai L, Deng S, Reboulet R, Mathew R, Teyton L, Savage PB, et al. (NKT)-B-cell interactions promote prolonged antibody responses and long-term memory to pneumococcal capsular polysaccharides. *Proc Natl Acad Sci U S A*. (2013) 110:16097–102. doi: 10.1073/pnas.1303218110

56. Kamii Y, Hayashizaki K, Kanno T, Chiba A, Ikegami T, Saito M, et al. IL-27 regulates the differentiation of follicular helper NKT cells via metabolic adaptation of mitochondria. *Proc Natl Acad Sci U S A*. (2024) 121:e2313964121. doi: 10.1073/pnas.2313964121

57. Shute T, Amiel E, Alam N, Yates JL, Mohrs K, Dudley E, et al. Glycolipid-Containing Nanoparticle Vaccine Engages Invariant NKT Cells to Enhance Humoral Protection against Systemic Bacterial Infection but Abrogates T-Independent Vaccine Responses. *J Immunol*. (2021) 206:1806–16. doi: 10.4049/jimmunol.2001283

58. Tsuji M, Nair MS, Masuda K, Castagna C, Chong Z, Darling TL, et al. An immunostimulatory glycolipid that blocks SARS-CoV-2, RSV, and influenza infections in vivo. *Nat Commun*. (2023) 14:3959. doi: 10.1038/s41467-023-39738-1

59. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res*. (2002) 8:3702–9.

60. Padte NN, Boente-Carrera M, Andrews CD, McManus J, Grasperge BF, Gettie A, et al. A glycolipid adjuvant, 7DW8-5, enhances CD8+ T cell responses induced by an adenovirus-vectored malaria vaccine in non-human primates. *PloS One*. (2013) 8:e78407. doi: 10.1371/journal.pone.0078407

61. Rey-Jurado E, Bohmwald K, Galvez NMS, Becerra D, Porcelli SA, Carreno LJ, et al. Contribution of NKT cells to the immune response and pathogenesis triggered by respiratory viruses. *Virulence*. (2020) 11:580–93. doi: 10.1080/21505594.2020.1770492

62. Traves R, Opadchy T, Slobedman B, Abendroth A. Varicella zoster virus downregulates expression of the non-classical antigen presentation molecule CD1d. *J Infect Dis*. (2023). doi: 10.1093/infdis/jiad512

63. Gensollen T, Lin X, Zhang T, Pyzik M, See P, Glickman JN, et al. Embryonic macrophages function during early life to determine invariant natural killer T cell levels at barrier surfaces. *Nat Immunol*. (2021) 22:699–710. doi: 10.1038/s41590-021-00934-0

64. Wang WB, Lin YD, Zhao L, Liao C, Zhang Y, Davila M, et al. Developmentally programmed early-age skin localization of iNKT cells supports local tissue development and homeostasis. *Nat Immunol*. (2023) 24:225–38. doi: 10.1038/s41590-022-01399-5

65. Luo J, Chen Z, Castellano D, Bao B, Han W, Li J, et al. Lipids regulate peripheral serotonin release via gut CD1d. *Immunity*. (2023) 56:1533–47 e7. doi: 10.1016/j.immuni.2023.06.001

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 24 June 2024
DOI 10.3389/fimmu.2024.1424987

OPEN ACCESS

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RECEIVED 29 April 2024
ACCEPTED 12 June 2024
PUBLISHED 24 June 2024

CITATION
Ito E and Yamasaki S (2024) Regulation of MAIT cells through host-derived antigens. *Front. Immunol.* 15:1424987. doi: 10.3389/fimmu.2024.1424987

Regulation of MAIT cells through host-derived antigens

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Mucosal-associated invariant T (MAIT) cells are a major subset of innate-like T cells that function at the interface between innate and acquired immunity. MAIT cells recognize vitamin B2-related metabolites produced by microbes, through semi-invariant T cell receptor (TCR) and contribute to protective immunity. These foreign-derived antigens are presented by a monomorphic antigen presenting molecule, MHC class I-related molecule 1 (MR1). MR1 contains a malleable ligand-binding pocket, allowing for the recognition of compounds with various structures. However, interactions between MR1 and self-derived antigens are not fully understood. Recently, bile acid metabolites were identified as host-derived ligands for MAIT cells. In this review, we will highlight recent findings regarding the recognition of self-antigens by MAIT cells.

KEYWORDS

MAIT cell, MR1 ligand, bile acids, T cell development, self-antigen

1 Introduction

Mucosal-associated invariant T (MAIT) cells are the most abundant T cell subset in humans (1). They recognize non-peptidic antigens presented on a monomorphic antigen presenting molecule, MR1 (2–4). T cell receptor (TCR) repertoires of MAIT cells are composed of restricted TCRα and β chains (mice, TRAV1- TRAJ9/12/33–TRBV13/19; human, TRAV1-2- TRAJ12/20/33–TRBV6/20) that recognize riboflavin-based metabolites produced in microbes but not in mammals (2, 5, 6).

MAIT cells are positively selected in the thymus through the interaction with MR1-expressing double-positive (DP) thymocytes (7) and/or thymic epithelial cells (8). The development of MAIT cells is severely impaired in germ-free (GF) mice and microbiota-derived antigen 5-OP-RU is reported to contribute to their thymic selection (8, 9). Judging from its structure, 5-OP-RU is unlikely to cross the plasma membrane and, as no transporter proteins have been identified thus far, it is still unclear how unstable 5-OP-RU is transferred from gut to the thymus. Additionally, a small but significant number of MAIT cells have been detected in the thymi of GF mice (5, 8, 10), potentially suggesting the presence of endogenous antigen(s) that influence thymic development of MAIT cells (11).

Conventional T cells, which recognize peptidic antigens presented by classical MHC molecules using variant TCRs, are positively selected by weak affinity of self-peptides (12). In the periphery, self-peptides also contribute to the survival and maintenance of conventional T cells (13–15). Since the affinity of bacteria-derived 5-OP-RU to MAIT TCR is strong enough to induce negative selection (8), it is possible that host-derived weak antigen(s) may also be involved in the development and/or maintenance of MAIT cells. However, it is still unclear whether the strength of antigen affinity can differentially regulate the fate and function of MAIT cells, as is reported for conventional T cells.

One of the tissues in which human MAIT cells are most abundant is the liver, particularly in the hepatic sinusoid around bile ducts (16–19). Liver MAIT cells constitutively express activation markers, suggesting that MAIT cells receive continuous TCR signaling even in a steady state (20, 21). Thus, the unique localization and/or maintenance of tissue residency may also be regulated by tissue-derived endogenous factor(s) abundant in the liver. However, such self-antigens have not been identified thus far.

2 Diverse ligands presented by MR1

2.1 Diversity and specificity of MR1 ligands

MR1 is a well-conserved MHC class I-like molecule and present small compounds unlike CD1 molecules which can accommodate large lipids. For ligand binding, MR1 utilizes the A-pocket, which is flexible and accommodates a large variety of ligands (22, 23). Within the A-pocket, K43 mediates a covalent bond with some typical antigens (5-OP-RU, 6-FP and Ac-6-FP) (24, 25). Neutralization of this positively charged K43 is required for the stabilization of MR1 (22). However, some other ligands (RL-7-Me, RL-6, 7-diMe, diclofenac (DCF), DB28 and NV18.1) non-covalently bind to MR1 (2, 22, 23, 26, 27). It is unknown whether these 'non-covalent' ligands induce MR1 stabilization beyond K43 neutralization. MR1 additionally requires the ligand to possess a hydroxy group to be 'pinched' by two Arg residues found on MR1 (R9 and R94) (2, 6, 22). However, a comprehensive screening of potential MR1 ligands demonstrates that MR1 can actually present a notably broader range of small molecules regardless of these requirements, including mono- and multi-cyclic chemical compounds (22, 23). It is therefore possible that MR1 can bind to previously unappreciated endogenous metabolites.

2.2 Self recognition by MAIT cells

There are some studies that support the possibility of self-recognition by MAIT cells. Young et al. reports that a cell line expressing a MAIT TCR was activated in the presence of MR1-expressing antigen presenting cells (APC) in the absence of infection (28). Additionally, cancer cells have been shown to be targeted by MAIT cells utilizing an MR1-dependent mechanism, although with an unidentified ligand (29). Some atypical MR1-related T cells (MR1T cells) are reported to respond to self-derived antigen(s) (30, 31). Recently, Chancellor et al. discovered the rare occurrence of self-reactive MAIT cells that display unique T-helper functions (32). However, endogenous MAIT cell antigen(s) presented by MR1 are yet to be identified.

3 Bile acid metabolites as host-derived ligands

3.1 Cholic acid 7-sulfate is a MAIT cell ligand

We recently purified and identified cholic acid 7-sulfate (CA7S) as a host-derived ligand for MAIT cells (33). CA7S has a structure that is distinct from those of previously-reported MR1 ligands in that it uniquely possesses four rings. In addition to previously-demonstrated small ligands (2, 6, 22), a large cholane skeleton can also be accommodated within the MR1 pocket. Notably, a competition assay (34) revealed that CA7S binds to the A-pocket of the MR1 molecule (33) similar to known ligands (2, 25). However, the chemical structure of CA7S and its ability to increase surface expression of MR1 lacking K43 (K43A) suggested that CA7S could bind to MR1 without forming a Schiff base. Indeed, the relative affinity of CA7S to MR1 was estimated to be as weak as that of DCF (33), which is also a non-covalent ligand (22). One of the structural characteristics of CA7S is the presence of carboxy group at position 24. This moiety might be interacted with cationic residues in the A-pocket, which warrants further structural analysis. Although we reported a potential function of CA7S on MAIT cell development, comprehensive understanding of its role in MAIT cell biology need further investigation.

3.2 Role of CAS in bile acid metabolic pathway

CA7S is a primary bile acid produced from cholic acid by sulfotransferase 2a (Sult2a), which is bile acid-specific sulfotransferases (35–37) (Figure 1). As amphiphilic bile acids are sometimes toxic, sulfotransferase function is essential for neutralization and detoxification of bile acids like cholic acid. To do this, Sult2a adds a hydrophilic SO_3^- group to the hydrophobic cholane skeleton, which generates sulfated cholic acids for excretion in feces and urine (35, 38). Thus, the significance of CA7S was previously thought to be as an excreted bile acid metabolite, and only a few additional roles were reported (39, 40).

Although CA7S is biosynthesized in the host, levels of CA7S were decreased in GF mice (33). This is likely due to the lack of deconjugation of tauro-CA7S (TCA7S) by symbiotic bacteria (Figure 1), as TCA7S was increased in GF mice (33, 41). Thus, CA7S is an endogenous metabolite, but its quantities are largely influenced by symbiotic bacteria. This is consistent with the observation that MAIT cells dramatically decreased in GF mice (5, 8, 9). These results suggest that the reduction in CA7S levels might also contribute to the impairment of MAIT cell development in GF mice in combination with the lack of microbial antigens like 5-OP-RU.

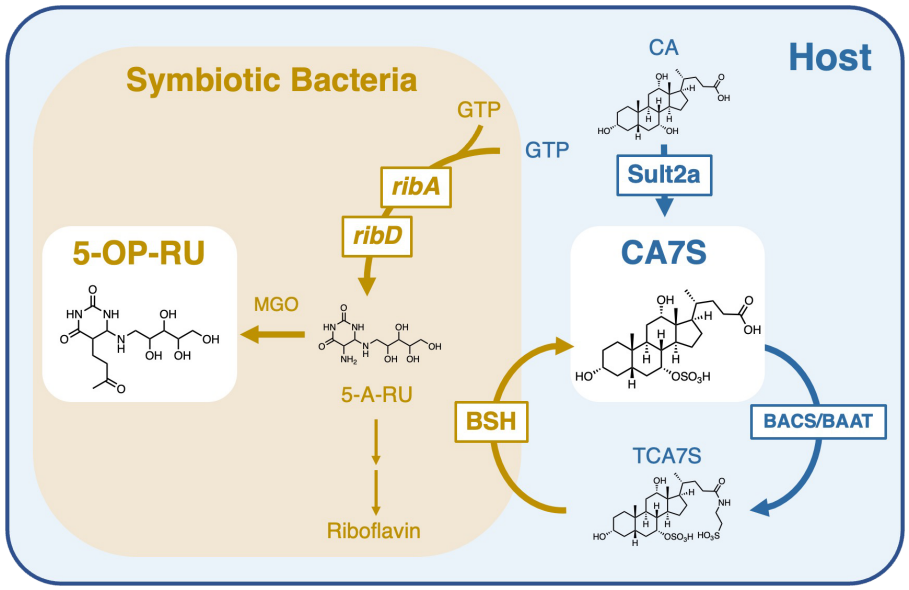

FIGURE 1
Role of symbiotic bacteria in the generation and modification of MAIT cell antigens. Bacterial riboflavin biosynthesizing enzymes, such as *ribA* and *ribD*, generate 5-A-RU, which is converted to 5-OP-RU in the presence of methylglyoxal (MGO) (left). CAS is produced by sulfation of cholic acid (CA) by sulfotransferase 2a (Sult2a) in the host. CAS is further taurine-conjugated in the host by bile acid–CoA:amino acid N-acyltransferase (BAAT) or bile acid–CoA synthetase (BACS) to generate TCA7S. Most intestinal bacteria have deconjugation enzymes, bile salt hydrolases (BSH), which metabolize TCA7S to CA7S. Symbiotic bacteria are therefore required for the maintenance of both 5-OP-RU and CA7S.

3.3 Role of CAS in MAIT cell development

As CA7S is weakly recognized by MAIT cells, it may contribute to the development of MAIT cells in the thymus (Figure 2). CA7S was detected in the thymus, and in mice lacking all Sult2a isoforms, thymic development of MAIT cells was impaired (33). Among thymic MAIT cells, the most mature stage, stage 3 ($CD44^{+}CD24^{-}$) (10), was severely affected. In mature thymic MAIT cells, MAIT17 occurs more frequently than MAIT1, whereas MAIT17 was fewer than MAIT 1 in Sult2a-deficient mice (33). These phenotypes were similar to those of GF mice (8, 10). While CA7S has been detected in the thymus, it is still unclear whether CAS is taken up and presented by thymocytes (Figure 2). Alternatively, Sult2a is also expressed in thymus as well as hepatocytes, implying that CAS might be synthesized within thymocytes when its substrate CA is available (Figure 2). Conditional deletion of all Sult2a isoforms will answer the key question whether CAS is *de novo* generated in thymocytes.

3.4 Effect of CA7S on MAIT cells in peripheral tissues

The role of CA7S in MAIT cells in peripheral tissues is not well understood. Unlike GF mice, the number of MAIT cells in the liver was not significantly decreased in Sult2a-deficient mice (33), implying that the antigen(s) utilized for the maintenance of MAIT cells vary by tissue. However, among liver-resident T cells, only MAIT cells lost the expression of multiple T cell signature genes, which is in contrast to invariant natural killer T (iNKT) cells and conventional T cells. Thus, CA7S might play an important role in shaping the identity of MAIT cells in the liver. MAIT cell-dependent protective immunity in the absence of CA7S function would be a worthy subject of further investigation utilizing infection models. Liver MAIT cells localize in the hepatic sinusoid, close to bile duct where CA7S is abundantly present; however, it is currently unknown whether bile acid metabolites play a role in MAIT cells in 'distant' tissues, such as the skin or lungs. Quantitative analysis of bile acid metabolites in these tissues would be required for further clarification. Although Sult2a is mainly expressed in hepatocytes, its transcript is also highly detected in other tissues (42), such as in some regions of the small intestine (Figure 2) (42, 43). CA7S produced in non-liver tissues might contribute to the maintenance of MAIT cells locally, which is a potential area of further investigation.

In humans, instead of CA7S, CA3S is an abundant cholic acid sulfate (35). Although the position of sulfation is different, CA3S can be presented by MR1 and recognized by MAIT TCR. In contrast to 5-OP-RU which triggers proliferation of peripheral MAIT cells, CA3/7S only induces survival, not proliferation. Thus, CA3/7S appears to induce qualitatively distinct MAIT cell responses. Indeed, while 5-OP-RU upregulates pro-inflammatory genes, CA3/7S induces gene signatures characterized by homeostatic and tissue repair responses (33). Among these is *CXCR4*, which contributes to migration and residency of lymphocytes in the tissues. It is therefore possible that CA3/7S, which is abundant in bile, may contribute to the residency of MAIT cells in the liver sinusoid where MAIT cells are most enriched

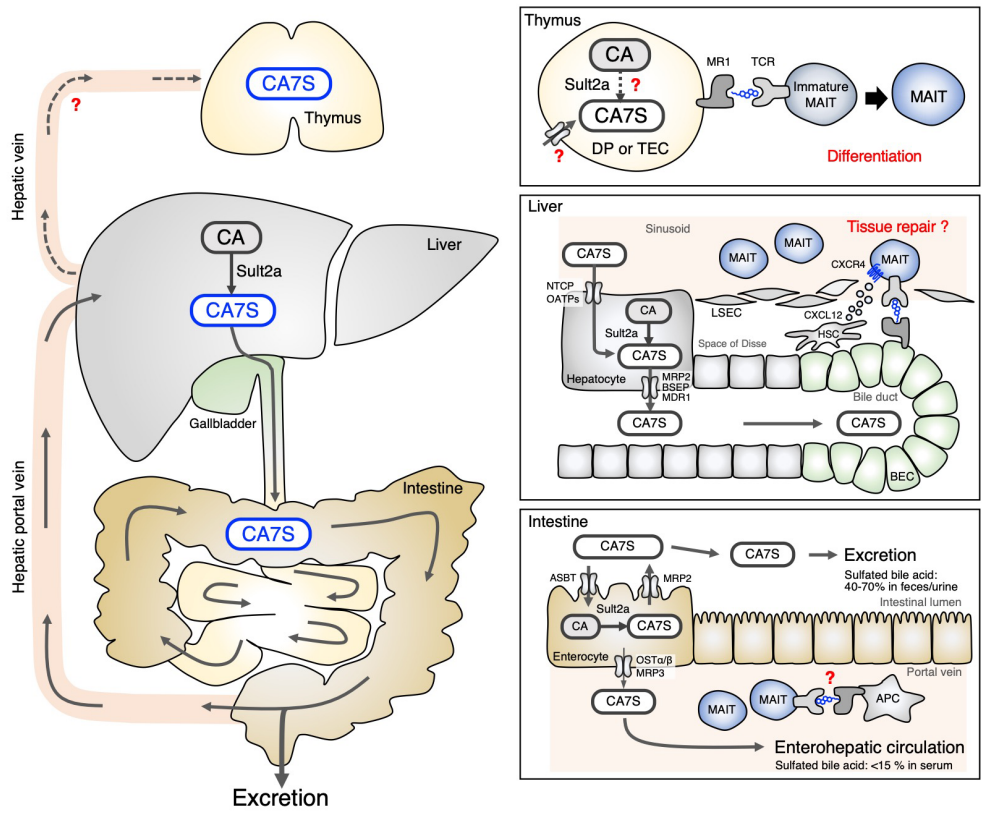

FIGURE 2
Circulation of CA7S in the body. CA7S is mainly biosynthesized in the liver from CA, stored in the gallbladder and excreted through the intestine (left). The source of thymic CA7S is unknown (Thymus). CA7S in the bile duct may be presented to liver MAIT cells surrounding biliary epithelial cells (BEC) expressing MR1 (Liver). Sult2a expressed by enterocytes can locally produce CA7S in some intestinal areas, which might control the homeostasis of intestinal MAIT cells. Enterocyte-derived CA7S may also be transported through the enterohepatic vein (Intestine). .

particularly in humans (16, 18, 19). Since the barrier composed of liver sinusoidal endothelial cells (LSEC) is fragile, upon liver damage, MAIT cells will likely come into contact with cholangiocytes/biliary epithelial cells (BEC) that express MR1 (16, 44). One might speculate that bile acid metabolites presented by MR1 on BEC promote tissue repair responses. Additionally, MAIT cells may act as a sensor of bile acid homeostasis.

Recently, roles of secondary bile acid metabolites, which are produced by the microbiota from primary bile acids, have been highlighted in T cell development. 3-oxo-litho cholic acid (3-oxo-LCA) has been shown to inhibit Th17 differentiation through the interaction with RORγt (45). Furthermore, the 3-oxo-LCA derivative, iso-allo-LCA, promotes regulatory T cell (Treg) differentiation via mitochondria-mediated epigenetic regulation (45) through Nr4a1 (46), which is supported by bacterial genetics (47). A similar secondary bile acid, iso-deoxycholic acid (iso-DCA), antagonizes FXR and the impairs immunogenic properties of dendritic cells, leading to pTreg maturation (48). In contrast to these indirect effects of secondary bile acids on T cells, CA3S and CA7S are endogenous primary bile acids that are directly recognized by TCR as antigens. Nevertheless, since CA3/7S also potentially act on nuclear receptor and/or GPCR families (39), sulfated bile acids may serve unknown pleiotropic functions within the immune system.

3.5 Role of CAS in disease settings

Thus far we have discussed CAS in a homeostatic context. However, quantitative variations in CAS have been reported in several diseases (35, 49–51). Furthermore, the expression of SULT2A1 is reported to be decreased in cholestatic diseases (52–55). Examination of the involvement of the CAS-MAIT cell axis in these disorders would be intriguing (18, 56). In particular, cholestatic autoimmune diseases, such as primary biliary cholangitis (PBC) and primary sclerosis cholangitis (PSC), have been associated with MAIT cells (57–60). The role of MAIT cells in immune diseases related to bile duct abnormalities is therefore an exciting area for future research.

4 Future perspective

Why should bile acids metabolites be recognized by MAIT cells? Currently, there is still no clear answer to this teleological question. The correlation of CAS-rich sites (bile duct) and MAIT cell-rich sites (liver sinusoid) raise several hypotheses regarding their role in tissue residency. As bile acids can sometimes harm the body, excessive cholic acids are continuously excreted as sulfated forms. Excreted forms are therefore stably and abundantly present in the

body, which make them effective for the maintenance of a host cell lineage. Moreover, recycling 'waste' metabolites is considered as an efficient strategy to make use of limited metabolites in the body. Nevertheless, at present, the localization of MAIT cells in other tissues, such as lung and skin where CAS is presumably less abundant, cannot be simply explained by bile acids. Additionally, the discovery of the 'peculiar' antigenic structure of the bile acid skeleton may suggest that MR1 can present a far greater variety of molecules than previously assumed, and that the MAIT TCR, despite its lack of diversity, can recognize complexes of such diverse antigens with MR1. It is exciting to imagine that further diverse self-antigen(s) for MAIT cells are present in different tissues and regulate their tissue-specific adaptation, which warrants future studies.

Author contributions

EI: Writing – original draft, Writing – review & editing. SY: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. EI is supported by JSPS (JP21J22863) and SY is supported by JSPS (JP22H05183) and AMED (JP21gm0910010, JP23fk0108608, JP23wm0325054, JP233fa727002, JP23jk0210005).

Acknowledgments

We thank I. C. Cabebe and C. R. Schutt for discussion.

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

References

1. Godfrey DI, Uldrich AP, Mccluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nat Immunol.* (2015) 16:1114–23. doi: 10.1038/ni.3298

2. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature.* (2012) 491:717–23. doi: 10.1038/nature11605

3. Godfrey DI, Koay HF, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. *Nat Immunol.* (2019) 20:1110–28. doi: 10.1038/s41590-019-0444-8

4. Legoux F, Salou M, Lantz O. MAIT cell development and functions: the microbial connection. *Immunity.* (2020) 53:710–23. doi: 10.1016/j.immuni.2020.09.009

5. Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F, et al. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature.* (2003) 423:164–9. doi: 10.1038/nature01700

6. Corbett AJ, Eckle SBG, Birkinshaw RW, Liu L, Patel O, Mahony J, et al. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature.* (2014) 509:361–5. doi: 10.1038/nature13160

7. Seach N, Guerri L, Le Bourhis L, Mburu Y, Cui Y, Bessoles S, et al. Double positive thymocytes select mucosal-associated invariant T cells. *J Immunol.* (2013) 191:6002–9. doi: 10.4049/jimmunol.1301212

8. Legoux F, Bellet D, Daviaud C, El Morr Y, Darbois A, Niort K, et al. Microbial metabolites control the thymic development of mucosal-associated invariant T cells. *Science.* (2019) 366:494–9. doi: 10.1126/science.aaw2719

9. Constantinides MG, Link VM, Tamoutounour S, Wong AC, Perez-Chaparro PJ, Han SJ, et al. MAIT cells are imprinted by the microbiota in early life and promote tissue repair. *Science.* (2019) 366:445. doi: 10.1126/science.aax6624

10. Koay HF, Gherardin NA, Enders A, Loh L, Mackay LK, Almeida CF, et al. A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage. *Nat Immunol.* (2016) 17:1300–11. doi: 10.1038/ni.3565

11. Bugaut H, El Morr Y, Mestdagh M, Darbois A, Paiva RA, Salou M, et al. A conserved transcriptional program for MAIT cells across mammalian evolution. *J Exp Med.* (2024) 221:e20231487. doi: 10.1084/jem.20231487

12. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. *Cell.* (1994) 76:17–27. doi: 10.1016/0092-8674(94)90169-4

13. Tanchot C, Lemonnier FA, Pérarnau B, Freitas AA, Rocha B. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science.* (1997) 276:2057–62. doi: 10.1126/science.276.5321.2057

14. Viret C, Wong FS, Janeway CA. Designing and maintaining the mature TCR repertoire. *Immunity.* (1999) 10:559–68. doi: 10.1016/s1074-7613(00)80055-2

15. Pais Ferreira D, Silva JG, Wyss T, Fuertes Marraco SA, Scarpellino L, Charmoy M, et al. Central memory CD8+ T cells derive from stem-like Tcf7hi effector cells in the absence of cytotoxic differentiation. *Immunity.* (2020) 53:985–1000. doi: 10.1016/j.immuni.2020.09.005

16. Jeffery HC, Van Wilgenburg B, Kurioka A, Parekh K, Stirling K, Roberts S, et al. Biliary epithelium and liver B cells exposed to bacteria activate intrahepatic MAIT cells through MR1. *J Hepatol.* (2016) 64:1118–27. doi: 10.1016/j.jhep.2015.12.017

17. Böttcher K, Rombouts K, Saffioti F, Roccarina D, Rosselli M, Hall A, et al. MAIT cells are chronically activated in patients with autoimmune liver disease and promote profibrogenic hepatic stellate cell activation. *Hepatology.* (2018) 68:172–86. doi: 10.1002/hep.29782

18. Hegde P, Weiss E, Paradis V, Wan J, Mabire M, Sukriti S, et al. Mucosal-associated invariant T cells are a profibrogenic immune cell population in the liver. *Nat Commun.* (2018) 9:1–12. doi: 10.1038/s41467-018-04450-y

19. Lett MJ, Mehta H, Keogh A, Jaeger T, Jacquet M, Powell K, et al. Stimulatory MAIT cell antigens reach the circulation and are efficiently metabolised and presented by human liver cells. *Gut.* (2022) 71:2526–38. doi: 10.1136/gutjnl-2021-324478

20. Dusseaux M, Martin E, Serriari N, Péguillet I, Premel V, Louis D, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161 hi IL-17-secreting T cells. *Blood.* (2011) 117:1250–9. doi: 10.1182/blood-2010-08-303339

21. Tang X-Z, Jo J, Tan AT, Sandalova E, Chia A, Tan KC, et al. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. *J Immunol.* (2013) 190:3142–52. doi: 10.4049/jimmunol.1203218

22. Keller AN, Eckle SBG, Xu W, Liu L, Hughes VA, Mak JYW, et al. Drugs and drug-like molecules can modulate the function of mucosal-associated invariant T cells. *Nat Immunol.* (2017) 18:402–11. doi: 10.1038/ni.3679

23. Harriff MJ, McMurtrey C, Froyd CA, Jin H, Cansler M, Null M, et al. MR1 displays the microbial metabolome driving selective MR1-restricted T cell receptor usage. *Sci Immunol.* (2018) 3:eaao2556. doi: 10.1126/sciimmunol.aao2556

24. Patel O, Kjer-Nielsen L, Le Nours J, Eckle SBG, Birkinshaw R, Beddoe T, et al. Recognition of vitamin B metabolites by mucosal-associated invariant T cells. *Nat Commun*. (2013) 4:1–9. doi: 10.1038/ncomms3142

25. Eckle SBG, Birkinshaw RW, Kostenko L, Corbett AJ, McWilliam HEG, Reantragoon R, et al. A molecular basis underpinning the T cell receptor heterogeneity of mucosal-associated invariant T cells. *J Exp Med*. (2014) 211:1585–600. doi: 10.1084/jem.20140484

26. Salio M, Awad W, Veerapen N, Gonzalez-Lopez C, Kulicke C, Waithe D, et al. Ligand-dependent downregulation of MR1 cell surface expression. *Proc Natl Acad Sci U. S. A*. (2020) 117:10465–75. doi: 10.1073/pnas.2003136117

27. McWilliam HEG, Villadangos JA. MR1 antigen presentation to MAIT cells and other MR1-restricted T cells. *Nat Rev Immunol*. (2023) 24:178–92. doi: 10.1038/s41577-023-00934-1

28. Young MH, U'Ren L, Huang S, Mallevaey T, Scott-Browne J, Crawford F, et al. MAIT cell recognition of MR1 on bacterially infected and uninfected Cells. *PloS One*. (2013) 8:6–11. doi: 10.1371/journal.pone.0053789

29. Crowther MD, Dolton G, Legut M, Caillaud ME, Lloyd A, Attaf M, et al. Genome-wide CRISPR–Cas9 screening reveals ubiquitous T cell cancer targeting via the monomorphic MHC class I-related protein MR1. *Nat Immunol*. (2020) 21:178–85. doi: 10.1038/s41590-019-0578-8

30. Lepore M, Kalinichenko A, Calogero S, Kumar P, Paleja B, Schmaler M, et al. Functionally diverse human T cells recognize non-microbial antigens presented by MR1. *Elife*. (2017) 6:1–22. doi: 10.7554/eLife.24476

31. Shibata K, Motozono C, Nagae M, Shimizu T, Ishikawa E, Motooka D, et al. Symbiotic bacteria-dependent expansion of MR1-reactive T cells causes autoimmunity in the absence of Bcl11b. *Nat Commun*. (2022) 13:1–5. doi: 10.1038/s41467-022-34802-8

32. Chancellor A, Alan Simmons R, Khanolkar RC, Nosi V, Beshirova A, Berloffa G, et al. Promiscuous recognition of MR1 drives self-reactive mucosal-associated invariant T cell responses. *J Exp Med*. (2023) 220:e20221939. doi: 10.1084/jem.20221939

33. Ito E, Inuki S, Izumi Y, Takahashi M, Dambayashi Y, Ciacchi L, et al. Sulfated bile acid is a host-derived ligand for MAIT cells. *Sci Immunol*. (2024) 9:eade6924. doi: 10.1126/sciimmunol.ade6924

34. Wang CJH, Awad W, Liu L, Mak JYW, Veerapen N, Illing PT, et al. Quantitative af fi nity measurement of small molecule ligand binding to major histocompatibility complex class-I – related. *J Biol Chem*. (2022) 298:1–12. doi: 10.1016/j.jbc.2022.102714

35. Alnouti Y. Bile acid sulfation: A pathway of bile acid elimination and detoxification. *Toxicol Sci*. (2009) 108:225–46. doi: 10.1093/toxsci/kfn268

36. Shimohira T, Kurogi K, Liu MC, Suiko M, Sakakibara Y. The critical role of His48 in mouse cytosolic sulfotransferase SULT2A8 for the 7α-hydroxyl sulfation of bile acids. *Biosci Biotechnol Biochem*. (2018) 82:1359–65. doi: 10.1080/09168451.2018.1464897

37. Teramoto T, Nishio T, Kurogi K, Sakakibara Y, Kakuta Y. The crystal structure of mouse SULT2A8 reveals the mechanism of 7α-hydroxyl, bile acid sulfation. *Biochem Biophys Res Commun*. (2021) 562:15–20. doi: 10.1016/j.bbrc.2021.04.113

38. Dawson PA, Karpen SJ. Intestinal transport and metabolism of bile acids. *J Lipid Res*. (2015) 56:1085–99. doi: 10.1194/jlr.R054114

39. Chaudhari SN, Harris DA, Aliakbarian H, Luo JN, Henke MT, Subramaniam R, et al. Bariatric surgery reveals a gut-restricted TGR5 agonist with anti-diabetic effects. *Nat Chem Biol*. (2021) 17:20–9. doi: 10.1038/s41589-020-0604-z

40. Chaudhari SN, Luo JN, Harris DA, Aliakbarian H, Yao L, Paik D, et al. A microbial metabolite remodels the gut-liver axis following bariatric surgery. *Cell Host Microbe*. (2021) 29:408–24. doi: 10.1016/j.chom.2020.12.004

41. Cai J, Sun L, Gonzalez FJ. Gut microbiota-derived bile acids in intestinal immunity, inflammation, and tumorigenesis. *Cell Host Microbe*. (2022) 30:289–300. doi: 10.1016/j.chom.2022.02.004

42. Teubner W, Meinl W, Florian S, Kretzschmar M, Glatt H. Identification and localization of soluble sulfotransferases in the human gastrointestinal tract. *Biochem J*. (2007) 404:207–15. doi: 10.1042/BJ20061431

43. Alnouti Y, Klaassen CD. Tissue distribution and ontogeny of sulfotransferase enzymes in mice. *Toxicol Sci*. (2006) 93:242–55. doi: 10.1093/toxsci/kfl050

44. Banales JM, Huebert RC, Karlsen T, Strazzabosco M, LaRusso NF, Gores GJ. Cholangiocyte pathobiology. *Nat Rev Gastroenterol Hepatol*. (2019) 16:269–81. doi: 10.1038/s41575-019-0125-y

45. Hang S, Paik D, Yao L, Kim E, Jamma T, Lu J, et al. Bile acid metabolites control TH17 and Treg cell differentiation. *Nature*. (2019) 576:143–8. doi: 10.1038/s41586-019-1785-z

46. Li W, Hang S, Fang Y, Bae S, Zhang Y, Zhang M, et al. A bacterial bile acid metabolite modulates Treg activity through the nuclear hormone receptor NR4A1. *Cell Host Microbe*. (2021) 29:1366–1377.e9. doi: 10.1016/j.chom.2021.07.013

47. Song X, Sun X, Oh SF, Wu M, Zhang Y, Zheng W, et al. Microbial bile acid metabolites modulate gut RORγ+ regulatory T cell homeostasis. *Nature*. (2020) 577:410–5. doi: 10.1038/s41586-019-1865-0

48. Campbell C, McKenney PT, Konstantinovsky D, Isaeva OI, Schizas M, Verter J, et al. Bacterial metabolism of bile acids promotes generation of peripheral regulatory T cells. *Nature*. (2020) 581:475–9. doi: 10.1038/s41586-020-2193-0

49. Shinohara T, Muraji T, Tsugawa C, Nishijima E, Satoh S, Takamizawa S. Efficacy of urinary sulfated bile acids for diagnosis of bacterial cholangitis in biliary atresia. *Pediatr Surg Int*. (2005) 21:701–4. doi: 10.1007/s00383-005-1493-7

50. Nanashima A, Obatake M, Sumida Y, Abo T, Yamane Y, Nomura M, et al. Clinical significance of measuring urinary sulfated bile acids in adult patients with hepatobiliary diseases. *Hepato-Gastroenterology J Clin Res Pract*. (2009) 56:299–302.

51. Li W, Alamoudi JA, Gautam N, Kumar D, Olivera M, Gwon Y, et al. Urinary BA indices as prognostic biomarkers for complications associated with liver diseases. *Int J Hepatol*. (2022) 2022:5473752. doi: 10.1155/2022/5473752

52. Elekima OTL, Mills CO, Ahmad A, Skinner GRB, Ramsden DB, Bown J, et al. Reduced hepatic content of dehydroepiandrosterone sulphotransferase in chronic liver diseases. *Liver*. (2000) 20:45–50. doi: 10.1034/j.1600-0676.2000.020001045.x

53. Yalcin EB, More V, Neira KL, Lu ZJ, Cherrington NJ, Slitt AL, et al. Downregulation of sulfotransferase expression and activity in diseased human livers. *Drug Metab Dispos*. (2013) 41:1642–50. doi: 10.1124/dmd.113.050930

54. Chai J, Feng X, Zhang L, Chen S, Cheng Y, He X, et al. Hepatic expression of detoxification enzymes is decreased in human obstructive cholestasis due to gallstone biliary obstruction. *PloS One*. (2015) 10:1–14. doi: 10.1371/journal.pone.0120055

55. Wunsch E, Klak M, Wasik U, Milkiewicz M, Blatkiewicz M, Urasinska E, et al. Liver expression of sulphotransferase 2A1 enzyme is impaired in patients with primary sclerosing cholangitis: lack of the response to enhanced expression of PXR. *J Immunol Res*. (2015) 2015:571353. doi: 10.1155/2015/571353

56. Jiang X, Peng Y, Liu L, Wang Y, Li M, Li W, et al. MAIT cells ameliorate liver fibrosis by enhancing the cytotoxicity of NK cells in cholestatic murine models. *Liver Int*. (2022) 42:2743–58. doi: 10.1111/liv.15445

57. Jiang X, Lian M, Li Y, Zhang W, Wang Q, Wei Y, et al. The immunobiology of mucosal-associated invariant T cell (MAIT) function in primary biliary cholangitis: regulation by cholic acid-induced interleukin-7. *J Autoimmun*. (2018) 90:64–75. doi: 10.1016/j.jaut.2018.01.007

58. Setsu T, Yamagiwa S, Tominaga K, Kimura N, Honda H, Kamimura H, et al. Persistent reduction of mucosal-associated invariant T cells in primary biliary cholangitis. *J Gastroenterol Hepatol*. (2018) 33:1286–94. doi: 10.1111/jgh.14076

59. von Seth E, Zimmer CL, Reuterwall-Hansson M, Barakat A, Arnelo U, Bergquist A, et al. Primary sclerosing cholangitis leads to dysfunction and loss of MAIT cells. *Eur J Immunol*. (2018) 48:1997–2004. doi: 10.1002/eji.201847608

60. Valestrand L, Zheng F, Hansen SH, Øgaard J, Hov JR, Björkström NK, et al. Bile from patients with primary sclerosing cholangitis contains mucosal-associated invariant T-cell antigens. *Am J Pathol*. (2022) 192:629–41. doi: 10.1016/j.ajpath.2021.12.008

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 19 August 2024
DOI 10.3389/fimmu.2024.1457771

OPEN ACCESS

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RECEIVED 01 July 2024
ACCEPTED 02 August 2024
PUBLISHED 19 August 2024

CITATION
Takami M and Motohashi S (2024) Comparative assessment of autologous and allogeneic iNKT cell transfer in iNKT cell-based immunotherapy.
Front. Immunol. 15:1457771.
doi: 10.3389/fimmu.2024.1457771

Comparative assessment of autologous and allogeneic iNKT cell transfer in iNKT cell-based immunotherapy

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Invariant natural killer T (iNKT) cells are a small subset of T lymphocytes that release large amounts of cytokines such as IFN-γ and exhibit cytotoxic activity upon activation, inducing strong anti-tumor effects. Harnessing the anti-tumor properties of iNKT cells, iNKT cell-based immunotherapy has been developed to treat cancer patients. In one of the iNKT cell-based immunotherapies, two approaches are utilized, namely, active immunotherapy or adoptive immunotherapy, the latter involving the *ex vivo* expansion and subsequent administration of iNKT cells. There are two sources of iNKT cells for adoptive transfer, autologous and allogeneic, each with its own advantages and disadvantages. Here, we assess clinical trials conducted over the last decade that have utilized iNKT cell adoptive transfer as iNKT cell-based immunotherapy, categorizing them into two groups based on the use of autologous iNKT cells or allogeneic iNKT cells.

KEYWORDS

invariant natural killer T cells, adoptive immunotherapy, cancer immunotherapy, allogeneic cells, induced pluripotent stem cells

1 Introduction

Invariant natural killer T (iNKT) cells were identified in the late 1980s as a distinct population of T cells expressing both T-cell receptor (TCR) and NK cell markers (1–3). iNKT cells express invariant TCRs, consisting of a Vα24-Jα18 chain and Vβ11 chain in humans, and a Vα14-Jα18 chain and Vβ8.2, Vβ7, or Vβ2 chain in mice. These TCRs recognize their cognate glycolipid presented on the MHC class I-like molecule CD1d, and can produce large amounts of cytokines including IFN-γ. Meanwhile, iNKT cells directly exert tumoricidal activity upon activation (4, 5). The discovery of iNKT cells' potent immunomodulatory effects sparked interest in their therapeutic potential. Furthermore, the development of iNKT cell-based immunotherapy was accelerated upon the discovery of the cognate ligand α-galactosylceramide (α-GalCer) (2).

Clinical trials have been initiated to evaluate the safety and efficacy of iNKT cell-targeted therapies in humans. About 20 years ago, we embarked on a phase I clinical study of iNKT cell-based immunotherapy targeting non-small cell lung cancer (NSCLC) patients (6). In that study, autologous α-GalCer-pulsed antigen-presenting cells (APCs) were generated *ex vivo* and infused into patients, with the expectation that they would activate endogenous iNKT cells, which would subsequently trigger other anti-tumor immune responses in patients to fight cancer. This work showed promising results, so other clinical trials using similar methods targeting NSCLCs and head and neck cancers (HNCs) were conducted at our facility (7–12). In 2020, the results of a single-arm phase II clinical trial of α-GalCer-pulsed APCs as a second-line treatment for advanced or recurrent NSCLC was reported (13). The intravenous injection of α-GalCer-pulsed APCs was well-tolerated and was accompanied by prolonged overall survival. The median overall survival time of all 35 enrolled patients was 21.9 months, which was better than the expected survival time. However, we encountered challenges such as limited clinical efficacy due to a lower percentage of iNKT cells in cancer patients than in healthy donors (14–16). To begin with, a certain number of autologous iNKT cells have to be present in the body in the case of active immunotherapy. In adoptive immunotherapy, iNKT cells can be expanded *ex vivo* before infusion into patients, increasing the likelihood of inducing iNKT cell responses upon activation. In adoptive transfer therapy, there are two types of iNKT cells that can be infused: autologous or allogeneic. While the adoptive transfer of *ex vivo* expanded iNKT cells was not effective as transferring α-GalCer-pulsed APCs in our past clinical trials, adoptive transfer of iNKT cell therapy is gaining attention with the advancement of combination therapies and novel techniques such as transducing chimeric antigen receptor (CAR). In this review, we discuss the advantages and disadvantages of adoptive iNKT cell-based immunotherapy using autologous or allogeneic iNKT cells.

2 Autologous iNKT cell transfer

The main advantage of using autologous iNKT cells for adoptive transfer therapy is the avoidance of rejection via host immune responses; thus, transferred iNKT cells are expected to persist longer than allogeneic iNKT cells. In addition, autologous iNKT therapy allows us to establish personalized treatment approaches tailored to individual patients' characteristics, including HLA type and immune status. Moreover, autologous iNKT therapy avoids the risk of infection from donor cells. However, there are disadvantages including difficulty obtaining a sufficient number of functional autologous iNKT cells, and the fact that the procedure is time-consuming and expensive because of the need to manufacture cell products individually (Table 1).

2.1 Expansion of autologous iNKT cells

We performed a phase I clinical trial of autologous iNKT cell infusion targeting recurrent or advanced NSCLC patients (n=6) (9). iNKT cells were expanded from peripheral blood mononuclear cells (PBMCs) of patients in the presence of α-GalCer and IL-2 *ex vivo*. A bulk population of cultured cells including iNKT cells (0.3% to 21.5%) was then infused into the patients intravenously. No adverse events were observed in this study. Adverse event was defined based on common terminology criteria for adverse events (CTCAE, https://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm). In general, adverse events are classified by their severity from Grade 1 (mild) to Grade 5 (death). Furthermore, some of the patients showed increased proportions of iNKT cells and IFN-γ-producing cells among PBMCs, indicating the immune responses after infusion of the iNKT cells. Based on these promising findings, we also conducted phase I and phase II clinical trials, investigating adoptive transfer of both iNKT cells and α-GalCer-pulsed APCs for HNC patients (n=18) (11, 12). iNKT cells were injected into a tumor-feeding artery. This combined therapy was associated with a serious adverse event in 1 patient (phase I) with a pharyngo-cutaneous fistula related to local tumor reduction. The remaining 17 patients experienced only mild adverse events and 8 patients (3 patients in phase I and 5 patients in phase II) achieved partial responses, suggesting the enhancement of anti-tumor immune responses.

There are several ways of expanding iNKT cells and the proportion of iNKT cells also differs among individuals. Exley et al. expanded and generated iNKT cells at much higher purity for adoptive therapy. In a phase I clinical study, the expanded autologous iNKT cells with much higher purity (13%–87%) were used for adoptive transfer into advanced melanoma patients (n=9) (17). In the study, iNKT cells were isolated using a monoclonal antibody (6B11) against the invariant TCRα chain expressed by these cells, followed by expansion using anti-CD3 antibody in the presence of IL-2. Grade 1–2 toxicities were observed, while an increased number of iNKT cells were detected after infusion.

2.2 Global clinical trials of iNKT cell-based immunotherapy

In the last 10 years, various clinical studies have been performed on adoptive autologous iNKT cell therapy globally. Gao et al. reported on the adoptive transfer of autologous iNKT cells (85%–95%) targeting advanced hepatocellular carcinoma (n=10) (18). In that study, PBMCs were stimulated with α-GalCer in the presence

TABLE 1 Comparison of autologous and allogeneic iNKT cells.

Origin of iNKT cells	Advantages	Disadvantages
Autologous	• Avoidance of rejection • Avoidance of infection from donor cells • Tailored to individuals	• Time consuming • Expensive
Allogeneic	• Easy to handle • Enables off-the-shelf therapy • Avoidance of mmune exhaustion	• Shorter persistence • Risk of infection from donor cells

of IL-2 for the first round of expansion, followed by the sorting of iNKT cells using magnetic beads. Then, purified iNKT cells were co-cultured with autologous mature DCs as the second round of expansion. Grade 1–2 toxicities were observed in most of the patients, while grade 3 adverse events were reported in three patients, suggesting that infusion of autologous iNKT cells was safe and well tolerated. The latest study by the same group was a phase II clinical trial (NCT04011033, n=54) investigating the combination therapy of autologous iNKT cell infusion with trans arterial embolization for hepatocellular carcinoma (19). Here, patients were randomly assigned to either trans arterial embolization alone or in combination with iNKT cell infusion. For the combination treatment, expanded iNKT cells (purity >95%) were infused into patients along with trans arterial embolization treatment. The results indicated that the infusion of iNKT cells significantly improved progression-free survival, overall response rate, disease control, and quality of life while keeping toxicity at manageable levels. Additionally, other combination therapies involving iNKT cell infusion have been reported. For example, a phase I/II clinical trial of combination therapy of autologous iNKT cells and PD-1$^+$CD8$^+$ T-cell infusion targeting advanced NSCLC patients was conducted (NCT03093688, 2017–2022) (20). Autologous iNKT cells were expanded from PBMCs with the stimulation of α-GalCer in the presence of IL-2 and IL-7, followed by the addition of α-GalCer-pulsed DCs. Autologous PD1$^+$CD8$^+$ T cells sorted from PBMCs were expanded using anti-CD3/anti-CD28-coated beads in the presence of IL-2, IL7, IL-15, and TLR agonists. Three patients were enrolled in this study and autologous iNKT cells (purity 13% to 88%) and PD-1$^+$CD8$^+$ T cells (purity >95%) were infused into them for multiple cycles (6, 10, or 16 cycles). In that study, the infusion of iNKT cells and PD-1$^+$CD8$^+$ T cells induced grade 1–2 toxicities, but the patients tolerated it well. The same group has also conducted the combination therapy of autologous iNKT cells and PD-1$^+$CD8$^+$ T-cell infusion for patients with advanced pancreatic cancer (NCT03093688) (21). In that study, nine patients received at least three cycles of cell infusion, with no adverse events observed. Furthermore, overall survival of 5 patients were over 15 month and showed a potentially promising prolonged overall survival time compared to patients who received general chemo therapies (22).

2.3 Utilizing autologous iNKT cells for CAR-NKT cell therapy

Chimeric antigen receptor T-cell (CAR T-cell) therapy is one of the major immunotherapeutic options for certain cancer patients and has shown remarkable success for hematological malignancies. CAR consists of an extracellular antigen recognition domain derived from a monoclonal antibody, which recognizes a specific antigen expressed on cancer cells. CAR-NKT cell therapy has been developed and a phase I/II clinical trial of therapy with GD2 CAR NKT cells expressing IL-15 has been performed (NCT03294954) (23, 24). NKT cells with median purity of 93% were retrovirally transduced with GD2 CAR and IL-15 with median 60.1% efficiency. While this study is ongoing, preliminary results indicate a 25% response rate among neuroblastoma patients (n=12) who received this therapy, with one patient demonstrating a complete response, affirming the effective trafficking of CAR-NKT cells to the tumor site.

Other clinical trials of autologous NKT cell infusion targeting NSCLC, renal cell carcinoma, and melanoma have been registered at ClinicalTrials.gov, although no results have been reported yet (e.g., NCT02562963, NCT03198923, NCT06182735, NCT02619058).

3 Allogeneic iNKT cell transfer

In recent years, adoptive transfer of allogeneic iNKT cells has been developed as a new therapeutic tool to treat cancer patients in parallel with autologous iNKT cell therapy. There are at least three advantages of using allogeneic iNKT cells compared with autologous iNKT cells in cell therapy (Table 1). First, the use of allogeneic iNKT cells enables the development of off-the-shelf therapy. Off-the-shelf therapy can reduce the cost and streamlines the process, shortening the time from cell preparation to shipment, since there is no need to customize or individualize the preparation of cells. Second, the use of allogeneic iNKT cells allows the simple preparation of a sufficient number of iNKT cells. It has been reported that the proportion of iNKT cells in the peripheral blood of cancer patients is decreased compared with that in healthy donors (14–16), so obtaining enough autologous iNKT cells for treatment has often been challenging. Meanwhile, the expansion of iNKT cells derived from healthy donors is relatively easy. Moreover, cord blood, which is comparatively accessible and contains a large number of T cells, can be used as a source of allogeneic iNKT cells. Third, allogeneic iNKT cells derived from healthy donors enable the avoidance of immune exhaustion. T cells in the tumor microenvironment are exhausted, as characterized by the expression of PD-1, TIM-3, and LAG-3, while iNKT cells derived from a healthy donor are expected to be less so (25). In addition, TCR expressed on iNKT cells, unlike TCR expressed on conventional T cells, does not recognize antigens in an MHC-restricted manner, which is a significant advantage as it reduces the risk of developing graft-versus-host disease (GVHD).

Meanwhile, a potential disadvantage of using allogeneic iNKT cells is shorter persistence, compared with that of autologous iNKT cells after infusion into the host, because of host immune responses of allogeneic reaction.

3.1 Utilizing allogeneic iNKT cells from healthy donors

MINK Therapeutics developed the protocol of allogeneic iNKT cell expansion for off-the-shelf therapy to use in clinical trials (26). Briefly, iNKT cells isolated from healthy donors were expanded under stimulation with α-GalCer in the presence of cytokines. The function of these cells was confirmed by cytotoxicity assay and cytokine production against a tumor cell line. Unmodified allogeneic iNKT cells derived from healthy donors were stimulated with α-GalCer in the presence of cytokines for

expansion and adoptively transferred to 34 solid tumor patients, including those with NSCLC, pancreatic, rectal, cholangiocarcinoma/biliary duct, and other cancers (NCT05108623) (27). Whereas allogeneic iNKT cells were expected to be eliminated via an allogeneic reaction by host immune cells, peripheral allogeneic iNKT cells were detected at a consistent level up to 8 weeks after the adoptive transfer. The combination of allogeneic iNKT cell therapy with pembrolizumab or nivolumab, both of which are immune check point inhibitors targeting programmed cell death 1 receptor (PD-1), was also examined in six patients, along with monotherapy of allogeneic iNKT cell transfer in 26 patients (28). The study suggested that both this monotherapy and these two combination therapies were well tolerated. Notably, one patient with gastric cancer who was resistant to nivolumab treatment showed a partial response, while increased immune infiltration, activation, and Th-1 polarization were observed after iNKT cell infusion (29).

Other combination therapies with allogeneic iNKT cell transfer have also been performed in China. Yu et al. reported on a phase I/II clinical trial targeting non-small-cell lung cancer patients. NSCLC patients bearing epidermal growth factor receptor (EGFR) mutation received allogeneic iNKT cell infusion along with gefitinib, an EGFR inhibitor (30). Thirty patients were randomly assigned to receive either monotherapy of gefitinib or combination therapy of gefitinib with allogeneic iNKT cell infusion. One EGFR mutation-positive NSCLC patient who received this combination therapy showed a delay in the development of molecular-targeted drug resistance with no adverse events (31).

iNKT cells play an important role not only for fighting tumor cells but also for clearing bacterial and viral infections. A phase I/II clinical trial of allogeneic iNKT cell therapy for treating acute respiratory distress syndrome (ARDS) patients secondary to SARS-CoV-2 infection, has been performed (n=20) (NCT04582201) (32). That study showed the safety of treatment

TABLE 2 Clinical trials of iNKT cell adoptive transfer.

References	Source	Expansion methods from PBMCs	Purity of iNKT cells (%)	Type of cancer	Number of patients	Phase	Combination
(9)	Autologous	▼expansion (+ α-GalCer, IL-2)	0.1-25	NSCLC	6	I	
(10)			1-59	HNC	8	I	α-GalCer-APCs
(11)			0.1-6.2	HNC	10	II	α-GalCer-APCs
(17)		▼sorting iNKT cells ▼expansion (+anti-CD3, IL-2)	13-87	Melanoma	9	I	
(18)		▼expansion (+α-GalCer, IL-2) ▼sorting iNKT cells ▼coculture (+autologous DCs)	85-95	HCC	10	I	
(19)			N/A	HCC	27 27 (Ctrl)	II	Trans-arterial embolization
(20)		▼expansion (+α-GalCer) ▼+IL-2, IL-7 ▼coculture (+autologous DCs) ▼+IL-12 ▼+IL-15	13-88	NSCLC	3	I/II	$PD1^+CD8^+$ T cells
(21)			5-65	PC	9	I	$PD1^+CD8^+$ T cells
(23, 24)		▼sorting iNKT cells ▼expansion (+irradiated PBMCs, α-GalCer, IL-2, IL-21) ▼transducing GD2-CAR-IL-15	74-97	NB	12	I	
(27)	Allogeneic	▼sorting iNKT cells ▼expansion (+irradiated PBMCs, α-GalCer, IL-2)	>99	Solid tumor	34	I	
(28)			>99	Solid tumor	26 6 (Combo)	I	Pembrolizumab or Nivolumab
(30)		N/A	>20	NSCLC	15 15 (Ctrl)	I/II	Gefitinib
(33)		▼transducing CD19-CAR-IL-15, HLA ablation	N/A	NHL, ALL	9	I	
N/A		▼expansion (+α-GalCer, IL-2) ▼expansion (+mouse DCs, IL-2, IL-7, IL-15) ▼inducing iPS cell-derived NKT cells	>80	HNC	10	I	

NSCLC, non-small cell lung cancer; HNC, head and neck cancer; PC, pancreatic cancer; NB, neuroblastoma; NHL, non-Hodgkin lymphoma; ALL, acute lymphoblastic leukemia; N/A, not available; iPS, induced pluripotent stem; Ctrl, control.

without toxicities and the persistence of allogeneic iNKT cells in patients infused with iNKT cells. They also showed that injected allogeneic iNKT cells rescued exhausted T cells and killed M2 macrophages promoting infection. Infused iNKT cells induced an anti-inflammatory systemic response, which contributed to the stabilization of ARDS.

In addition to combination therapy, CAR therapy using allogeneic iNKT cells has been investigated. Ramos et al. performed clinical studies to examine allogeneic iNKT cells transduced with CD19 CAR along with shRNA targeting β2 microglobulin and CD74, which downregulate HLA class I and class II and thereby avoid elimination by host immune responses via an allogeneic reaction. Seven patients with relapsed/refractory B-cell non-Hodgkin lymphoma (NHL) and two patients with relapsed B-cell lymphoblastic leukemia (ALL) were enrolled (NCT00840853) (33). In that study, two NHL patients and one ALL patient exhibited complete responses, suggesting that allogeneic CAR iNKT cells induce strong anti-tumor immunity.

3.2 Utilizing allogeneic iPS cell-derived iNKT cells

Induced pluripotent stem cells (iPSCs) are reprogrammed adult cells that regain pluripotency, allowing them to differentiate into various cell types, including immune cells. Yamada et al. established techniques to generate iPSC-NKT cells from adult iNKT cells and confirmed that iPSC-NKT cells possess anti-tumor activity matching that of the primary iNKT cells (34). The iPSC technique offers a significant advantage by enabling the mass expansion of a limited number of iNKT cells. Based on these results, we are currently conducting the first-in-human clinical trial using allogeneic iPSC-iNKT cells as a monotherapy for treating head and neck cancer patients (jRCT2033200116). iPSC-NKT cells derived from HLA-mismatched donors were infused into patients with advanced HNC to examine the safety and clinical efficacy of this treatment. The safety of administrating high doses of iPSC-NKT cells has been confirmed so far.

4 Discussion

iNKT cell-based immunotherapy has advanced dramatically over the last 20 years, with new clinical trials, including combination therapies, continually emerging. The use of allogeneic iNKT cells for such therapy was a particular breakthrough, overcoming the key weakness of iNKT cells of a low cell number and further accelerating clinical trials with greater potential. Both autologous and allogeneic iNKT cell transfer have advantages and advantages as listed in Table 1. In all of the above-mentioned clinical trials on the adoptive transfer of either autologous and allogeneic iNKT cells, safety and a certain level of immunological responses with some clinical efficacy were confirmed, suggesting the promise of these treatment options for cancer patients (Table 2). In a pre-clinical study using murine models, Li et al. reported that allogeneic CAR-iNKT cells derived from hematopoietic stem cells became resistant to host cell-mediated allorejection by editing the HLA gene (35). They recently improved the CAR-iNKT cell culture method using feeder free system and brought the technology closer to clinical application (36). In addition, Heczey et al. showed that knocking down BTG proliferation factor 1 (BTG1) rescued the hyporesponsiveness of CAR-NKT cells (24). These findings can be applied for the next step of new iNKT-cell-based therapy to enhance the anti-tumor function and persistence of iNKT cells. These gene editing techniques hold the potential to significantly enhance the efficacy of iNKT cell-based immunotherapy, thereby improving outcomes in the fight against various types of cancers.

Author contributions

MT: Conceptualization, Data curation, Investigation, Writing – original draft. SM: Supervision, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

We thank Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

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

References

1. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1+ T lymphocytes. *Science*. (1995) 268:863–5. doi: 10.1126/science.7538697

2. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science*. (1997) 278:1626–9. doi: 10.1126/science.278.5343.1626

3. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol*. (2003) 21:483–513. doi: 10.1146/annurev.immunol.21.120601.141057

4. Taniguchi M, Seino K, Nakayama T. The NKT cell system: bridging innate and acquired immunity. *Nat Immunol*. (2003) 4:1164–5. doi: 10.1038/ni1203-1164

5. Rossjohn J, Pellicci DG, Patel O, Gapin L, Godfrey DI. Recognition of CD1d-restricted antigens by natural killer T cells. *Nat Rev Immunol*. (2012) 12:845–57. doi: 10.1038/nri3328

6. Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, et al. A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res*. (2005) 11:1910–7. doi: 10.1158/1078-0432.CCR-04-1453

7. Motohashi S, Nagato K, Kunii N, Yamamoto H, Yamasaki K, Okita K, et al. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol*. (2009) 182:2492–501. doi: 10.4049/jimmunol.0800126

8. Nagato K, Motohashi S, Ishibashi F, Okita K, Yamasaki K, Moriya Y, et al. Accumulation of activated invariant natural killer T cells in the tumor microenvironment after alpha-galactosylceramide-pulsed antigen presenting cells. *J Clin Immunol*. (2012) 32:1071–81. doi: 10.1007/s10875-012-9697-9

9. Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, et al. A phase I study of in *vitro* expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res*. (2006) 12:6079–86. doi: 10.1158/1078-0432.CCR-06-0114

10. Uchida T, Horiguchi S, Tanaka Y, Yamamoto H, Kunii N, Motohashi S, et al. Phase I study of alpha-galactosylceramide-pulsed antigen presenting cells administration to the nasal submucosa in unresectable or recurrent head and neck cancer. *Cancer Immunol Immunother*. (2008) 57:337–45. doi: 10.1007/s00262-007-0373-5

11. Kunii N, Horiguchi S, Motohashi S, Yamamoto H, Ueno N, Yamamoto S, et al. Combination therapy of in *vitro*-expanded natural killer T cells and alpha-galactosylceramide-pulsed antigen-presenting cells in patients with recurrent head and neck carcinoma. *Cancer Sci*. (2009) 100:1092–8. doi: 10.1111/j.1349-7006.2009.01135.x

12. Yamasaki K, Horiguchi S, Kurosaki M, Kunii N, Nagato K, Hanaoka H, et al. Induction of NKT cell-specific immune responses in cancer tissues after NKT cell-targeted adoptive immunotherapy. *Clin Immunol*. (2011) 138:255–65. doi: 10.1016/j.clim.2010.11.014

13. Toyoda T, Kamata T, Tanaka K, Ihara F, Takami M, Suzuki H, et al. Phase II study of alpha-galactosylceramide-pulsed antigen-presenting cells in patients with advanced or recurrent non-small cell lung cancer. *J Immunother Cancer*. (2020) 8. doi: 10.1136/jitc-2019-000316

14. Motohashi S, Kobayashi S, Ito T, Magara KK, Mikuni O, Kamada N, et al. Preserved IFN-alpha production of circulating Valpha24 NKT cells in primary lung cancer patients. *Int J Cancer*. (2002) 102:159–65. doi: 10.1002/ijc.10678

15. Kawano T, Nakayama T, Kamada N, Kaneko Y, Harada M, Ogura N, et al. Antitumor cytotoxicity mediated by ligand-activated human V alpha24 NKT cells. *Cancer Res*. (1999) 59:5102–5.

16. Crough T, Purdie DM, Okai M, Maksoud A, Nieda M, Nicol AJ. Modulation of human Valpha24(+)Vbeta11(+) NKT cells by age, Malignancy and conventional anticancer therapies. *Br J Cancer*. (2004) 91:1880–6. doi: 10.1038/sj.bjc.6602218

17. Exley MA, Friedlander P, Alatrakchi N, Vriend L, Yue S, Sasada T, et al. Adoptive transfer of invariant NKT cells as immunotherapy for advanced melanoma: A phase I clinical trial. *Clin Cancer Res*. (2017) 23:3510–9. doi: 10.1158/1078-0432.CCR-16-0600

18. Gao Y, Guo J, Bao X, Xiong F, Ma Y, Tan B, et al. Adoptive transfer of autologous invariant natural killer T cells as immunotherapy for advanced hepatocellular carcinoma: A phase I clinical trial. *Oncologist*. (2021) 26:e1919–30. doi: 10.1002/onco.13899

19. Guo J, Bao X, Liu F, Guo J, Wu Y, Xiong F, et al. Efficacy of invariant natural killer T cell infusion plus transarterial embolization vs transarterial embolization alone for hepatocellular carcinoma patients: A phase 2 randomized clinical trial. *J Hepatocell Carcinoma*. (2023) 10:1379–88. doi: 10.2147/JHC.S416933

20. Cheng X, Wang J, Qiu C, Jin Y, Xia B, Qin R, et al. Feasibility of iNKT cell and PD-1+CD8+ T cell-based immunotherapy in patients with lung adenocarcinoma: Preliminary results of a phase I/II clinical trial. *Clin Immunol*. (2022) 238:108992. doi: 10.1016/j.clim.2022.108992

21. Wang J, Cheng X, Jin Y, Xia B, Qin R, Zhang W, et al. Safety and clinical response to combined immunotherapy with autologous iNKT cells and PD-1(+)CD8(+) T cells in patients failing first-line chemotherapy in stage IV pancreatic cancer. *Cancer Res Commun*. (2023) 3:991–1003. doi: 10.1158/2767-9764.CRC-23-0137

22. Nichetti F, Rota S, Ambrosini P, Pircher C, Gusmaroli E, Droz Dit Busset M, et al. NALIRIFOX, FOLFIRINOX, and gemcitabine with nab-paclitaxel as first-line chemotherapy for metastatic pancreatic cancer: A systematic review and meta-analysis. *JAMA Netw Open*. (2024) 7:e2350756. doi: 10.1001/jamanetworkopen.2023.50756

23. Heczey A, Courtney AN, Montalbano A, Robinson S, Liu K, Li M, et al. Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: an interim analysis. *Nat Med*. (2020) 26:1686–90. doi: 10.1038/s41591-020-1074-2

24. Heczey A, Xu X, Courtney AN, Tian G, Barragan GA, Guo L, et al. Anti-GD2 CAR-NKT cells in relapsed or refractory neuroblastoma: updated phase 1 trial interim results. *Nat Med*. (2023) 29:1379–88. doi: 10.1038/s41591-023-02363-y

25. Thommen DS, Schumacher TN. T cell dysfunction in cancer. *Cancer Cell*. (2018) 33:547–62. doi: 10.1016/j.ccell.2018.03.012

26. Yigit B, Michelet X, Kharkwal S, Chantzoura E, Bergmann J, Yague AS, et al. Agent-797, a novel allogeneic and 'off-the shelf' inkt cell therapy promotes effective tumor killing. *J Immunother Cancer*. (2020) 8. doi: 10.1136/jitc-2020-SITC2020.0164

27. Moon J, Ryu S, Ramos IR, Garmezy B, Hamm J, Carneiro B, et al. Peripheral and tissue persistence of agent-797, an allogeneic inkt cell-based cell therapy for the treatment of cancer. *J Immunother Cancer*. (2023) 11. doi: 10.1136/jitc-2023-SITC2023.0735

28. Carneiro B, Garmezy B, Hamm HT, Sanborn RE, Draper TW, Khoueiry AE, et al. Phase 1 clinical update of allogeneic invariant natural killer T cells (iNKTs), agenT-797, alone or in combination with pembrolizumab or nivolumab in patients with advanced solid tumors. *Cancer Res*. (2023) 83 doi: 10.1158/1538-7445.am2023-ct275

29. Hadfield MJ, Safran H, Purbhoo MA, Grossman JE, Buell JS, Carneiro BA. Overcoming resistance to programmed cell death protein 1 (PD-1) blockade with allogeneic invariant natural killer T-cells (iNKT). *Oncogene*. (2024) 43:758–62. doi: 10.1038/s41388-024-02948-y

30. Yu W, Ye F, Yuan X, Ma Y, Mao C, Li X, et al. A phase I/II clinical trial on the efficacy and safety of NKT cells combined with gefitinib for advanced EGFR-mutated non-small-cell lung cancer. *BMC Cancer*. (2021) 21:877. doi: 10.1186/s12885-021-08590-1

31. Yu W, Yuan X, Ye F, Mao C, Li J, Zhang M, et al. Role of allogeneic natural killer T cells in the treatment of a patient with gefitinib-sensitive lung adenocarcinoma. *Immunotherapy*. (2022) 14:1291–6. doi: 10.2217/imt-2022-0178

32. Hammond TC, Purbhoo MA, Kadel S, Ritz J, Nikiforow S, Daley H, et al. A phase 1/2 clinical trial of invariant natural killer T cell therapy in moderate-severe acute respiratory distress syndrome. *Nat Commun*. (2024) 15:974. doi: 10.1038/s41467-024-44905-z

33. Ramos PD, Courtney AN, Lulla PD, Hill LC, Kamble RT, Carrum G, et al. S, off-the-shelf CD19-specific CAR-NKT cells in patients with relapsed or refractory B-cell Malignancies. *Transplant Cell Ther*. (2024) 30:S41–2. doi: 10.1016/j.jtct.2023.12.072

34. Yamada D, Iyoda T, Vizcardo R, Shimizu K, Sato Y, Endo TA, et al. Efficient regeneration of human valpha24(+) invariant natural killer T cells and their anti-tumor activity in vivo. *Stem Cells*. (2016) 34:2852–60. doi: 10.1002/stem.2465

35. Li YR, Zhou Y, Kim YJ, Zhu Y, Ma F, Yu J, et al. Development of allogeneic HSC-engineered iNKT cells for off-the-shelf cancer immunotherapy. *Cell Rep Med*. (2021) 2:100449. doi: 10.1016/j.xcrm.2021.100449

36. Li YR, Zhou Y, Yu J, Zhu Y, Lee D, Zhu E, et al. Engineering allorejection-resistant CAR-NKT cells from hematopoietic stem cells for off-the-shelf cancer immunotherapy. *Mol Ther*. (2024) 32: 1849–74. doi: 10.1016/j.ymthe.2024.04.005

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 22 August 2024
DOI 10.3389/fimmu.2024.1462209

OPEN ACCESS

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RECEIVED 09 July 2024
ACCEPTED 05 August 2024
PUBLISHED 22 August 2024

CITATION
Szoke-Kovacs R, Khakoo S, Gogolak P and Salio M (2024) Insights into the CD1 lipidome. *Front. Immunol.* 15:1462209. doi: 10.3389/fimmu.2024.1462209

Insights into the CD1 lipidome

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CD1 isoforms are MHC class I-like molecules that present lipid-antigens to T cells and have been associated with a variety of immune responses. The lipid repertoire bound and presented by the four CD1 isoforms may be influenced by factors such as the cellular lipidome, subcellular microenvironment, and the properties of the binding pocket. In this study, by shotgun mass spectrometry, we performed a comprehensive lipidomic analysis of soluble CD1 molecules. We identified 1040 lipids, of which 293 were present in all isoforms. Comparative analysis revealed that the isoforms bind almost any cellular lipid.CD1a and CD1c closely mirrored the cellular lipidome, while CD1b and CD1d showed a preference for sphingolipids. Each CD1 isoform was found to have unique lipid species, suggesting some distinct roles in lipid presentation and immune responses. These findings contribute to our understanding of the role of CD1 system in immunity and could have implications for the development of lipid-based therapeutics.

KEYWORDS

CD1, antigen presentation, self-lipid antigens, lipid mass-spectrometry, lipidome

Introduction

The Cluster of Differentiation 1 molecules (CD1a, CD1b, CD1c, and CD1d) are Major Histocompatibility Complex (MHC) class I-like molecules that bind endogenous and exogenous lipids (1). They can activate subsets of T cells by presenting lipids on the cell surface of antigen-presenting cells. Depending on their ability to activate adaptive or innate-like T cells, CD1 molecules are divided into group 1 (CD1a, b, c) and group 2 (CD1d), respectively. A fifth molecule, CD1e, is located in the endo-lysosomes and assists lipid loading on CD1b (2). The most well-studied innate-like CD1-restricted T cells are the invariant Natural Killer T cells (iNKT), restricted to CD1d (3). Some CD1-restricted T lymphocytes recognize exogenous microbial lipids and lipopeptide antigens presented by group 1 CD1 molecules (4), while others recognize self glycosphingolipids (5, 6). Additionally, some CD1-restricted T-cell clones have been found to exhibit a dual response to both self- and microbial antigens (7). By virtue of their ability to modulate dendritic cell (DC) function, CD1-restricted T cells may play a role in both the early and late stages of the immune response (8). Recognition of microbial derived lipid antigens through CD1 molecules suggests a function of CD1-restricted T cells in anti-microbial immunity (9). Conversely, recognition of CD1-associated self-lipids is important in maintaining tissue homeostasis, and dysregulation contributes to autoimmunity (10).

Little is known about what determines the lipid repertoire captured by the different CD1 isoforms. Like classical MHC-I, CD1 molecules are synthesized in the endoplasmic reticulum, assisted by several chaperones (11, 12), and are loaded with self-lipids while they traffic to the cell surface. They are subsequently recycled through the endocytic pathway, depending on their cytoplasmic motifs (13), which might enable them to come into contact with unique lipid antigens (13). CD1a molecules primarily recycle within the early endosomes (14). This isoform has the smallest binding pocket which allows for the binding of small lipids, often from the extracellular milieu (4). CD1b molecules traffic through all endo-lysosomal compartments to the lysosomes (15, 16). CD1b has the largest binding pocket, allowing the binding of larger lipids, such as complex mycobacterial antigens (17). CD1c and CD1d both have intermediate-size binding grooves and follow similar trafficking routes, reaching the late endosomes (18, 19).

The cellular lipidome, subcellular microenvironment and the properties of the binding pocket influence the lipid repertoire bound and presented by the different CD1 isoforms. Earlier studies have characterized lipids bound to individual soluble or cleavable CD1d or CD1c molecules (20–25). Recently, using a non-targeted approach based on high-resolution mass spectrometry, Huang et al. investigated the lipidome of the four CD1 isoforms and provided a comprehensive map of self-lipid display (24, 26).

In this paper, to understand the diversity of the CD1 lipidome, we used a targeted approach, shotgun mass spectrometry, which allows the precise identification and quantification of a large number of lipids (27). Shotgun lipidomics detects the most abundant lipid contained in a sample and identifies the targeted lipids with the use of internal standards (28). In addition, this fast and highly sensitive method produces highly accurate and replicable results using minimal sample quantities (29).

We focused our investigation on soluble CD1 molecules, as the recent work by Huang et al. (26) demonstrated a significant overlap between the lipidomes eluted from recycling and non-recycling CD1a and CD1b molecules, pointing to recycling as a way to present exogenous antigens. Similar results were also observed comparing the lipidome of recycling and non-recycling murine CD1d molecules (21).

We observed that the four CD1 isoforms share a significant amount of the cellular lipidome while deviating in their preference towards certain lipid species and chain lengths. Capture of unique lipid species by each CD1 isoform implies some level of specialization in lipid presentation and specific roles during immune responses: CD1a prefers mid-sized lipids, CD1c binds a wide range of lipids including gangliosides, and CD1d uniquely captures cholesterol. These findings provide insights into lipid-antigen presentation by the CD1 molecules and help to understand how these molecules contribute to immune responses in health and disease.

Materials and methods

Molecular cloning and expression of CD1 molecules

The human HLA-A*02:01, CD1a (NM_001763), CD1b (NM_001764.3), CD1c (NM_001765.3) and CD1d (NM_001766.3) protein α1, α2 and α3 domains were linked to the human β2-microgobulin (β2m) via a glycine-serine linker (GGGGSGGSGSGGGSS) followed by a rigid peptide linker (PPTPSTPPT), linked C-terminal Avi-Tag™ and 6xHis tag (Supplementary Figure 1). They were synthesized as a single chain construct by GeneArt Gene Synthesis (Invitrogen) and subcloned into pCDNA3.1 episomal expression vector (Invitrogen), with expression driven by the IL-2 leader sequence. The pCDNA3.1 vector was engineered to co-express the BirA enzyme for biotinylation of the recombinant CD1 protein on the AviTag (Supplementary Figure 1). Expi293F cells (Thermo Fisher Scientific A14527) were cultivated in Expi293™ Expression Medium (Thermo FisherA1435101) and were transfected with 1μg/ml plasmid following the ExpiFectamine™ 293 Transfection Kit protocol (Life Technologies A14636). Biotinylated CD1 monomers loaded with endogenous lipids (CD1-endo) were expressed and secreted into the cell medium for five days post-transfection. They were then purified by Ni-Affinity chromatography on HisTrap excel (5ml) column (Cytiva 17371205) followed by Size Exclusion chromatography on Superdex200 increase column (Cytiva 28990944). The lipidome of the tissue culture medium is not published and we did not assess it experimentally. We cannot rule out that lipid exchange could have happened after secretion of the CD1 moleucles, however we believe high concentrations of lipids and low pH would be needed for passive ligand exchange.

Lipid extraction for mass spectrometry lipidomics

Mass spectrometry-based lipid analysis of purified molecules was performed by Lipotype GmbH (Dresden, Germany) as described (30). Lipids were extracted using a two-step chloroform/methanol procedure (31). Gangliosides were extracted from the water phase of the preceding chloroform/methanol extraction with a solid-phase extraction protocol. Blank elution buffer was used as background control. Before extraction, samples were spiked with an internal lipid standard mixture containing: cardiolipin 14:0-14:0-14:0-14:0 (CL), ceramide 18:1;2-17:0 (Cer), diacylglycerol 17:0-17:0 (DAG), hexosylceramide 18:1;2-12:0 (HexCer), dihexosylceramide 18:1;2-12:0 (DiHexCer), Globoside 3 18:1;2-17:0 (Gb3), GM3-d3 18:1;2-18:0 (GM3), GM1-D3 18:1;2-18:0 (GM1), lyso-phosphatidate 17:0 (LPA), lyso-phosphatidylcholine 12:0 (LPC), lyso-phosphatidylethanolamine 17:1 (LPE), lyso-phosphatidylglycerol 17:1 (LPG), lyso-phosphatidylinositol 17:1 (LPI), lyso-phosphatidylserine 17:1 (LPS), phosphatidate 17:0-17:0 (PA), phosphatidylcholine 17:0-17:0 (PC), phosphatidylethanolamine 17:0-17:0 (PE), phosphatidylglycerol 17:0-17:0 (PG), phosphatidylinositol 16:0-16:0 (PI), phosphatidylserine 17:0-17:0 (PS), cholesterol ester 16:0 (CE), sphingomyelin 18:1;2-12:0;0 (SM), sulfatide 18:1;2-12:0;0 (Sulf), triacylglycerol 17:0-17:0-17:0 (TAG) and cholesterol D6 (Chol). To extract lipids from Expi293F cells, a mix of internal lipid standards was added to 1 million cells in 300μl of PBS. After extraction, the organic phase was transferred to an infusion plate

and dried in a speed vacuum concentrator. The first step dry extract was re-suspended in 7.5 mM ammonium acetate in chloroform/methanol/propanol (1:2:4; V:V:V) and the second step dry extract in 33 percent ethanol solution of methylamine in chloroform/methanol (0.003:5:1; V:V:V). All liquid handling steps were performed using the Hamilton Robotics STARlet robotic platform with the Anti Droplet Control feature for organic solvent pipetting.

Lipid identification and quantification by shotgun lipidomics

After drying and resuspension in MS acquisition mixture, lipid extracts were subjected to mass spectrometric analysis. To overcome possible MS limitations of detection due to high abundant species dominating the spectrum and reducing low abundant species with similar m/z ratio to noise, samples were analysed in replicates at high and low concentration. Two replicates of CD1 protein were analyzed in 100µg and 500µg quantities from the same protein batch. These concentrations were chosen based on an initial feasibility experiment conducted by Lipotype GmbH, where the optimal amount of 500µg of CD1 protein were identified for mass-spectrometry lipidomics. The additional 100µg replicates were analyzed to identify occurrent lipid species masked by more abundant species in the 500µg replicate. Samples were analysed by direct infusion on the QExactive hybrid quadrupole/Orbitrap mass spectrometer (Thermo Scientific) equipped with the TriVersa NanoMate automated nano-flow electrospray ion source (Advion Biosciences). All samples were analysed in both positive and negative ion modes with a resolution of $R_{m/z\ 200}$ = 280000 for MS and $R_{m/z\ 200}$ = 17500 for MS/MS experiments, in a single acquisition. MS/MS was triggered by an inclusion list encompassing corresponding MS mass ranges scanned in 1 Da increments (32). Both MS and MS/MS data were combined to monitor CE, Chol, DAG and TAG ions as ammonium adducts; PC and PC O-, as acetate adducts; and CL, PA, PE, PE O-, PG, PI and PS as deprotonated anions. MS only was used to monitor Gb3, Gb4, GM4, GM3, GM2, GM1, GD3, GD2, GD1, GT3, GT2, GT1, GQ1, Sulf, LPA, LPE, LPE O-, LPI and LPS as deprotonated anions, and Cer, HexCer, DiHexCer, SM, LPC and LPC O- as acetate adducts. The list of analysed lipid classes in MS and MS/MS mode can be found in supplementary data (Supplementary Table 1).

Data analysis and post-processing

Data were analysed with a lipid identification software developed by Lipotype, based on LipidXplorer (33, 34). Lipid identification using LipotypeXplorer was performed on unprocessed (*.raw format) mass spectra. For MS-only mode, lipid identification was based on the molecular masses of the intact molecules. MS/MS mode included the collision-induced fragmentation of lipid molecules and lipid identification was based on both the intact masses and the masses of the fragments. Prior to normalization and further statistical analysis performed by Lipotype GmbH, lipid identified were filtered according to mass accuracy, occupation threshold, noise, and background. The lists of identified lipids and their intensities were stored in a database optimized for the particular structure inherent to lipidomic datasets. The intensity of lipid class-specific internal standards was used for lipid quantification. Data post-processing and normalization were performed at Lipotype using an in-house developed data management system. Only lipid identified with a signal-to-noise ratio >5, and a signal intensity 5-fold higher than in corresponding blank samples were considered for further data analysis. The dynamic range for cell culture samples was determined prior to analysis (30). Based on these data, limits of quantification and coefficients of variation for the different lipid classes were determined. Limits of quantification were in the lower µM to sub-µM range, depending on the lipid class. The average coefficient of variation for a complete set of quantified lipid classes was around 10-15%. Each analysis was accompanied by a set of blank samples to control for a background and a set of quality control reference samples to control for intra-run reproducibility and sample specific issues. The identified lipid molecules were quantified by normalization to a lipid class-specific internal standard.

The amounts in pmoles of individual lipid molecules (species or subspecies) of a given lipid class were summed to yield the total amount of the lipid class. The amounts of the lipid classes were normalized to the total lipid amount yielding mol% per total lipids. For lipid classes that were analysed semi-quantitatively, peak intensities were normalized to the intensity of an internal standard which did not belong to the respective lipid class (normalized intensities). Additionally, normalized intensities were further standardized to total lipid content of each sample (normalized relative abundance).

We next developed an R workflow to analyse the data received from Lipotype using R version R-4.3.2. The source code is available on GitHub at: https://github.com/ritaszokekovacs/CD1-lipidome17052024.git. The R-script has functionalities for data transformation, data exploration using unsupervised learning and lipid enrichment analysis. We performed an unsupervised clustering analysis to evaluate the structure of complex data with consideration for the connections between variables. Principal component analysis (PCA) was used for exploratory data analysis. Ggfortify (0.4.17) ggcorrplot (0.1.4.1), corrr (0.4.4), FactoMineR (2.11) and factoextra (1.0.7) packages were used for PCA analysis. We generated Venn Diagrams using the ggVennDiagram (1.5.2) package. To achieve consistent visualization, plots were generated with ggplot2 (3.5.0) and viridis (0.6.5), a colour palette robust to colour blindness and greyscale printing, whenever possible.

Data filtering and HLA-A*02:01 background

With only 81 features, which is less than 6% of the total features detected from HLA-A*02:01 controls, we assumed that the lipids detected in the CD1 samples are likely eluted from the lipid-binding groove (Supplementary Figure 2).

Results

Analysis of the lipid profiles eluted from CD1molecules: the overlapping lipidome of CD1 isoforms

We expressed four CD1 isoforms (CD1a, CD1b, CD1c and CD1d) in mammalian, Expi293F cells as soluble molecules, a format allowing detergent-free protein purification to avoid the loss of lipid-ligands. We also expressed the classical antigen-presenting molecule HLA-A*02:01 in the same cell line and analysed the lipid content eluted from it as a negative control. HLA-A*02:01 is similar in size and possesses a similar structure as CD1 molecules. However, HLA-A*02:01 captures and presents peptide antigens instead of lipids, representing a good control for non-specific binding. After affinity and size exclusion purification of the soluble molecules from the cell supernatants, lipids were eluted and profiled by mass spectrometry as described in the methods.

We identified a total of 1040 lipids belonging to 39 classes, of which 29 at species level, and 10 at subspecies level in MS/MS mode (Table 1). As an overview of the data and to convey the most variation in the dataset, we performed a principal component analysis (PCA) on the lipid dataset, standardized to mol percentage (Figure 1A). We observed the largest variation in the CD1a lipidome. The lipid species that had the most influence on the principal component analysis were PC(16:0;0-18:1;0), PC(18:1;0-18:1;0), PC(16:0;0-16:1;0), SM(34:1;2), PE(18:1;0-18:1;0) and GM(34:1;2).

The different lipid classes were non-uniformly represented across the four CD1 isoforms, with a preference of phospholipids for CD1a, gangliosides for CD1b, phosphatidylcholine for CD1c and HexCer and sphingomyelin for CD1d (Supplementary Figure 3). We therefore first evaluated the lipidomic overlap between the CD1 isoforms and divided the lipids into bins according to the occurrence of each feature. We observed a high lipidomic overlap between the CD1 isoforms (Figure 1B), suggesting broad sampling of the cellular lipidome, in agreement with recent data (26). Of the total 1040 lipids identified in the CD1 lipidome, 293 were present in all CD1 isoforms. Each isoform shared a similar level of overlap, with an average 366 shared lipids between any two isoforms; we observed the largest shared lipidome (474 species) between CD1a and CD1d molecules (Table 1; Figure 1B).

We next compared the classes of the 293 lipids shared between CD1 isoforms with the total cellular lipidome of Expi293F cells, and we found that – with some exceptions - the CD1 isoforms remarkably bound almost every cellular lipid species (Figure 2).

TABLE 1 CD1-associated ligands.

Isotype	Unique	Total
CD1a	128	648
CD1b	25	428
CD1c	33	487
CD1d	57	605

1040 lipid species were identified from the CD1 lipidome of which 293 were present in all CD1 isoforms.

However, while the profiles of the CD1a and CD1c lipidome were close to the cellular background, with the main enrichment found in phospholipids, the lipid profiles of CD1b and CD1d diverged: in addition to the dominant PC species, an enrichment in sphingolipid classes and sphingomyelin (SM) was also observed.

The common features present in all CD1 isoforms were 12 Ceramides (Cer), 15 diacylglycerols (DAG), 3 hexosylceramides (HexCer), 4 lyso-phosphatidyl-cholines (LPC), 4 lyso-phosphatidyl-ethanolamines (LPE), 3 ether-linked lyso-phosphatidyl-ethanolamines (LPE O-), 74 phosphatidylcholines (PC), 68 ether-linked Phosphatidylcholines (PC O-), 34 phosphatidylethanolamines (PE), 59 ether-linked phosphatidylethanolamines (PE O-), 7 sphingomyelins (SM), 4 Sulfatide species 1 phosphatidylglycerol PG (16:0;0-18:0;0) and 1 monosialodihexosylganglioside GM3 (34:1;2).

To further understand the correlation between cellular abundance and capture by CD1 isoform (normalised to mol%), we restricted the analysis to the nine most abundant lipid classes (Figure 3). PC and ether-PC (PC O-) were present in all isoforms (Figures 3A, B), however, PE and ether-PE (PE O-) were under-captured by most isoforms (Figures 3C, D). Moderate levels of SM were captured by most isoforms, with the exception of CD1a (Figure 3E) (35). HexCer was only moderately over-captured by CD1d (Figure 3F). GM3, a headed sphingolipid was enriched 10-fold in CD1b and CD1c and 26-fold in CD1d (Figure 3G). Headless lipids that don't protrude from the CD1 binding pockets like ceramides, were enriched ~2-3 fold in CD1 a, b and c and 9-fold in CD1d (Figure 3H). DAG was enriched 3-fold in CD1a and 6-fold in CD1b (Figure 3I).

Analysis of the lipid profiles eluted from CD1 molecules: unique features detected in individual CD1 isoforms

Although we found a high level of overlap in the CD1 lipidomes, we also asked whether we could identify any isoform-specific lipid species in our dataset. To answer this question, we extracted the lipid species from the Venn diagram intersections unique for each isoform. The highest count of individual lipids was observed in the CD1a lipidome with 128 individual lipids; CD1d had 57, CD1c had 33 and CD1b contained 25 unique lipids (Table 1; Figure 1B).

We observed different distributions of lipid classes amongst the unique eluate of the four CD1 isoforms (Figure 4; Supplementary Figure 4). Phosphatidylserine (18:1;0-20:2;0) (PS) was the most prominent feature amongst the CD1a-specific lipids, followed by similar chain length phosphatidylinositol (18:1;0-20:2;0) (PI) and phosphatidylglycerol (18:0;0-18:1;0) (PG). A second cluster of unique CD1a lipids comprised phosphatidic acid species (PA) as well as headless lipid species of DAG. The CD1b-specific lipid group contained 25 individual lipids, mainly lyso- and phospholipids with (18:1;0-18:1;0) and (16:1;0-18:0;0) PG and (36:1;2) and (38:0;2) sulfatide (Sulf) showing the biggest enrichment. In the CD1c-specific lipidome (33 lipids) gangliosides species GM2 (36:1;2), GD1, GD2 and GD3 were the most enriched. CD1c also uniquely accommodated shorter chain

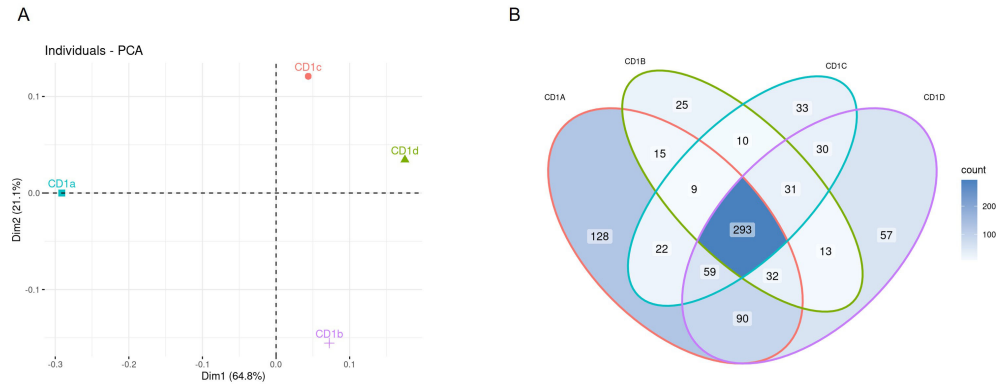

FIGURE 1
Lipidome of CD1 isoforms **(A)** Principal Component Analysis of the lipid classes bound to each CD1 isoform. **(B)** Venn diagram of overlapping lipid classes between the CD1 isoforms. The diagram shows the number events detected in each isoform.

lengths (16:0;0–16:1;0) PI and PS, lysolipids (LPC, LPA, LPI and (32:2;3) and (34:2;3) sphingomyelin (SM). (Figure 4; Supplementary Figure 4).

CD1d bound to 57 unique lipids, of which cholesterol (Chol) was the most abundant. The second most abundant species bound to CD1d was the ganglioside GM3.

Lipid chain lengths captured by CD1 isoforms

Crystal structures have shown that lipid-ligands are anchored in the CD1 molecules' hydrophobic binding pockets (36). As the CD1 isoforms possess different structures of binding pockets (36), they can accommodate lipid antigens with acyl chains of different length and saturation. We therefore queried the acyl-chain length at the species and subspecies levels of phospholipids and glycolipids and at the species level of sphingolipids.

When we analysed the lipid chain length captured by the shared CD1 lipidome (Figure 5), we observed that CD1c molecules bound lipids similar in length to the cellular lipidome (with an enrichment for C32, C34 and C36). In the CD1a- and CD1d-specific lipid pool, we mainly found mid-sized (C34-C38) species, in agreement with previous findings (26). CD1d molecules showed an additional preference for longer C42 lipids. Compared to the cellular profile, and in agreement with previous findings (4, 17), CD1b molecules over captured short chain length lipids (C16-C20), that might be suitable spacer lipids for dual lipid presentation (37). In addition, CD1b molecules captured C32-C36 species (Figure 5). Short (C14-C18) lysolipids, possibly spacer lipids accommodated in the F'

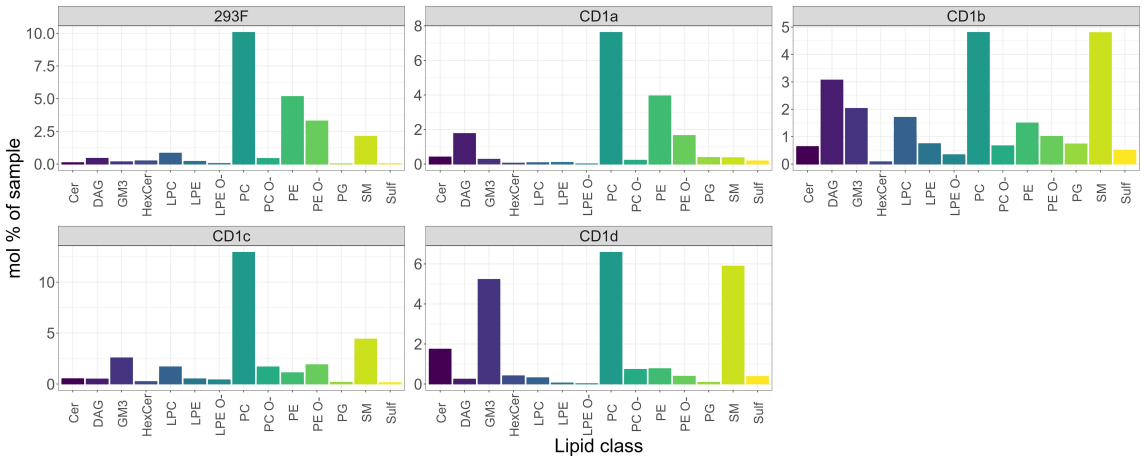

FIGURE 2
Shared cellular lipids captured by the CD1 isoforms. Mol percentage of the 293F shared lipid classes captured by the indicated CD1 isoforms in comparison with the 293F cellular lipidome. The graphs show only the proportion of lipids that are present in all isoforms. The identified lipid molecules were quantified by normalization to a lipid class-specific internal standard. The amounts in pmoles of individual lipid molecules (species of subspecies) of a given lipid class were summed to yield the total amount of the lipid class. The amounts of the lipid classes were then normalized to the total lipid amount yielding mol% per total lipids. Ceramide (Cer), Diacylglycerol (DAG), Monosialodihexosylganglioside (GM3), hexosylceramide (HexCer), lyso-phosphatidyl-cholines (LPC), ether-linked lyso-phosphatidyl-ethanolamine (LPE O-), phosphatidylcholine (PC), ether-linked Phosphatidylcholine (PC O-), phosphatidylethanolamine (PE), ether-linked phosphatidylethanolamine (PE O-), phosphatidylglycerol (PG), sphingomyelin (SM), Sulfatide (Sulf).

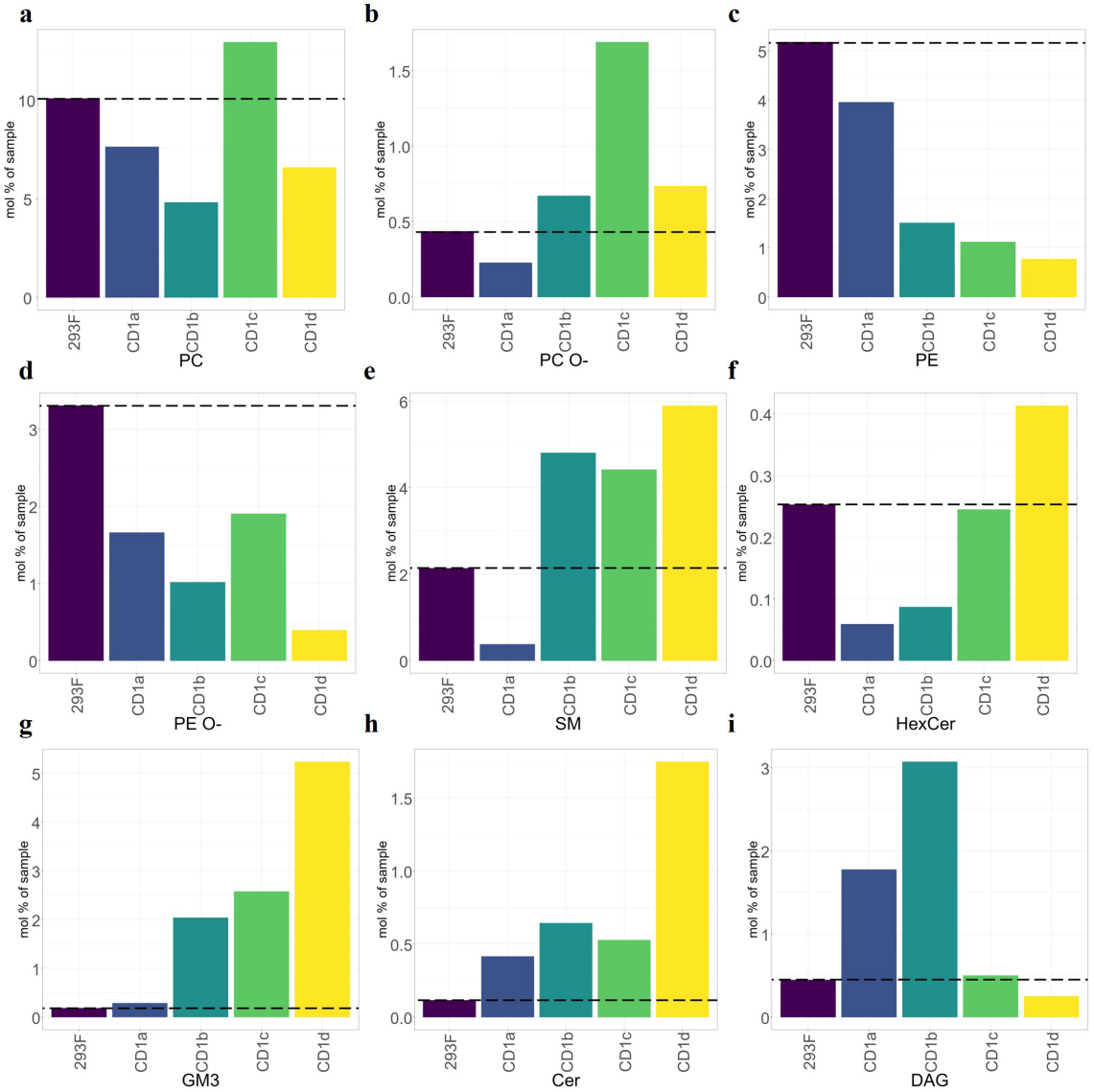

FIGURE 3
Over capture analysis of the shared CD1 lipidomes. Mol percentage of the nine most abundant shared lipid classes eluted from CD1 molecules, compared to the cellular background (dashed line). **(A-D)** Phospholipids. **(E-G)** Headed sphingolipids. **(H, I)** Headless lipids. Ceramides showed strong capture by CD1d but lower capture by other isoforms. SMs were over-captured by CD1b, CD1c and CD1d. General pattern of under-capture of phospholipids (PE, EPE, and EPC). GM3 was over-captured by CD1b, CD1c and CD1d.

pocket (36), were also found in the CD1c lipidome. Altogether, these results suggest that the binding pocket size and structure might have a unique influence in driving the ligand capture over the cellular background.

The chain length capture analysis of the whole lipidome was comparable to that of the shared CD1 lipidome discussed above (Supplementary Figure 4). In addition, larger lipids like cardiolipin (C60) and triacylglycerol (C56) were found in CD1a molecules and larger lipids (C40-C42) were captured in CD1b molecules. Large (C42) species dominated the isoform specific lipidome of CD1b and CD1d (Figure 6).

We next compared the acyl-chain length distribution of the most abundant phospholipid species across CD1 isoforms. These species include PC and its ether-bound format PC O- (Figure 7) and SM and GM3 (Figure 8). We assigned z-scores to the mol% values of the lipid abundance and plotted the values on a bubble heat map. The size of the bubble on the heat map indicates the abundance of the lipid in the isoforms' lipidome. In agreement with the above analysis, CD1a bound PC chains of middle length (C38–C40) and larger SM species (C40-C42). CD1b captured the shortest chain length of PC (C30–C38) and SMs (C34). CD1d bound long-chain PCs (C42– C46), without any preference towards SM chain length. CD1c bound to C34-C36 SMs and captured the full spectrum of PC species (C30–C46). Our data showed no preference towards specific acyl chair pairing or saturation.

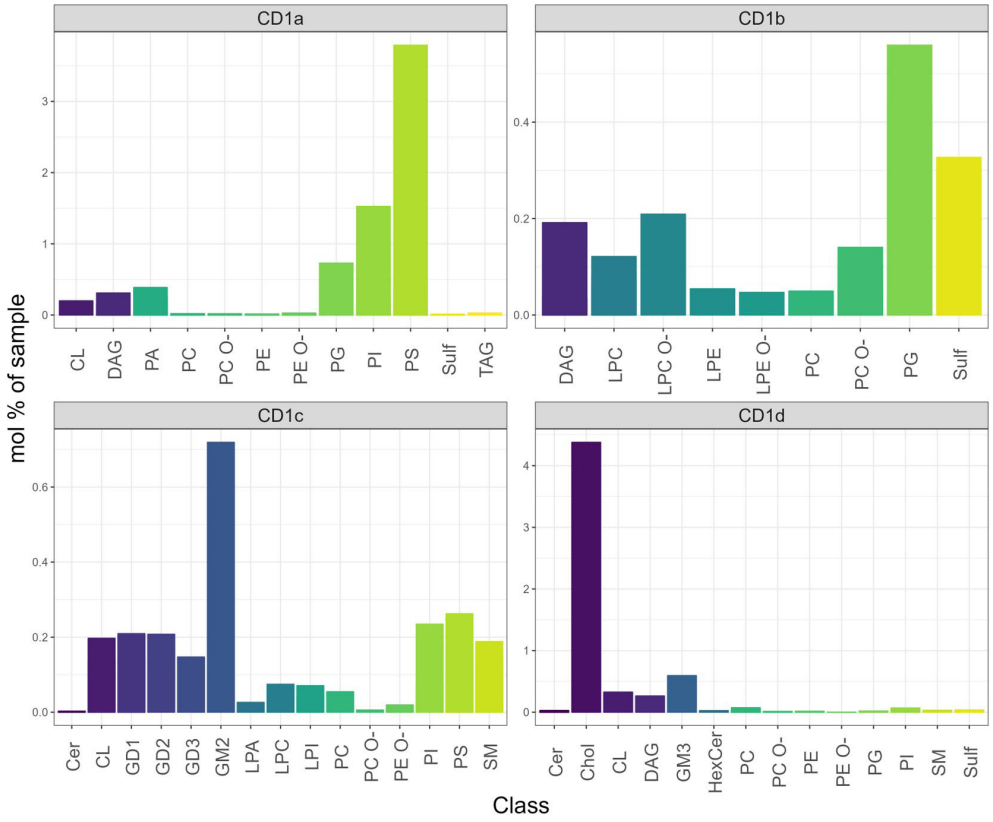

FIGURE 4
Mol% of unique lipids of the CD1 isoforms. The CD1a unique features were PI and PS; the CD1b lipidome was enriched in PG and sulfatide; the CD1c-specific lipidome was enriched in GM2 (36:1;2), GD1, and GD2 species; Cholesterol was enriched in the CD1d unique lipidome.

Discussion

In this study, by shotgun mass spectrometry, we performed a comprehensive analysis of the lipidome captured by soluble CD1 molecules.

The shotgun lipidomic approach only identifies lipids based on internal standards. No modified lipids nor novel targets can be identified with this method. The methods used in the recent Huang study (26) are more developed, yet we demonstrate an overlap of the two analysis platforms. Accordingly, in both datasets, the four CD1

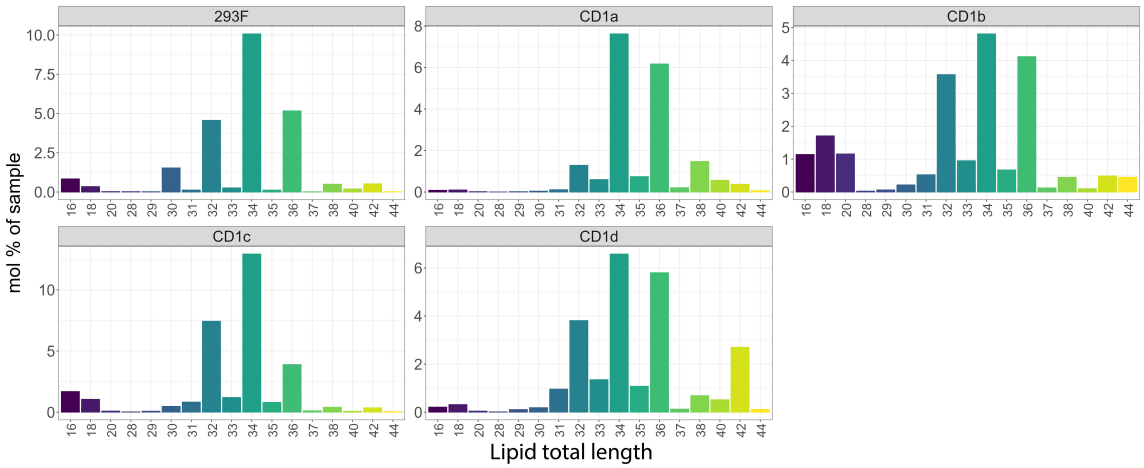

FIGURE 5
Chain length profile of the lipids captured by the CD1 isoforms. Lipid chain length captured in the shared CD1 lipidome expressed as a mol percentage of sample.

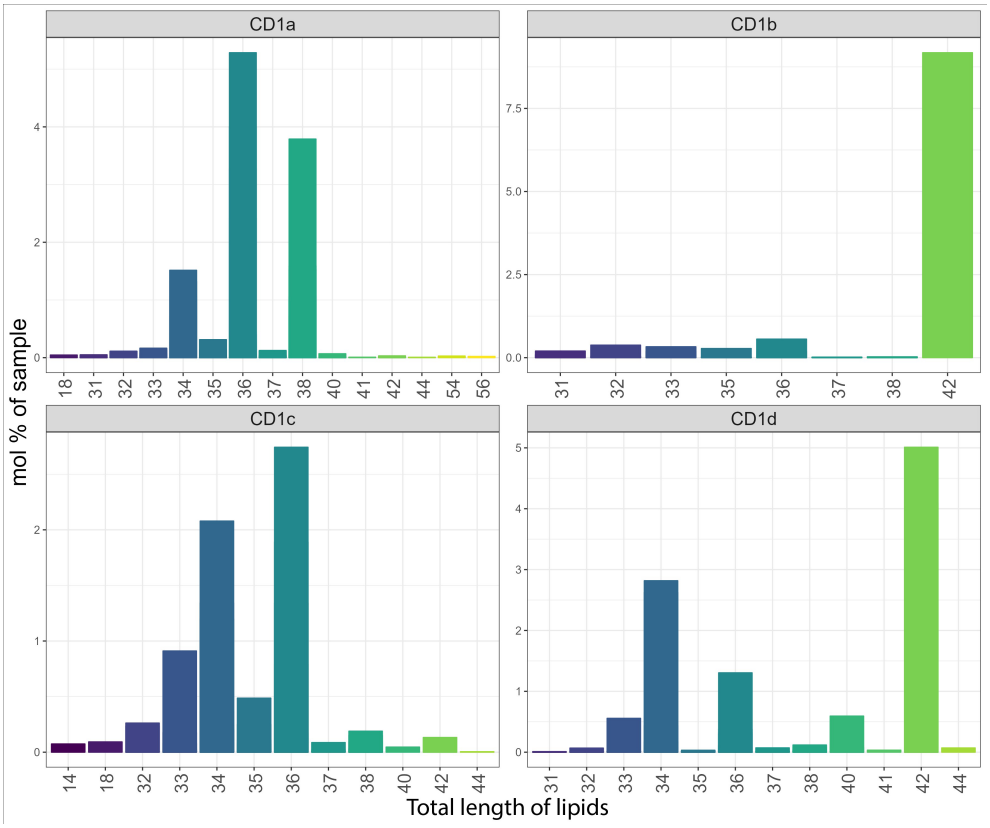

FIGURE 6
Total length of unique lipids captured by the CD1 isoforms. Lipid chain length captured in the CD1 isoform-specific lipidome expressed as a mol percentage of sample.

isoforms were found to bind almost any lipids from the cell, with some lipids deviating strongly from the cellular background. We reported that the lipid profiles of CD1a and CD1c were closest to the cellular background, with the main enrichment found in phospholipids. We also observed a high level of overlap, in that 28% of the CD1 lipidome was present in all isoforms and 45-68% of the CD1 isoform's lipidome consisted of shared lipids. However, in our dataset we also identified isoform-specific lipid species. CD1a was found to have the highest count of individual lipids (128), followed by CD1d, CD1c, and CD1b. These unique isoforms were enriched in certain lipid species, suggesting that each isoform may have a unique role in lipid presentation and subsequent immunological function.

It has been previously suggested that the small binding pocket of CD1a amongst the CD1 isoforms might limit the size of lipid-ligands captured (36). Consistent with that report, in our dataset, we observed a lipid chain length skewed towards mid-sized lipids. The lipidome eluted from the CD1a isoform closely followed the reference lipidome of Expi293F cells, which is enriched in so-called non-permissive phospholipids (PI, PS), as they have been shown to block activation of autoreactive T cell clones, with their polar head protruding from the antigen binding groove (38). In the CD1a-specific lipidome, we also identified permissive headless lipid species (DAG, CL) that might allow TCR binding via the A' roof, in a relative lipid agnostic manner (38).

Huang and colleagues have elegantly uncovered a mismatch between size of the antigen binding groove of some CD1 molecules and chain length of the accommodated lipids (26). In our dataset, we observed that different CD1 isoforms showed a tendency towards varying lipid chain lengths: this was true at a class more than at a species level, where we did not identify preferences towards acyl chain lengths or saturation. In their study, Huang et al. demonstrated CD1 isoform specificity towards lipids with similar structures, based on lipid chain-length. Remarkably, these capture patterns were different from the order of the binding groove sizes: accordingly, we observed that both CD1b and CD1c could accommodate, in addition to C32-C36 lipids, short chain lipids (C16-C18). The presence of these small lipids in the antigen binding groove has been confirmed in several crystal structures (37, 39–41), and it has been suggested that may function as spacer lipids, preserving the fold of the CD1 molecule bound to intermediate length lipids. CD1c has a unique structure with interconnected hydrophobic channels. Fully or partially enclosed pockets and accessible portals that can accommodate structurally different lipids (36). In agreement, we observed that the lipidome eluted from the CD1c molecules mirrored the cellular lipid background and the wide spectrum of lipid chain lengths captured by this molecule suggest flexible ligand binding capabilities. Gangliosides (GD1, GD2, GD3 and GM2) were the most prominent CD1c-

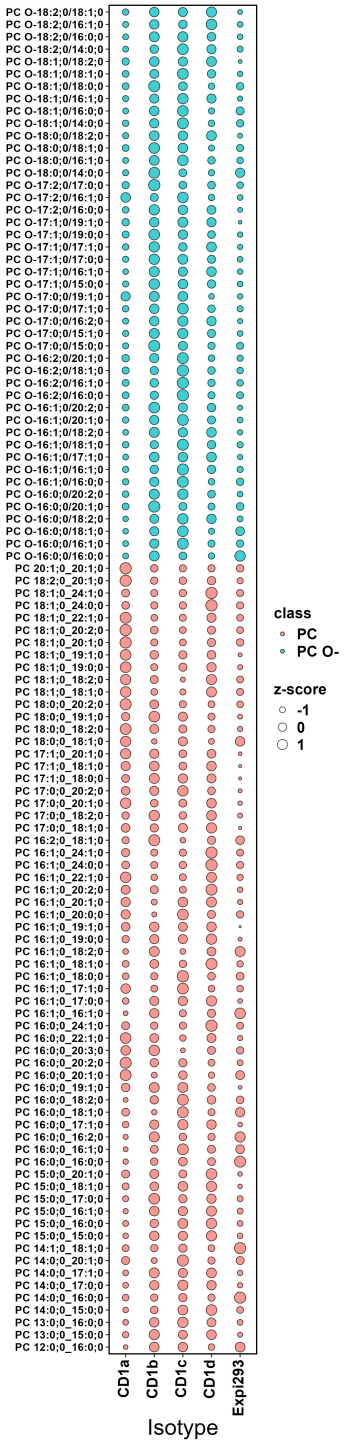

FIGURE 7
Abundance analysis of PC and PC O- species across CD1 isoforms. The bubble size indicates the abundance of the lipid species in each isoforms lipidome expressed as z-score (describing the positive or negative deviance from the mean in standard deviation units). CD1a bound PC chains of middle length (C38–C40); CD1b captured the shortest chain length of PC (C30–C38); CD1d skewed towards long-chain PCs (C42– C46); CD1c bound to the full spectrum of PC species (C30–C46).

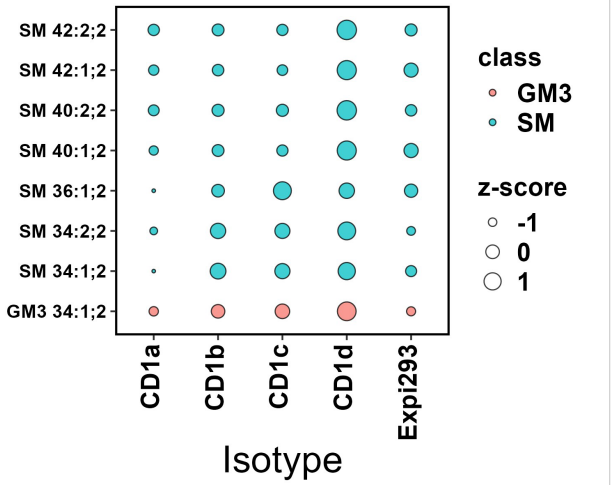

FIGURE 8
Abundance analysis of selected Sphingolipids in CD1 isoforms. The bubble size indicates the abundance of the lipid species in each isoforms' lipidome expressed as z-score. CD1a bound to larger SM species (C40-C42); CD1b captured the shortest chain length of SMs (C34); CD1c bound to C34-C36 SMs and CD1d didn't show any SM chain length preference. GM3 was most abundantly captured by CD1d.

bound lipids, and unpublished data presented at the 2024 CD1 MR1 conference suggest they may even wrap around the alpha-helices and could aid the binding of additional lipids.

Interestingly, CD1b and CD1d diverged from the cellular lipid profile showing main enrichment in sphingolipids. These results are in agreement with findings published by Rudolph et al. (21), demonstrating that the spectrum and abundance of CD1d-associated lipids are not representative of the total cellular lipidome but rather characterized by preferential binding to long-chain sphingolipids and glycerophospholipids (21). Strikingly, CD1d was the only isoform that over-captured cholesterol. This finding is consistent with CD1d being a surface receptor for oxidized cholesterol, mediating induction, and activation of the transcription factor peroxisome proliferator-activated receptor-γ (42). Previously, cholesteryl esters had been shown to bind CD1c and stabilise its conformation to promote recognition by autoreactive T cells (39). GM3 was over captured by CD1b, CD1c and CD1d. GM3 was identified as an inhibitory natural killer T-cell ligand in 2008 (43) and may act as a modulator to fine-tune and perhaps inhibit the reactivity of Th2 NKT-cells towards self-glycolipids.

It has been demonstrated by Cotton et al. that CD1a captures endogenous sphingomyelins (C42:2), which block tetramer binding to TCRs of autoreactive T cells (35). Although we didn't observe SM over capture by CD1a, we detected selective CD1a binding to larger SM species (C40-C42). Furthermore, in our dataset, we detected SM over capture by CD1b, c and d. By shotgun lipidomics of the CD1d associated lipidome, long chain sphingolipids, such as (SM42:2) were also detected by Rudolph et al. (21). Similarly, our data also indicated preferential binding of CD1d to longer chain SMs (C40:1,2; C40:2,2; C421,2;

C42:2,2). Our recent analysis reaffirms these previously published findings, providing support for the selective binding of long chain SMs, which may represent natural endogenous T cell inhibitors (21, 44).

In conclusion, our lipidomic analysis provides valuable insights into the lipids captured by the four soluble CD1 isoforms. It highlights the unique and overlapping features of the CD1 isoforms, their preferences for certain types of lipids, and the influence of lipid chain lengths on their binding capacity. These findings contribute to our understanding of their role in immunity and could have implications for the development of lipid-based therapeutics to modulate the function of lipid reactive T cells. Indeed, lipidomic analysis represents the first step towards understanding the biological outcome of lipid antigen presentation, be it stimulatory (by so called permissive lipids), or inhibitory (presentation of non-permissive lipids, or displacement of agonist lipids). Lipidomic analysis, coupled with crystallographic studies has provided evidence for the different models of CD1-lipid recognition by T cell receptors: co-recognition of CD1 and lipid, as in the case of αβ–peptide-MHC recognition, and exemplified by CD1-αGalCer-iNKT recognition (45); absence of interference or recognition of the CD1 scaffold irrespective of the cargo, as shown for self-reactive CD1a and CD1c T cell clones (38, 46). Further lipidomic studies should encompass CD1 molecules purified from cells exposed to microbial lipids or inflamed tissues and may provide more insights into how the immune system responds to various diseases.

Limitations of this study

While we analysed the lipidome of soluble molecules, we have not addressed the precise site of lipid loading. The lipid composition of mammalian cell organelles is not yet fully characterised, and it varies across cell types, tissues and in health versus diseased conditions. Nevertheless, it is understood that most lipids (including diacylglycerol, cholesterol, PC, PE, PI and the ceramide backbone) are synthesized in the ER and further modified (to sphingomyelin, glycosphingolipids or gangliosides) and sorted in the secretory pathway, mainly within the Golgi apparatus. This is reflected in the lipids identified in our soluble molecules, and we speculate they would be loaded in the ER and the Golgi compartments (47).

CD1 molecules were expressed as soluble proteins. These molecules do not recycle through the endo-lysosomal compartments to load lipids from these compartments. However, neither Huang and colleagues (26) nor Rudolph and colleagues (21) identified differences between the lipidome of truncated and full-length CD1 molecules.

We only expressed CD1 molecules in Expi293F cells, and we cannot exclude that molecules expressed in other cell types/states may capture additional lipid species. It would be of particular interest to characterise the lipidome of natural CD1 antigen presenting cells, such as thymocytes, dendritic cells, B cells and Langerhans cells, at steady state and in disease. These experiments are currently not possible for technical limitations.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://github.com/ritaszokekovacs/CD1-lipidome17052024.

Author contributions

RS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. SK: Formal analysis, Software, Visualization, Writing – review & editing. PG: Supervision, Writing – review & editing. MS: Conceptualization, Investigation, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study received funding from Immunocore Ltd. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

Acknowledgments

The authors acknowledge Hussein Al-Mossawi and Ricardo Da Silva Carreira for critical reading of the manuscript.

Conflict of interest

MS, RK and SK were employed Immunocore Ltd.

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

The author MS declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1462209/full#supplementary-material

References

1. Salio M, Silk JD, Jones EY, Cerundolo V. Biology of CD1- and MR1-restricted T cells. *Immunology*. (2014) 32:323–66. doi: 10.1146/annurev-immunol-032713-120243

2. de la Salle H, Mariotti S, Angenieux C, Gilleron M, Garcia-Alles L-F, Malm D, et al. Assistance of microbial glycolipid antigen processing by CD1e. *Science*. (2005) 310:1321–4. doi: 10.1126/science.1115301

3. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol*. (2007) 25:297–336. doi: 10.1146/annurev.immunol.25.022106.141711

4. Libero GD, Mori L. Recognition of lipid antigens by T cells. *Nat Rev Immunol*. (2005) 5:485–96. doi: 10.1038/nri1631

5. Shamshiev A, Donda A, Prigozy TI, Mori L, Chigorno V, Benedict CA, et al. The αβ T cell response to self-glycolipids shows a novel mechanism of CD1b loading and a requirement for complex oligosaccharides. *Immunity*. (2000) 13:255–64. doi: 10.1016/S1074-7613(00)00025-X

6. Shamshiev A, Gober H-J, Donda A, Mazorra Z, Mori L, Libero GD. Presentation of the same glycolipid by different CD1 molecules. *J Exp Med*. (2002) 195:1013–21. doi: 10.1084/jem.20011963

7. Vincent MS, Xiong X, Grant EP, Peng W, Brenner MB. CD1a-, b-, and c-Restricted TCRs Recognize Both Self and Foreign Antigens. *J Immunol*. (2005) 175:6344–51. doi: 10.4049/jimmunol.175.10.6344

8. Brigl M, Bry L, Kent SC, Gumperz JE, Brenner MB. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol*. (2003) 4:1230–7. doi: 10.1038/ni1002

9. Kinjo Y, Takatsuka S, Kitano N, Kawakubo S, Abe M, Ueno K, et al. Functions of CD1d-restricted invariant natural killer T cells in antimicrobial immunity and potential applications for infection control. *Front Immunol*. (2018) 9:1266. doi: 10.3389/fimmu.2018.01266

10. Ye JH, Chen Y-L, Ogg G. CD1a and skin T cells: a pathway for therapeutic intervention. *Clin Exp Dermatol*. (2024) 49:450–8. doi: 10.1093/ced/llad460

11. Dougan SK, Salas A, Rava P, Agyemang A, Kaser A, Morrison J, et al. Microsomal triglyceride transfer protein lipidation and control of CD1d on antigen-presenting cells. *J Exp Med*. (2005) 202:529–39. doi: 10.1084/jem.20050183

12. Brozovic S, Nagaishi T, Yoshida M, Betz S, Salas A, Chen D, et al. CD1d function is regulated by microsomal triglyceride transfer protein. *Nat Med*. (2004) 10:535–9. doi: 10.1038/nm1043

13. Moody DB, Porcelli SA. Intracellular pathways of CD1 antigen presentation. *Nat Rev Immunol*. (2003) 3:11–22. doi: 10.1038/nri979

14. Cernadas M, Cavallari M, Watts G, Mori L, Libero GD, Brenner MB. Early recycling compartment trafficking of CD1a is essential for its intersection and presentation of lipid antigens. *J Immunol*. (2010) 184:1235–41. doi: 10.4049/jimmunol.0804140

15. Briken V, Jackman RM, Dasgupta S, Hoening S, Porcelli SA. Intracellular trafficking pathway of newly synthesized CD1b molecules. *EMBO J*. (2002) 21:825–34. doi: 10.1093/emboj/21.4.825

16. Sugita M, Jackman RM, Donselaar van Behar E SM, Rogers RA, Peters PJ, et al. Cytoplasmic tail-dependent localization of CD1b antigen-presenting molecules to MIICs. *Science*. (1996) 273:349–52. doi: 10.1126/science.273.5273.349

17. Moody DB. How T cells grasp mycobacterial lipid antigens. *Proc Natl Acad Sci*. (2017) 114:13312–4. doi: 10.1073/pnas.1719260115

18. Geho DH, Fayen JD, Jackman RM, Moody DB, Porcelli SA, Tykocinski ML. Glycosyl-phosphatidylinositol reanchoring unmasks distinct antigen-presenting pathways for CD1b and CD1c. *J Immunol*. (2000) 165:1272–7. doi: 10.4049/jimmunol.165.3.1272

19. Rodionov DG, Nordeng TW, Pedersen K, Balk SP, Bakke O. A critical tyrosine residue in the cytoplasmic tail is important for CD1d internalization but not for its basolateral sorting in MDCK cells. *J Immunol (Baltim Md: 1950)*. (1999) 162:1488–95. doi: 10.4049/jimmunol.162.3.1488

20. Haig NA, Guan Z, Li D, McMichael A, Raetz CRH, Xu X-N. Identification of self-lipids presented by CD1c and CD1d proteins*. *J Biol Chem*. (2011) 286:37692–701. doi: 10.1074/jbc.M111.267948

21. Rudolph M, Wang Y, Simolka T, Huc-Claustre E, Dai L, Grotenbreg G, et al. Sortase A-cleavable CD1d identifies sphingomyelins as major class of CD1d-associated lipids. *Front Immunol*. (2022) 13:897873. doi: 10.3389/fimmu.2022.897873

22. Huang S, Cheng T-Y, Altman J, Moody DB. Comparative lipidomics reveals a global sampling of major cellular membrane lipids by human CD1 proteins (P5006). *J Immunol*. (2013) 190:41.4–4. doi: 10.4049/jimmunol.190.Supp.41.4

23. Yuan W, Kang S-J, Evans JE, Cresswell P. Natural lipid ligands associated with human CD1d targeted to different subcellular compartments. *J Immunol*. (2009) 182:4784–91. doi: 10.4049/jimmunol.0803981

24. Correction for Muindi, et al. Activation state and intracellular trafficking contribute to the repertoire of endogenous glycosphinogolipids presented by CD1d. *Proc Natl Acad Sci*. (2010) 107:6118–8. doi: 10.1073/pnas.1001448107

25. Cox D, Fox L, Tian R, Bardet W, Skaley M, Mojsilovic D, et al. Determination of cellular lipids bound to human CD1d molecules. *PloS One*. (2009) 4:e5325. doi: 10.1371/journal.pone.0005325

26. Huang S, Shahine A, Cheng T-Y, Chen Y-L, Ng SW, Balaji GR, et al. CD1 lipidomes reveal lipid-binding motifs and size-based antigen-display mechanisms. *Cell*. (2023) 186:4583–4596.e13. doi: 10.1016/j.cell.2023.08.022

27. Züllig T, Trötzmüller M, Köfeler HC. Lipidomics from sample preparation to data analysis: a primer. *Anal Bioanal Chem*. (2020) 412:2191–209. doi: 10.1007/s00216-019-02241-y

28. Hsu F-F. Mass spectrometry-based shotgun lipidomics – a critical review from the technical point of view. *Anal Bioanal Chem*. (2018) 410:6387–409. doi: 10.1007/s00216-018-1252-y

29. Wang J, Han X. Analytical challenges of shotgun lipidomics at different resolution of measurements. *TrAC Trends Anal Chem*. (2019) 121:115697. doi: 10.1016/j.trac.2019.115697

30. Surma MA, Gerl MJ, Herzog R, Helppi J, Simons K, Klose C. Mouse lipidomics reveals inherent flexibility of a mammalian lipidome. *Sci Rep*. (2021) 11:19364. doi: 10.1038/s41598-021-98702-5

31. Ejsing CS, Sampaio JL, Surendranath V, Duchoslav E, Ekroos K, Klemm RW, et al. Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proc Natl Acad Sci*. (2009) 106:2136–41. doi: 10.1073/pnas.0811700106

32. Surma MA, Herzog R, Vasilj A, Klose C, Christinat N, Morin-Rivron D, et al. An automated shotgun lipidomics platform for high throughput, comprehensive, and quantitative analysis of blood plasma intact lipids. *Eur J Lipid Sci Technol*. (2015) 117:1540–9. doi: 10.1002/ejlt.201500145

33. Herzog R, Schuhmann K, Schwudke D, Sampaio JL, Bornstein SR, Schroeder M, et al. LipidXplorer: A software for consensual cross-platform lipidomics. *PloS One*. (2012) 7:e29851. doi: 10.1371/journal.pone.0029851

34. Herzog R, Schwudke D, Schuhmann K, Sampaio JL, Bornstein SR, Schroeder M, et al. A novel informatics concept for high-throughput shotgun lipidomics based on the molecular fragmentation query language. *Genome Biol*. (2011) 12:R8. doi: 10.1186/gb-2011-12-1-r8

35. Cotton RN, Wegrecki M, Cheng T-Y, Chen Y-L, Veerapen N, Nours JL, et al. CD1a selectively captures endogenous cellular lipids that broadly block T cell response. *J Exp Med*. (2021) 218:e20202699. doi: 10.1084/jem.20202699

36. Ly D, Moody DB. The CD1 size problem: lipid antigens, ligands, and scaffolds. *Cell Mol Life Sci*. (2014) 71:3069–79. doi: 10.1007/s00018-014-1603-6

37. Garcia-Alles LF, Versluis K, Maveyraud L, Vallina AT, Sansano S, Bello NF, et al. Endogenous phosphatidylcholine and a long spacer ligand stabilize the lipid-binding groove of CD1b. *EMBO J*. (2006) 25:3684–92. doi: 10.1038/sj.emboj.7601244

38. Birkinshaw RW, Pellicci DG, Cheng T-Y, Keller AN, Sandoval-Romero M, Gras S, et al. αβ T cell antigen receptor recognition of CD1a presenting self lipid ligands. *Nat Immunol*. (2015) 16:258–66. doi: 10.1038/ni.3098

39. Mansour S, Tocheva AS, Cave-Ayland C, Machelett MM, Sander B, Lissin NM, et al. Cholesteryl esters stabilize human CD1c conformations for recognition by self-reactive T cells. *Proc Natl Acad Sci*. (2016) 113:E1266–75. doi: 10.1073/pnas.1519246113

40. Garcia-Alles LF, Collmann A, Versluis C, Lindner B, Guiard J, Maveyraud L, et al. Structural reorganization of the antigen-binding groove of human CD1b for presentation of mycobacterial sulfoglycolipids. *Proc Natl Acad Sci*. (2011) 108:17755–60. doi: 10.1073/pnas.1110118108

41. Gadola SD, Zaccai NR, Harlos K, Shepherd D, Castro-Palomino JC, Ritter G, et al. Structure of human CD1b with bound ligands at 2.3 Å, a maze for alkyl chains. *Nat Immunol*. (2002) 3:721–6. doi: 10.1038/ni821

42. Rosales C, Tang D, Geng Y-J. CD1d serves as a surface receptor for oxidized cholesterol induction of peroxisome proliferator-activated receptor-γ. *Atherosclerosis*. (2015) 239:224–31. doi: 10.1016/j.atherosclerosis.2015.01.004

43. Park J, Wu DY, Prendes M, Lu SX, Ragupathi G, Schrantz N, et al. Fine specificity of natural killer T cells against GD3 ganglioside and identification of GM3 as an inhibitory natural killer T-cell ligand. *Immunology*. (2008) 123:145–55. doi: 10.1111/j.1365-2567.2007.02760.x

44. Melum E, Jiang X, Baker KD, Macedo MF, Fritsch J, Dowds CM, et al. Control of CD1d-restricted antigen presentation and inflammation by sphingomyelin. *Nat Immunol*. (2019) 20:1644–55. doi: 10.1038/s41590-019-0504-0

45. Koch M, Stronge VS, Shepherd D, Gadola SD, Mathew B, Ritter G, et al. The crystal structure of human CD1d with and without α-galactosylceramide. *Nat Immunol*. (2005) 6:819–26. doi: 10.1038/ni1225

46. Wun KS, Reijneveld JF, Cheng T-Y, Ladell K, Uldrich AP, Nours JL, et al. T cell autoreactivity directed toward CD1c itself rather than toward carried self lipids. *Nat Immunol*. (2018) 19:397–406. doi: 10.1038/s41590-018-0065-7

47. Sarmento MJ, Llorente A, Petan T, Khnykin D, Popa I, Perkovic MN, et al. The expanding organelle lipidomes: current knowledge and challenges. *Cell Mol Life Sci*. (2023) 80:237. doi: 10.1007/s00018-023-04889-3

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 17 September 2024
DOI 10.3389/fimmu.2024.1472854

OPEN ACCESS

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RECEIVED 30 July 2024
ACCEPTED 26 August 2024
PUBLISHED 17 September 2024

CITATION
Rajashekar V, Stern L, Almeida CF, Slobedman B and Abendroth A (2024) The surveillance of viral infections by the unconventional Type I NKT cell.
Front. Immunol. 15:1472854.
doi: 10.3389/fimmu.2024.1472854

The surveillance of viral infections by the unconventional Type I NKT cell

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Type I NKT cells, also known as Invariant Natural Killer T (iNKT) cells, are a subpopulation of unconventional, innate-like T (ILT) cells which can proficiently influence downstream immune effector functions. Type I NKT cells express a semi-invariant αβ T cell receptor (TCR) that recognises lipid-based ligands specifically presented by the non-classical cluster of differentiation (CD1) protein d (CD1d) molecule. Due to their potent immunomodulatory functional capacity, type I NKT cells are being increasingly considered in prophylactic and therapeutic approaches towards various diseases, including as vaccine-adjuvants. As viruses do not encode lipid synthesis, it is surprising that many studies have shown that some viruses can directly impede type I NKT activation through downregulating CD1d expression. Therefore, in order to harness type I NKT cells for potential anti-viral therapeutic uses, it is critical that we fully appreciate how the CD1d-iNKT cell axis interacts with viral immunity. In this review, we examine clinical findings that underpin the importance of type I NKT cell function in viral infections. This review also explores how certain viruses employ immunoevasive mechanisms and directly encode functions to target CD1d expression and type I NKT cell function. Overall, we suggest that the CD1d-iNKT cell axis may hold greater gravity within viral infections than what was previously appreciated.

KEYWORDS

type I NKT cell, viral infections, CD1d-iNKT cell axis, unconventional innate-like T cell, type I iNKT cell function

1 Introduction

The innate immune system mounts a rapid and widespread response to an array of diverse pathogens or danger signals, but is not considered as efficient at forming a memory response for subsequent pathogen exposure. In contrast, the adaptive immune system recognises specific antigenic signatures, albeit with a slower response, but is pivotal for

long-term pathogen control. Conventional T cells are mostly thought of as part of the adaptive immune system and mount highly specific, but slow responses. Natural killer T (NKT) cells are a population of unconventional, innate-like T (ILT) cells that can rapidly respond in an innate-like manner, but also in an adaptive-like manner to further enact more antigen-specific responses. Thus, NKT cells are thought to influence and bridge both arms of the immune system.

Unlike conventional αβ T cells that recognise peptide antigens bound by major histocompatibility complex (MHC) molecules, the NKT cell T cell receptor (TCR) exclusively recognises foreign and self-lipid-based antigens presented by the non-classical cluster of differentiation (CD1) protein d (CD1d) molecule. NKT cells are composed of two subpopulations: type I NKT cells, also commonly known as 'invariant' NKT (iNKT) cells, which are the best characterised and predominantly explored for their immune-therapeutic potential; and type II NKT cells, which remain less studied and more poorly understood (1). The type I NKT TCR is a semi-invariant αβ TCR comprised of an invariant TCRα chain, Vα24Jα18, which predominantly pairs with Vβ11 TCRs in humans (2, 3). In mice, this TCR comprises of a Vα14Jα18 chain which typically pairs with Vβ2, Vβ7, or Vβ8 chains (4–6). In addition to murine models, swine have also been greatly valued as models for type I NKT cell research as they share a similar type I NKT cell frequency and tissue distribution to that found in humans (7). The TCR of pig type I NKT cells is characterised by a wide range of Vα, Jα, Vβ and Jβ segments, with a large majority of these corresponding to gene sequences recognised in humans (8).

All type I NKT cells share reactivity towards a common lipid antigen termed α-galactosylceramide (α-GalCer), a well-characterised agonist of type I NKT cell responses (9–11). This has enabled the development of CD1d-loaded α-GalCer tetramers, which have facilitated the specific identification of type I NKT cells and type I NKT cell effector function (6, 12, 13). In contrast, type II NKT cells do not express the semi-invariant type I NKT TCR α chain and do not respond to α-GalCer. Instead, type II NKT cells exhibit a greater TCR sequence diversity (14–16) and recognise other lipids and small sulfa-drug-like molecules, such as benzofuran sulfonates, bound by the CD1d molecule (16, 17). Due to a lack of reagents available to universally identify them, much less is known about type II NKT cell immune effector functions and their therapeutic potential remains understudied (15).

Type I NKT cells can be activated upon TCR engagement with a lipid-loaded CD1d molecule, or upon TCR-independent stimulation in response to innate cytokines such as interleukin (IL)-12 and IL-18 (18, 19). Upon activation, type I NKT cells are able to rapidly secrete a plethora of potent cytokines such as interferon (IFN)-γ, tumour necrosis factor (TNF) and IL-4 (20–24). Type I NKT cells can either directly target infected/cancer cells through cytotoxic activity or indirectly control the effector functions of other immune cells, including but not limited to helping B cells form highly specific antibodies (25–29). Due to their capacity to enhance downstream immune functions, type I NKT cells have been increasingly implicated in a variety of viral infections, with their anti-viral potential being a focal point of this review. Current research also harnesses type I NKT cells in multiple clinical settings including anti-cancer treatments, vaccine adjuvants, and cell-based therapies (30, 31).

Although viruses do not typically encode lipid antigens themselves, they are able to modulate the self-lipids expressed from host cells, which can be differentially recognised by certain NKT cell subsets and can affect the cytokine environment upon infection, thus influencing NKT cell responses (type I and type II) (32–34). In agreement, lipodomics studies have shown that viral infections trigger endoplasmic reticulum (ER) stress (35) which can in turn lead to the accumulation of certain CD1d-bound self-lipids (36) that are recognised by the type I NKT TCR (37). This suggests a role for type I NKT cells in viral surveillance through sensing cellular stress (35, 36). Thus, it could be possible that viruses may indirectly modulate CD1d antigen expression of these lipids and inhibit type I NKT cell function to circumvent their anti-viral capacity (38, 39). Considering the profound importance that type I NKT cells may play in viral infections, it is imperative to study the mechanisms through which viruses can either elicit or avoid immune responses through type I NKT cell interaction.

2 Type I NKT cells in viral infections

2.1 Deficiencies in CD1d molecule expression and Type I NKT cells predispose individuals to exacerbated viral infections

Severe viral infections are more commonly experienced in individuals with weakened and compromised immune capacities (40). Exacerbated symptoms following viral infection have been particularly observed in individuals with decreased CD1d molecule expression or type I NKT cell deficiencies (41–44), underscoring the importance of the CD1d-iNKT cell axis in controlling viral infections.

Varicella zoster virus (VZV) is a highly common alphaherpesvirus with an approximate 90% worldwide seroprevalence (45). In individuals who are latently infected with VZV, periods of diminished VZV-specific immunity results in VZV reactivation (46). VZV reactivation commonly manifests as a painful, unilateral rash (known as herpes zoster/shingles), which typically only occurs once or twice in an immunocompetent individuals' lifetime (47). Interestingly, individuals who have experienced multiple VZV reactivations exhibited a stark decrease in peripheral type I NKT cell numbers, with residual type I NKT cells skewed to an inhibitory phenotype by higher expression of the inhibitory receptor CD158a (43). IL-2 enhances the functional activity of NK cells and subsequently upregulates CD158a in an attempt to then regulate any cytotoxic repercussions of this activation (48). Interestingly, IL-2 has been readily detectable in varicella patients (49). Thus, the increased inhibitory profile of residual type I NKT cells in zoster patients may be IL-2-dependent and a possible consequence of repeated activation/stimulation. Aimed to increase VZV-specific immunity, primary varicella vaccines, such as Varivax, and booster doses are generally well-tolerated prophylaxis methods (50–52). It is mainly immunodeficient individuals that experience symptoms which are adverse and potentially life-threatening post-vaccination (53, 54).

Following vaccination with an attenuated Oka-strain varicella vaccine, two children experienced severe respiratory distress and painful papulovesicular rashes (41, 42). Upon lymphocyte analysis, it was revealed that both patients exhibited a genetic deficiency and dysfunction of type I NKT cell populations, with one patient also deficient in CD1d expression. These clinical findings are consistent with the proposal that the CD1d-iNKT cell axis commands a critical role in VZV resolution.

Mutations in the SH2D1A gene causes defective functioning of the signalling lymphocyte activation molecules (SLAM) -Associated Protein (SAP). SAP is necessary for T and NK cell function and has further been implicated in type I NKT cell development and function (55). Patients with X-linked lymphoproliferative (XLP) 1 disease who have a mutated SH2D1A gene, exhibited a stark absence of type I NKT cells, with no apparent paucity of other lymphocyte populations (55). A child with XLP1 had presented with Epstein-Barr virus (EBV) infection, which then rapidly developed into EBV encephalitis (44). This clinical finding suggests a correlation between the absence and dysfunction of type I NKT cells, and an exacerbated EBV infection.

Recent data has shown that NKT cells also hold great significance in viral control in the context of human transplantation, and thus transplantation success (56). Allografts with a higher abundance of type I NKT cells resulted in a decreased human cytomegalovirus (HCMV) reactivation rate post-allogeneic hematopoietic cell transplantation (HCT), with the association to CD1d expression in these allografts still unknown (56). Accordingly, the secretion of IFN-γ, perforin, and granzyme B from activated iNKT cells had lead to liver damage (57), but had also facilitated cytotoxic T cell activation and thus hepatitis B virus (HBV) inhibition (58). On the other hand, a rat model of hepatitis C virus (HCV) -related virus infection showed that type I NKT cells, which are biased to type 2 immunity, can limit liver injury while preventing infection (59). The role of type I NKT cells in HCV-related virus infection is further explored in Section 2.2 Type I NKT cell activation and function in viral infection. A murine study using NKT knockout mice showed that NKT cell populations and more specifically their IFN-γ production, are necessary for long-term cardiac allograft acceptance (60). In mice who were previously deficient in NKT cells, the adoptive transfer of NKT cells post-transplantation had ameliorated allograft rejection and prolonged cardiac allograft survival (60). Therefore, understanding what factors drive and control the different type I NKT cell subsets could inform how to safely mitigate viral infection severity in a transplant setting, and promote transplantation success.

The importance of CD1d expression and type I NKT cell activity within viral infections has also been supported by studies using murine models. In murine CMV (MCMV) infected mice which were either CD1d or Jα18 deficient, there was a significant suppression of myeloid progenitor cell numbers and proliferative ability (61). Remarkably, the adoptive transfer of type I NKT cells to Jα18 deficient mice, which were then intraperitoneally infected with MCMV, had rescued their myelosuppression profile and improved myeloid progenitor cell cycling status (61). This study suggests that the absence of CD1d molecule expression and type I NKT cell populations leaves myeloid progenitor cells vulnerable to MCMV-induced suppression.

Type I NKT cells have also demonstrated involvement in herpes simplex virus (HSV) type-1 (HSV-1) infection of mice. Following cutaneous inoculation of HSV-1, CD1d knockout mice exhibited an accelerated development of HSV-1 zosteriform skin lesions and a delayed clearance of virus when compared to wild-type mice (62). Jα281 knockout mice, lacking the type I NKT Vα14-Jα281 TCR, also revealed considerably higher viral loads with a diminished capacity to clear virus (62). However, a subsequent study revealed that the Jα281 knockout mice express lower TCR diversity, which can impact the viral-specific T cell repertoire and potentially other unconventional T cells too, such as Mucosal-Associated Invariant T (MAIT) cells (63). Nonetheless, in an alternate murine study, CD1d knockout mice infected with ocular HSV-1 infections displayed exacerbated eye inflammation with a delayed disease clearance (64). As the absence of CD1d molecule expression impedes type I NKT cell development in the thymus of mice (65) and pigs (66), these studies collectively suggest that a lack of CD1d molecule and NKT cell functionality enhances severe viral dissemination. The range of NKT cell functional responses against HSV infection is further explored later in this review.

Overall, many clinical findings and murine studies have established that a deficiency in CD1d molecule expression and type I NKT cell frequencies can leave hosts vulnerable to severe viral dissemination and reactivation. It is thus evident that the CD1d-iNKT cell axis instructs a profound immune response which may be necessary in defending the host from an aggravated viral infection.

2.2 Type I NKT cell activation and function in viral infections

The aforementioned studies suggest the involvement and importance of type I NKT cells in anti-viral immune responses. Conversely, multiple studies also report that viral infections modulate the activation and function of type I NKT cells.

2.2.1 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

SARS-CoV-2 is the virus responsible for COVID-19. A marked depletion of type I NKT cells was observed in the peripheral blood samples of SARS-CoV-2 infected patients, a finding which was found to be independent of CD1d downregulation during infection. This depletion of type I NKT cells was likely a result of SARS-CoV-2 spike protein binding to the type I NKT cell TCR, and causing cellular activation, exhaustion, and apoptosis since existing type I NKT cells expressed higher levels of the exhaustive marker Tim-3 (67). Further studies between convalescent and uninfected patient cohorts revealed that SARS-CoV-2 infected individuals specifically showed a striking reduction in type I NKT cell frequency (68). There was no reduction in conventional T cell frequencies which suggests that type I NKT cells may be more vulnerable to depletion in SARS-CoV-2 infection. In mouse models, the SARS-CoV-2 envelope (E) protein was also found to suppress activation and

effector function of type I NKT cells (39). However, when mice were treated with α-GalCer prior to SARS-CoV-2 intranasal infection, they exhibited a decreased viral titre and improved survival rates. Thus, although SARS-CoV-2 can substantially impede type I NKT cell functionality, these findings propose that activated type I NKT cells hold an immunoprotective role against SARS-CoV-2. Interestingly, type I NKT cells expressed a greater activation profile in severe COVID-19 patients (69). A higher CD69 expression level was positively correlated with plasma levels of IL-18, which has been established as a potent activator of type I NKT cells (69, 70). Albeit type I NKT cells from SARS-CoV-2-infected patients did produce less IFN-γ than those from healthy control donors, suggesting that despite a persistent activation profile, type I NKT cells expressed a mitigated functional profile. The substantial activation of type I NKT cells throughout SARS-CoV-2 disease progression is likely to be cytokine-dependent, as well as correlated to spike protein binding, and suggests an intricate balance between activation and functional loss of type I NKT cells in SARS-CoV-2 infection.

2.2.2 Herpes Simplex Virus type -1 (HSV-1)

Epidermal keratinocytes express substantial levels of CD1d and are a primary site of infection by the herpesvirus HSV-1. The co-culture of human type I NKT cells with HSV-1 infected human keratinocytes showed that HSV-1 was able to extensively shut down both the cytokine- and TCR-dependent activation of human type I NKT cells, resulting in an impaired cytokine output (38). However, HSV-1 infected keratinocytes do not exhibit CD1d downregulation which suggests that this weakened functional phenotype of type I NKT cells following contact with HSV-1 infected keratinocytes is independent of CD1d downregulation (38). In contrast to SARS-CoV-2 infection, type I NKT cell function is not rescued by α-GalCer treatment after co-culture with HSV-1 infected keratinocytes (38). Recent studies reveal that in HSV-1 infected human keratinocytes, the IL-15/IL-15 receptor-α (IL-15R-α) complex is rapidly upregulated and then subsequently downregulated with prolonged infection (71). Remarkably, the profound downregulation of the IL-15/IL-15R-α complex by HSV-1 infection was counteracted by IFN-γ production from type I NKT cells. The co-culture of type I NKT cells with HSV-1 infected keratinocytes also resulted in fewer keratinocytes expressing the HSV-1 envelope glycoprotein D (gD) (71). These novel reports represent a new perspective of how type I NKT cells may be able to counteract the modulatory mechanisms of viruses and exert anti-viral activity.

2.2.3 Hepatitis C virus (HCV)

The infection of liver tissue with HCV often results in detrimental inflammation and with no viral clearance, will eventually lead to chronic HCV (72). A patient cohort study indicated that the progression of acute HCV infection to chronic HCV infection is strongly correlated with an elevated activation profile of peripheral blood type I NKT cells as well as pro-inflammatory Type II NKT cells (73), which could possibly be a result of the upregulated CD1d expression present in HCV infection of the liver (74, 135). This suggests that the elevated proinflammatory cytokine milieu of type I NKT cells during HCV infection may contribute to the aggravated liver damage sustained during the progressing stages of HCV infection. However, recent studies have utilised a HCV-related hepacivirus murine model with CD1d knockout mice to explore how the type I NKT cell cytokine response could simultaneously mediate liver pathology and influence hepacivirus-specific $CD8^+$ T cells (59). Here, it was deduced that a paucity of liver type I NKT cells led to heightened tissue damage during hepacivirus infection despite previous findings indicating that type I NKT cell function could be associated with liver pathology. These type I NKT cell deficient mice also experienced an exacerbated $CD8^+$ T cell response, suggesting that type I NKT cells do offer an immunoprotective role during hepatic viral infection. Furthermore, the production of type 2 cytokines IL-4 and IL-13 from activated type I NKT cell subsets indicated a skewing towards an NKT2 profile (59). These results suggest that type I NKT cells could more specifically serve a regulatory role in viral infections such that the effector functions of hepacivirus-specific T cells, and potentially HCV-specific T cells too, are controlled to limit liver damage.

2.2.4 Influenza A virus (IAV)

IAV is a common respiratory virus that can efficiently infect swine, birds, and humans. From an evolutionary standpoint, the success of IAV within global populations is a result of the virus' ability to constantly and rapidly produce antigenically distinct viral strains (75). The morbidity and mortality associated with IAV is also related to a substantial viral titre and a destabilising overproduction of cytokines (76). To determine whether type I NKT cells could reduce IAV load and regulate the cytokine production in IAV infection, mice were intraperitoneally administered with α-GalCer concurrent to intranasal IAV inoculation (77). In treated mice, viral titre was significantly lower and body weight also remained more consistent over the course of infection compared to untreated mice. This improved disease outcome was likely a result of the activation and subsequent migration of type I NKT cells from the liver to the lungs, as there was a drop in liver type I NKT cell frequency but a significant increase in blood and lung type I NKT cell frequencies (77). The contribution of activated type I NKT cells to anti-viral immunity is similarly conveyed through IAV-infected CD1d knockout and type I NKT cell deficient mice experiencing an increased IAV titre compared to wild-type mice (78). This is suggested to be correlated to increased myeloid-directed suppressor cell (MDSC) activity, which are a cell-type capable of suppressing T cell functionality and thus cell-mediated anti-IAV immunity. Upon adoptive transfer of type I NKT cells into previously type I NKT cell deficient mice, the suppressive capacity of MDSCs was no longer observed as mice experienced a reduced viral titre, thus indicating the importance of type I NKT cells in controlling MDSC responses in viral environments (78). Type I NKT cells prepared from IAV-infected mice also expressed a higher level of IFN-γ and IL-22 transcripts. This functional output is subsequent to IAV-infected dendritic cells (DCs) activating type I NKT cells via toll-like receptor (TLR)-7/MYD88 signalling and type I NKT cell recognition of secreted IL-1β and IL-23 from IAV-infected DCs (79).

The release of IL-22, a critical Th17-related cytokine, from type I NKT cells had protected lung epithelial cells from IAV-mediated cell death whereas the depletion of IL-22 in mice had exacerbated the pathology of airway epithelium (79). In swine models, IAV infection had resulted in an increased frequency and activation of type I NKT cells within blood, lung lymph nodes, and broncho-alveolar lavage (80). It is likely that type I NKT cells may be instrumental in IAV infection of swine as these tissues are all notable in IAV pathology, however further research into the precise role of swine type I NKT cells in anti-IAV immunity is still necessary. These studies thus suggest that type I NKT cells are functionally dynamic in their ability to serve both an anti-viral and protective role against IAV infection.

3 Viral immunomodulation of the CD1d antigen presentation pathway

Due to the efficient viral clearance enacted by conventional $CD4^+$ and $CD8^+$ T cells, it is unsurprising that a myriad of viruses targets the classical antigen presentation pathways of both MHC class -I and -II molecules (81–84). Extensive research has shown that numerous viral infections also impact CD1d antigen presentation and thus type I NKT cell effector function, despite viruses not typically encoding lipid ligands (Figure 1).

SARS-CoV-2 is known to be highly successful at immune evasion and suppression. Observed in a human kidney epithelial cell-line, the SARS-CoV-2 envelope (E) protein was found to be responsible for the specific downregulation of mature CD1d molecules (39). As inhibition of proteasomal and lysosomal activity rescued the presence of mature CD1d, it was deduced that the downregulation of CD1d by SARS-CoV-2 is mediated by proteasomal and lysosomal-mediated degradation. However, as aforementioned, previous studies of human peripheral blood from SARS-CoV-2 infected patients showed no CD1d downregulation, thus prompting further research into SARS-CoV-2-mediated modulation of CD1d across different cell-types.

Human immunodeficiency virus (HIV) is an intensively researched virus which mainly infects $CD4^+$ T cells, leading to the destruction of cell-mediated immunity and thus impairing the body's overall immune response. In addition to the finding that $CD4^+$ type I NKT cells are also permissive to HIV-1 infection (85), it has also been shown that HIV-1 infection can interfere with CD1d expression and thus, CD1d-dependent activation of type I NKT cells (86, 87). Jurkat cells, which are an immortalised human T cell-line, were infected with GFP-HIV-1, resulting in CD1d being internalised and recycled back to the trans-golgi network (TGN) (86). Interestingly, in GFP-HIV-1 Nef deficient infections, there was minimal CD1d downregulation thus indicating that the immunomodulation of CD1d expression in HIV-1 infection is Nef-dependent. Upon replacing the tyrosine residues in the CD1d molecule cytoplasmic tail with alanine, CD1d expression was not impaired which proposes that the tyrosine-based residues of CD1d are the target of Nef-dependent CD1d internalisation. In HIV-1-infected DCs, the interaction of viral protein U (Vpu) with CD1d resulted in CD1d recycling and retention within the early endosome, thus inhibiting cell-surface presentation (87). Further

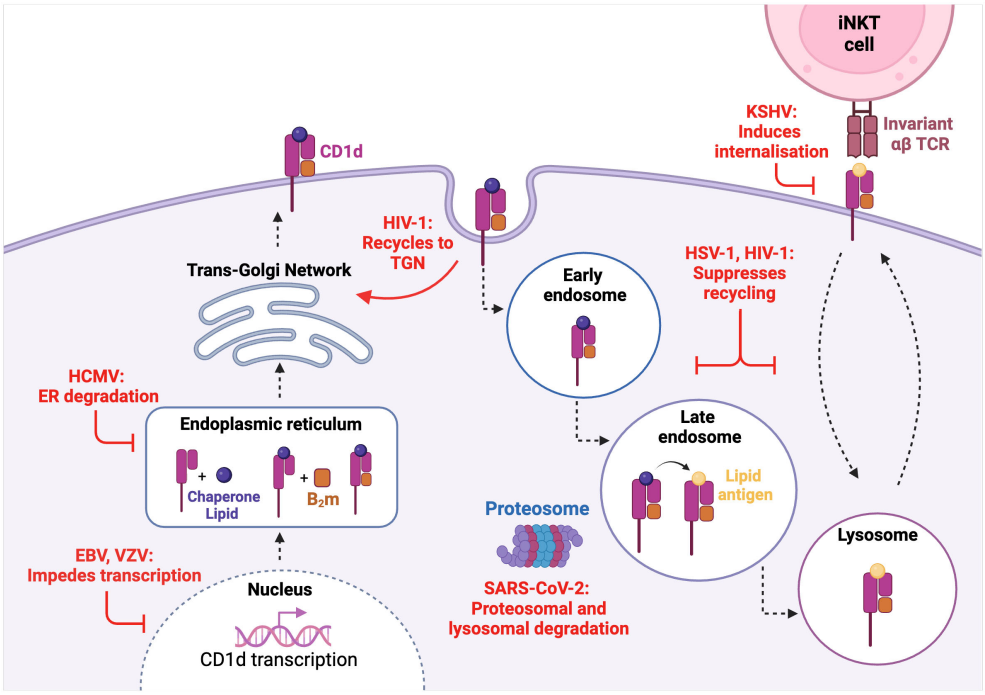

FIGURE 1
Viral interference of the CD1d molecule biosynthesis and recycling pathway. VZV and EBV impede CD1d transcription while HCMV induces CD1d molecular degradation in the enoplasmic reticulum (ER). HIV-1 and HSV-1 both suppress CD1d cell-surface recycling with HIV-1 also being shown to recycle CD1d back to the trans-golgi network (TGN). SARS-CoV-2 induces both proteasomal and lysosomal degradation of CD1d. KSHV induces CD1d endocytosis and internalisation.

investigation revealed that Vpu does not alter the rate of CD1d internalisation, but rather prevents the ability of CD1d to be subsequently recycled back to the cell-surface. Patients with HIV-1 are reported to display a reduced abundance of $CD4^+$ type I NKT cells (88, 89), which prompts that both the Nef- and Vpu-mediated retention of CD1d could be partially involved in lowered type I NKT cell activation.

The Herpesviruses family is highly ubiquitous and successful, a favourable outcome which is largely underscored by the ability of these viruses to manipulate and evade the host immune response to establish a life-long latent infection (90). Multiple herpesviruses target CD1d expression including HCMV, Kaposi-sarcoma associated herpesvirus (KSHV), EBV, VZV, and HSV-1. In contrast to SARS-CoV-2, the immature form of CD1d is more vulnerable to viral US2-mediated, ubiquitin-dependent proteasomal degradation in HCMV infection (91). The ubiquitination of the CD1d cytoplasmic tail by KSHV induces endocytosis and thus downregulation of cell-surface CD1d (92). Surprisingly, the modulator of immune recognition (MIR) -induced downregulation of CD1d in KSHV infection does not seem to heavily enhance lysosomal degradation, a mechanism that is commonly triggered upon ubiquitin-dependent internalisation (93). This suggests that although CD1d expression is hampered, KSHV has less involvement in its molecular degradation, which seems to be distinct to other viruses studied. During productive infection of human B cells with EBV, a gammaherpesvirus closely related to KSHV, the degradation of CD1d at a transcriptional level by the EBV shutoff protein BFL5 had been reported (94). Recently, VZV has also been shown to downregulate CD1d, which was evident at both a transcript and protein level (95). This downregulated phenotype was observed in both viral antigen-positive cells and VZV-exposed cells that remained viral antigen-negative, a phenomenon unique to VZV infection. This finding is of particular importance given that 'bystander' cells are also targeted by VZV in order to inhibit CD1d expression, implying that viral-mediated modulation is not restricted to VZV-infected cells only.

The viral HSV-1 proteins glycoprotein B (gB) and serine-threonine kinase (US3) have also been shown to inhibit the recycling capacity of CD1d in immortalised HeLa cells, thus suppressing type I NKT cell activation (96, 97). As previously discussed, HSV-1 infection of human keratinocytes does not downregulate cell-surface CD1d expression (38). Intriguingly, in human DCs infected with low titres of HSV-1, CD1d expression was upregulated (98). Downregulation of CD1d on HSV-1 infected DCs was only identified in cells with high viral titre (98). These results suggest that the viral-mediated modulation of CD1d is not only cell-type/virus dependent, but also reliant on viral titre.

The downregulation of CD1d by viruses has also been shown by human papilloma virus (HPV) (99), vaccinia virus (100, 101) and vesicular stomatitis virus (100). In contrast, HCV infection caused an upregulation of CD1d expression in chronically infected HCV-infected human liver tissue (74). This finding further suggests that other NKT cell types which hold a stronger CD1d- "self-reactive" profile, such as type II NKT cells, may play a role in anti-viral responses or in influencing type I NKT responses. Overall, it is highlighted that multiple viruses directly encode functions to target CD1d expression at various points in the biosynthesis and recycling pathway, which may contribute to the evolutionary success of certain viruses.

4 The interplay between lipid metabolism and viral infections

With such stark modulation of CD1d molecule expression and type I NKT cell function by viruses, the intricate relationship between the CD1d-iNKT cell axis and viral infections is evident. However, there remains postulation as to why viruses may target this unconventional immune cell axis despite its inability to recognise viral proteins. Interestingly, it has been recently reviewed that many viruses manipulate the lipid microenvironment of host cells to enhance the viral lifecycle, and that host lipid mediators may also play a role in the innate immune response to viral infections (102). Through manipulation of host lipid synthesis, it is possible that some viruses may indirectly modulate the presentation of endogenous lipid antigens on CD1d in infected cells, though this remains an understudied area that requires further research.

Hepatic steatosis is a common hallmark of pathology in chronic HCV infection and is characterised by the excess build-up of fat in liver cells. For HCV to efficiently replicate and spread, the HCV Core protein, the tail-interacting protein 47 (TIP47), and the non-structural viral protein 5A (NS5A) all cooperate to transfer viral RNA to lipid droplets (LDs) (103). These LDs act as sites for the construction and assembly of *de novo* virions, in which the Core-dependent recruitment of nonstructural (NS) proteins and replication complexes facilitates HCV production (104). Recent studies have established that although HCV infection does induce LD accumulation in a human hepatic cell line, the increased LD accumulation is not associated with greater levels of HCV Core protein activity (105). Therefore, the accumulation of intracellular LDs in HCV infection is not a direct result of HCV replication, which necessitates further investigation into the mechanisms behind the modulation of lipids during HCV infection. The increased presence of intracellular lipids upon HCV infection may ultimately increase the likelihood of the TCR-dependent activation of type I NKT cells, however, this remains undetermined and warrants further study. Given the potential regulatory role that type I NKT cells may play in HCV pathogenesis (59), we present a valid rationale as to why viruses such as HCV could target a lipid detecting effector cell or a lipid antigen presentation molecule, such as CD1d.

On a similar note, it has been demonstrated that SARS-CoV-2-infected primary human monocytes upregulate lipid metabolism and display an increased accumulation of intracellular LDs, which facilitate viral replication (106). Interestingly, the inhibition of LD synthesis decreases viral progeny production in SARS-CoV-2-infected monocytes and impedes SARS-CoV-2-induced cell death (106). This correlation between SARS-CoV-2-induced lipid accumulation and endogenous lipid ligand availability for type I NKT cell recognition is highly relevant. As mentioned earlier, type I NKT cells are also able to be activated via a cytokine-dependent manner, specifically through IL-12 and IL-18 detection (70). To this

end, SARS-CoV-2 infected monocytes exhibit an increased production of IL-12, while the inhibition of lipid synthesis downregulates IL-12 secretion (106). This suggests that the viral replication enabled by lipid synthesis may contribute to the IL-12 production from SARS-CoV-2 infected monocytes. Ultimately, the multi-faceted shut-down of the CD1d-iNKT cell axis by SARS-CoV-2 represents an immune evasion strategy to potentially counteract the increased lipid synthesis needed for viral spread, and thus the potential increase of type I NKT cell surveillance.

While some viruses may manipulate LD production to increase viral replication, it has recently been proposed that LD formation may also hold an anti-viral role too (107). Interestingly, the induction of LD formation following viral infection was exhibited by IAV, HSV-1, Zika virus (ZIKV), and Dengue virus (DENV). IAV infection of human THP-1 monocytes, and HSV-1, ZIKV, and DENV infection of immortalised astrocytes showed that an increased LD accumulation correlated with an enhanced IFN response and thus, a decrease in viral replication (107). Such findings implicate LD accumulation as a possible immune defence mechanism implemented to restrict viral replication rather than to solely facilitate it.

Although certain viruses exploit lipid synthesis to enable viral replication and dissemination, the connection to type I NKT cell activation must still be determined. Upon the detection of lipid ligands such as fatty acids, peroxisome proliferator-activated receptor (PPAR)γ, a lipid-activated transcription factor, is stimulated in DCs (108). PPARγ subsequentially triggers the transcription of retinaldehyde dehydrogenase type 2 (RALDH2) through the promotor activity of peroxisome proliferator responsive element (PPRE). This subsequently increases the abundance of all-trans retinoic acid (ATRA). ATRA then binds and activates the retinoic acid receptor (RAR)α which is found within the CD1d promoter site (109). Therefore, the binding of ATRA to RARα upregulates CD1d transcription and thus, molecular expression (Figure 2). As aforementioned, upregulated CD1d expression in a viral environment was indeed reported in HCV infection of human liver tissue (74). While increased lipid metabolism may facilitate *de novo* virion synthesis, the resulting LD accumulation may also indirectly lead to CD1d molecule upregulation and consequently, leave infected cells vulnerable to detection by type I NKT cells. Therefore, the immunomodulation of the CD1d-iNKT cell pathway by certain viruses may stand as an attempt to circumvent the anti-viral capacity of activated type I NKT cells.

5 Type I NKT cells in anti-viral immunotherapy and prophylaxis

The use of type I NKT cells in cancer therapeutic approaches is underpinned by their multifaceted ability to activate and enhance

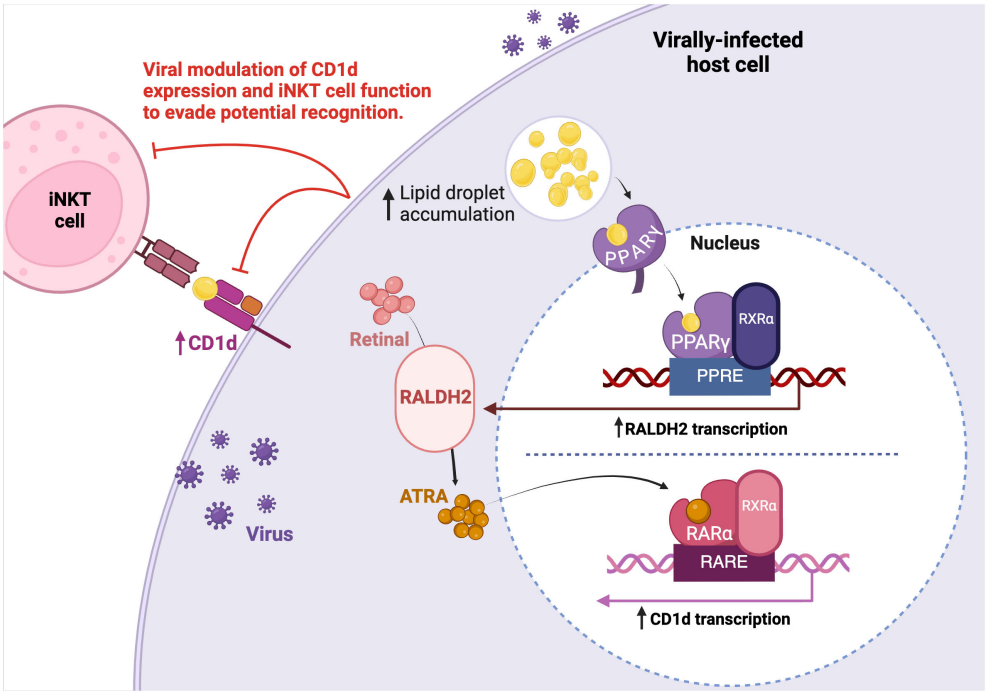

FIGURE 2
Viral modulation of host cell lipid metabolism. Multiple viruses have been identified to increase lipid droplet (LD) accumulation to facilitate replication. Upon increased LD accumulation, LDs bind to the PPARγ nuclear hormone receptor. PPARγ, which is associated to RXRα, binds to and enhances PPRE promoter activity to upregulate RALDH2 transcription. Increased retinal metabolism through RALDH2 activity leads to an increase of ATRA. ATRA activates the RARα receptor which is bound to the putative RARE promoter sequence found within the CD1d target gene, resulting in greater CD1d transcription. The increased lipid accumulation in consequence of viral replication may indirectly upregulate CD1d expression and thus, the viral modulation of lipid metabolism could promote TCR-dependent type I NKT cell recognition of an infected cell. Therefore, viruses may target CD1d expression and type I NKT cell function to evade potential recognition.

anti-tumour immunity. Such therapeutic advances have been seen through allogeneic human stem cell (HSC)-engineered type I NKT cells which have been able to induce potent anti-tumour NK cell activity (110). Moreover, type I NKT cells do not risk graft-versus-host disease (GVHD) following allogenic cancer therapy because they are unresponsive to mismatched MHC molecules between donor and patient, thus proving valuable in anti-tumour therapy applications (111). Such anti-tumour therapeutic advances also include generating chimeric antigen receptor (CAR)-type I NKT cells (30), in which these allogeneic CAR-type I NKT cells selectively target immunosuppressive cells in tumour environments (112), and also the *ex-vivo* expansion and activation of autologous iNKT cells (113). Induced pluripotent stem cell (iPSC)-derived type I NKT cells, which hold a similar genotype and functional profile to primary type I NKT cells, have also shown strong anti-tumour capacity and reduce the limitation of low type I NKT cell frequency in human peripheral blood (114).

Given the importance that type I NKT cells may also play in anti-viral immunity, type I NKT cells are now being harnessed for viral immunotherapy and prophylactic applications. As explored more specifically in this review, the anti-viral therapeutic use of type I NKT cells is being exploited through the adoptive administration of type I NKT cells to mediate viral pathology (115) and through the administration of glycolipid analogues as vaccine adjuvants (115–117).

Allogenic cell-based therapy, where a single donor's immune cells are modified and introduced back into multiple patients' blood, is emerging as a promising immunotherapy approach. AgenT-797 is an allogeneic, *ex-vivo* expanded type I NKT cell product (115). Preliminary clinical use against acute respiratory distress syndrome (ARDS) induced by SARS-CoV-2, has shown value in secondary infection prevention and rescue of exhausted T cells (115). Moreso, the key markers of cytokine response syndrome (CRS) were unchanged, with the general cytokine response post-administration favouring an anti-inflammatory profile. Such results indicate that agenT-797 has a dual role in preventing both virus and immune-mediated pathogenesis in SARS-CoV-2 induction of ARDS. The success of cellular therapeutic approaches for viral infections is underpinned by their longevity and persistence within the hosts' immune system post-administration. In this respect, agenT-797 remained detectable within patient blood and bronchoalveolar lavage (BAL) throughout hospitalisation, with patients who received cardiopulmonary bypass sustaining a stronger retention (115).

IL-4, initially coined as B cell growth factor-1 (BSF-1), plays a significant role in B cell activation and differentiation, and thus is partly responsible for antibody secretion (118, 119). During early stages of influenza infection, type I NKT cells have been found to comprise approximately 70% of the IL-4 producing cells in patient lymph node samples and thus are critical for infection resolution (120). The genetic patterns of type I NKT cells and IL-4 secretion have also corresponded with the abundance of antibodies in macaques infected with ZIKV (120). Recently, a glycolipid agonist adjuvanted to the SARS-CoV-2 RBD-Fc protein 'αGC-CPOEt' has shown promise as an effective SARS-CoV-2 vaccine adjuvant in murine models, with the ability to induce a greater secretion of IL-4 from type I NKT cells when compared to a vaccine adjuvanted by α-GalCer (116). Multiple administrations of αGC-CPOEt-adjuvanted vaccinations resulted in increased levels of neutralising antibodies against SARS-CoV-2 (116). This resolves a setback presented by α-GalCer adjuvanted vaccines as multiple exposures to α-GalCer may stun type I NKT cells into anergy and unresponsiveness (121).

A prominent hurdle of vaccine production is the constantly changing SARS-CoV-2 variants which hold distinct antigenic profiles from existing vaccine strains. To address this, a novel type I NKT cell agonist 7DW8-5 has recently shown protection against three antigenically distinct mouse-adapted SARS-CoV-2 strains when administered pre-infection (117). 7DW8-5 is an α-GalCer analogue, which through various biological assays was found to be more potent than α-GalCer at activation of type I NKT cells (122). Analysis of the cytokine profile post-administration of 7DW8-5 showed a skewing towards IFN-γ production from type I NKT, NK, T, and γδ T cells (117). Interestingly, in IFN-γ knockout mice, the anti-viral potential of 7DW8-5 was completely lost, which implies that the potent anti-viral effect of 7DW8-5 is dependent on the induction of an IFN-γ response. In testing whether 7DW8-5 induced anergy in type I NKT cells upon secondary administration, it was established that the repeated administration of 7DW8-5 at both low and high doses did not induce anergy and had maintained protective efficacy (117). Although this agonist still requires extensive clinical testing, *in vitro* testing in human type I NKT cells corroborates with the protective adjuvant activity of 7DW8-5 seen in HIV and malaria murine vaccines (122).

Similar to SARS-CoV-2, swine IAV inflicts a major disease burden in pig populations due to the virus' ability to evolve and develop drug resistance rapidly. Zoonotic IAV strains can also be transmitted to humans and therefore, swine IAV presents a substantial burden for human populations too (123). The intranasal administration of α-GalCer to H1N1 IAV-infected piglets resulted in complete amelioration of body weight, flu symptoms, and IAV-induced destruction of lung architecture (124). Moreover, α-GalCer-treated piglets had significantly reduced IAV titres compared to untreated piglets (124). This reinforces that activated type I NKT cells could serve as an effective, long-term therapeutic target against swine IAV infection, especially as it may prove difficult for IAV to adapt to the broad functionality of type I NKT cells. Within a prophylactic context, the intramuscular and intranasal administration of α-GalCer to pigs prior to IAV infection did not reduce subsequent viral replication or shedding (125). However, more recent studies have examined the efficacy of α-GalCer treatments for IAV infection in comparison to oseltamivir (126), a widely used anti-viral drug that blocks IAV virion release and spread (127). Here, α-GalCer treatment was ineffective at stimulating an anti-IAV immune response in pigs whereas oseltamivir was able to significantly reduce lung immunopathology and viral spread, suggesting that α-GalCer treatment for swine IAV infection may be highly variable (126). The intranasal co-immunisation of mice with α-GalCer and IAV hemagglutinin glytoprotein had offered substantial protection by inducing a strong mucosal

immune response (128). Pigs however represent a more translational animal model than mice, so the therapeutic potential of swine type I NKT cells may be a predictor of type I NKT cell therapy effectiveness in humans with IAV and warrants further investigation.

ABX196 is a variant of α-GalCer with a galactosyl 6-deoxy-6-N-acyl modification and produces a more potent agonistic activity in murine type I NKT cells when compared to the super agonist PBS-57 (129). Preclinical studies in ABX196-treated mice had indicated a large production of IFN-γ from type I NKT cells and NK cells, and did not generate substantial toxicity at any doses (129). Due to the monomorphic nature of the CD1d molecule, ABX196 was also able to be assessed in human subjects as a prophylactic vaccine in combination with HBV surface antigen (HBsAg). In a large portion of patients, an effective anti-HBV antibody response was generated, which is especially noteworthy given the poor immunogenicity of HBsAg. This agonist also established sufficient protective immunity against HBV after only one administration. Activation of liver type I NKT cells by ABX196 did induce cytotoxicity and cellular damage, however future studies can focus on altering the systemic delivery system to overcome this side effect. More recently, a conjugate vaccine platform has incorporated both α-GalCer and HBV viral antigens, such that antigen presenting cells are able to simultaneously activate type I NKT cells and HBV-specific $CD8^+$ T cells respectively (130). This co-delivery vaccine design had successfully improved viral clearance in a murine model of chronic HBV however efficacy and safety within human populations is still being evaluated (130). In previous human studies, the treatment of α-GalCer alone produced significant immune activation however this was not enough to efficiently clear HBV, suggesting that the co-delivery of α-GalCer and virus-specific antigens may be a better alternative (131). Unlike the polymorphic MHC molecules, CD1d is a monomorphic molecule and is highly conserved between species and individuals (132), thus representing a likely candidate to explore for future therapeutic gain.

Overall, NKT cells are a functionally dynamic and highly competent cell type that are well-documented in their ability to infiltrate tumour microenvironments and secrete anti-tumour cytokines. In addition, glycolipids are demonstrating significant protection and enhanced efficacy as adjuvants for vaccines against murine models of malaria (133) and as combination treatments with antibiotics against tuberculosis (134). Recent data has introduced a multitude of strategies to harness the multifaceted function of type I NKT cells in anti-viral prophylaxis or treatment, with each approach becoming increasingly valuable.

6 Concluding remarks

Type I NKT cells and CD1d antigen presentation molecules represent increasingly relevant players in host responses to viral infections. Although a direct causal relationship between the viral manipulation of host lipid metabolism and type I NKT cell function has yet to be meticulously explored, this review presents a perspective as to why viruses could view the CD1d presentation pathway and type I NKT cells as ideal targets for exploitation. In better understanding the intricate interaction between the CD1d-iNKT cell pathway and viral infections, type I NKT cells could be more prominently placed at the forefront of future viral prophylactic and therapeutic approaches, given their ability to quickly secrete cytokines and aid immune responses. Future exploration into this area may also divulge a better understanding of the unexplored role of type II NKT cells in viral infections and thus, reveal a new appreciation for NKT cells in viral infections.

Author contributions

VR: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. LS: Supervision, Writing – review & editing. CFA: Supervision, Writing – review & editing. BS: Supervision, Writing – review & editing, Funding acquisition. AA: Supervision, Writing – review & editing, Conceptualization, Funding acquisition, Project administration.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

All figures made with BioRender.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

References

1. Godfrey DI, Macdonald HR, Kronenberg M, Smyth MJ, Kaer LV. NKT cells: what's in a name? *Nat Rev Immunol*. (2004) 4:231–7. doi: 10.1038/nri1309

2. Lantz O, Bendelac A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med*. (1994) 180:1097–106. doi: 10.1084/jem.180.3.1097

3. Dellabona P, Padovan E, Casorati G, Brockhaus M, Lanzavecchia A. An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. *J Exp Med*. (1994) 180:1171–6. doi: 10.1084/jem.180.3.1171

4. Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med*. (1993) 178:1–16. doi: 10.1084/jem.178.1.1

5. Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, et al. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med*. (1998) 188:1521–8. doi: 10.1084/jem.188.8.1521

6. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol*. (2008) 20:358–68. doi: 10.1016/j.coi.2008.03.018

7. Thierry A, Robin A, Giraud S, Minouflet S, Barra A, Bridoux F, et al. Identification of invariant natural killer T cells in porcine peripheral blood. *Vet Immunol Immunopathol*. (2012) 149:272–9. doi: 10.1016/j.vetimm.2012.06.023

8. Yang G, Artiaga BL, Lomelino CL, Jayaprakash AD, Sachidanandam R, Mckenna R, et al. Next generation sequencing of the pig αβ TCR repertoire identifies the porcine invariant NKT cell receptor. *J Immunol*. (2019) 202:1981–91. doi: 10.4049/jimmunol.1801171

9. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of V(α)14 NKT cells by glycosylceramides. *Science*. (1997) 278:1626–9. doi: 10.1126/science.278.5343.1626

10. Kinjo Y, Kitano N, Kronenberg M. The role of invariant natural killer T cells in microbial immunity. *J Infect Chemother*. (2013) 19:560–70. doi: 10.1007/s10156-013-0638-1

11. Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, et al. Structure-Activity Relationship of.alpha.-Galactosylceramides against B16-Bearing Mice. *J Med Chem*. (1995) 38:2176–87. doi: 10.1021/jm00012a018

12. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med*. (2002) 195:625–36. doi: 10.1084/jem.20011786

13. Godfrey DI, Uldrich AP, Mccluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nat Immunol*. (2015) 16:1114–23. doi: 10.1038/ni.3298

14. Rhost S, Sedimbi S, Kadri N, Cardell SL. Immunomodulatory type II natural killer T lymphocytes in health and disease. *Scand J Immunol*. (2012) 76:246–55. doi: 10.1111/j.1365-3083.2012.02750.x

15. Singh AK, Tripathi P, Cardell SL. Type II NKT cells: an elusive population with immunoregulatory properties. *Front Immunol*. (2018) 9. doi: 10.3389/fimmu.2018.01969

16. Almeida CF, Smith DGM, Cheng T-Y, Harpur CM, Batleska E, Nguyen-Robertson CV, et al. Benzofuran sulfonates and small self-lipid antigens activate type II NKT cells via CD1d. *Proc Natl Acad Sci U.S.A*. (2021) 118:e2104420118. doi: 10.1073/pnas.2104420118

17. Van Rhijn I, Young DC, Im JS, Levery SB, Illarionov PA, Besra GS, et al. CD1d-restricted T cell activation by nonlipidic small molecules. *Proc Natl Acad Sci U.S.A*. (2004) 101:13578–83. doi: 10.1073/pnas.0402838101

18. Brigl M, Tatituri RVV, Watts GFM, Bhowruth V, Leadbetter EA, Barton N, et al. Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection. *J Exp Med*. (2011) 208:1163–77. doi: 10.1084/jem.20102555

19. Lezmi G, Leite-de-Moraes M. Invariant natural killer T and mucosal-associated invariant T cells in asthmatic patients. *Front Immunol*. (2018) 9:1766. doi: 10.3389/fimmu.2018.01766

20. Sag D, Özkan M, Kronenberg M, Wingender G. Improved detection of cytokines produced by invariant NKT cells. *Sci Rep*. (2017) 7:16607. doi: 10.1038/s41598-017-16832-1

21. Bai L, Constantinides MG, Thomas SY, Reboulet R, Meng F, Koentgen F, et al. Distinct APCs explain the cytokine bias of α-galactosylceramide variants *in vivo*. *J Immunol*. (2012) 188:3053–61. doi: 10.4049/jimmunol.1102414

22. Uldrich AP, Crowe NY, Kyparissoudis K, Pellicci DG, Zhan Y, Lew AM, et al. NKT cell stimulation with glycolipid antigen *in vivo*: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *J Immunol*. (2005) 175:3092–101. doi: 10.4049/jimmunol.175.5.3092

23. Gadola SD, Silk JD, Jeans A, Illarionov PA, Salio M, Besra GS, et al. Impaired selection of invariant natural killer T cells in diverse mouse models of glycosphingolipid lysosomal storage diseases. *J Exp Med*. (2006) 203:2293–303. doi: 10.1084/jem.20060921

24. Reilly EC, Wands JR, Brossay L. Cytokine dependent and independent iNKT cell activation. *Cytokine*. (2010) 51:227–31. doi: 10.1016/j.cyto.2010.04.016

25. Engel I, Seumois G, Chavez L, Samaniego-Castruita D, White B, Chawla A, et al. Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat Immunol*. (2016) 17:728–39. doi: 10.1038/ni.3437

26. Konishi J, Yamazaki K, Yokouchi H, Shinagawa N, Iwabuchi K, Nishimura M. The characteristics of human NKT cells in lung cancer—CD1d independent cytotoxicity against lung cancer cells by NKT cells and decreased human NKT cell response in lung cancer patients. *Hum Immunol*. (2004) 65:1377–88. doi: 10.1016/j.humimm.2004.09.003

27. Olson CM Jr., Bates TC, Izadi H, Radolf JD, Huber SA, Boyson JE, et al. Local production of IFN-γ by invariant NKT cells modulates acute lyme carditis1. *J Immunol*. (2009) 182:3728–34. doi: 10.4049/jimmunol.0804111

28. Xu Y, Wang Z, Du X, Liu Y, Song X, Wang T, et al. Tim-3 blockade promotes iNKT cell function to inhibit HBV replication. *J Cell Mol Med*. (2018) 22:3192–201. doi: 10.1111/jcmm.13600

29. Leadbetter EA, Brigl M, Illarionov P, Cohen N, Luteran MC, Pillai S, et al. NK T cells provide lipid antigen-specific cognate help for B cells. *Proc Natl Acad Sci U.S.A*. (2008) 105:8339–44. doi: 10.1073/pnas.0801375105

30. Liu Y, Wang G, Chai D, Dang Y, Zheng J, Li H. iNKT: A new avenue for CAR-based cancer immunotherapy. *Transl Oncol*. (2022) 17:101342. doi: 10.1016/j.tranon.2022.101342

31. Li Y-Q, Yan C, Luo R, Liu Z. iNKT cell agonists as vaccine adjuvants to combat infectious diseases. *Carbohydr Res*. (2022) 513:108527. doi: 10.1016/j.carres.2022.108527

32. Mattner J, Debord KL, Ismail N, Goff RD, Cantu C, Zhou D, et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature*. (2005) 434:525–9. doi: 10.1038/nature03408

33. Zeissig S, Murata K, Sweet L, Publicover J, Hu Z, Kaser A, et al. Hepatitis B virus–induced lipid alterations contribute to natural killer T cell–dependent protective immunity. *Nat Med*. (2012) 18:1060–8. doi: 10.1038/nm.2811

34. Holzapfel KL, Tyznik AJ, Kronenberg M, Hogquist KA. Antigen-dependent versus -independent activation of invariant NKT cells during infection. *J Immunol*. (2014) 192:5490–8. doi: 10.4049/jimmunol.1400722

35. He B. Viruses, endoplasmic reticulum stress, and interferon responses. *Cell Death Differ*. (2006) 13:393–403. doi: 10.1038/sj.cdd.4401833

36. Govindarajan S, Verheugen E, Venken K, Gaublomme D, Maelegheer M, Cloots E, et al. ER stress in antigen-presenting cells promotes NKT cell activation through endogenous neutral lipids. *EMBO Rep*. (2020) 21:e48927. doi: 10.15252/embr.201948927

37. Cheng T-Y, Praveena T, Govindarajan S, Almeida CF. Lipidomic scanning of self-lipids identifies headless antigens for Natural Killer T cells. *PNAS*. (2024) 121. doi: 10.1073/pnas.2321686121

38. Bosnjak L, Sahlström P, Paquin-Proulx D, Leeansyah E, Moll M, Sandberg JK. Contact-dependent interference with invariant NKT cell activation by herpes simplex virus-infected cells. *J Immunol*. (2012) 188:6216–24. doi: 10.4049/jimmunol.1100218

39. Lu H, Liu Z, Deng X, Chen S, Zhou R, Zhao R, et al. Potent NKT cell ligands overcome SARS-CoV-2 immune evasion to mitigate viral pathogenesis in mouse models. *PloS Pathog*. (2023) 19:e1011240. doi: 10.1371/journal.ppat.1011240

40. Dropulic LK, Cohen JI. Severe viral infections and primary immunodeficiencies. *Clin Infect Dis*. (2011) 53:897–909. doi: 10.1093/cid/cir610

41. Levy O, Orange JS, Hibberd P, Steinberg S, Larussa P, Weinberg A, et al. Disseminated Varicella infection due to the vaccine strain of varicella-zoster virus, in a patient with a novel deficiency in Natural Killer T Cells. *J Infect Dis*. (2003) 188:948–53. doi: 10.1086/378503

42. Banovic T, Yanilla M, Simmons R, Robertson I, Schroder WA, Raffelt NC, et al. Disseminated Varicella infection caused by Varicella vaccine strain in a child with low invariant Natural Killer T Cells and diminished CD1d expression. *J Infect Dis*. (2011) 204:1893–901. doi: 10.1093/infdis/jir660

43. Novakova L, Lehuen A, Novak J. Low numbers and altered phenotype of invariant natural killer T cells in recurrent varicella zoster virus infection. *Cell Immunol*. (2011) 269:78–81. doi: 10.1016/j.cellimm.2011.04.008

44. Mukai T, Waki K. X-linked lymphoproliferative syndrome associated with Epstein-Barr virus encephalitis and lymphoproliferative disorder. *Clin Case Rep*. (2023) 11:e7949. doi: 10.1002/ccr3.7949

45. Arvin AM. Varicella-zoster virus. *Clin Microbiol Rev*. (1996) 9:361–81. doi: 10.1128/CMR.9.3.361

46. Weinberg A, Lazar AA, Zerbe GO, Hayward AR, Chan IS, Vessey R, et al. Influence of age and nature of primary infection on varicella-zoster virus-specific cell-mediated immune responses. *J Infect Dis*. (2010) 201:1024–30. doi: 10.1086/651199

47. Kennedy PGE, Steiner I. A molecular and cellular model to explain the differences in reactivation from latency by herpes simplex and varicella–zoster viruses. *Neuropathol Appl Neurobiol*. (1994) 20:368–74. doi: 10.1111/j.1365-2990.1994.tb00982.x

48. Kogure T, Fujinaga H, Niizawa A, Hai LX, Shimada Y, Ochiai H, et al. Killer-cell inhibitory receptors, CD158a/b, are upregulated by interleukin-2, but not interferon-gamma or interleukin-4. *Mediators Inflammation*. (1999) 8:313–8. doi: 10.1080/09629359990324

49. Wallace MR, Woelfl I, Bowler WA, Olson PE, Murray NB, Brodine SK, et al. Tumor necrosis factor, interleukin-2, and interferon-gamma in adult varicella. *J Med Virol*. (1994) 43:69–71. doi: 10.1002/jmv.1890430113

50. Levin MJ, Smith JG, Kaufhold RM, Barber D, Hayward AR, Chan CY, et al. Decline in varicella-zoster virus (VZV)–specific cell-mediated immunity with increasing age and boosting with a high-dose VZV vaccine. *J Infect Dis*. (2003) 188:1336–44. doi: 10.1086/379048

51. Cenoz MG, Martínez-Artola V, Guevara M, Ezpeleta C, Barricarte A, Castilla J. Effectiveness of one and two doses of varicella vaccine in preventing laboratory-confirmed cases in children in Navarre, Spain. *Hum Vaccin Immunother*. (2013) 9:1172–6. doi: 10.4161/hv.23451

52. Bibi Z, Nawaz AD, Al Kurbi M, Fakhroo S, Ferih K, Al-Jaber N, et al. Real-world effectiveness of the varicella vaccine among children and adolescents in Qatar: A case-control study. *Vaccines (Basel)*. (2023) 11:1567. doi: 10.3390/vaccines11101567

53. Kramer JM, Larussa P, Tsai WC, Carney P, Leber SM, Gahagan S, et al. Disseminated vaccine strain varicella as the acquired immunodeficiency syndrome-defining illness in a previously undiagnosed child. *Pediatrics*. (2001) 108:e39. doi: 10.1542/peds.108.2.e39

54. Ghaffar F, Carrick K, Rogers BB, Margraf LR, Krisher K, Ramilo O. Disseminated infection with varicella-zoster virus vaccine strain presenting as hepatitis in a child with adenosine deaminese deficiency. *Pediatr Infect Dis*. (2000) 19:764. doi: 10.1097/00006454-200008000-00022

55. Nichols KE, Hom J, Gong SY, Ganguly A, Ma CS, Cannons JL, et al. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med*. (2005) 11:340–5. doi: 10.1038/nm1189

56. Schneidawind D, Duerr-Stoerzer S, Liewer S, Renner S, Sánchez Navarro B, Atar D, et al. Low graft invariant natural killer T-cell dose is a risk factor for cytomegalovirus reactivation after allogeneic hematopoietic cell transplantation. *Transpl Cell Ther*. (2022) 28:513.e1–4. doi: 10.1016/j.jtct.2022.05.011

57. Kaneko Y, Harada M, Kawano T, Yamashita M, Shibata Y, Gejyo F, et al. Augmentation of Valpha14 NKT cell-mediated cytotoxicity by interleukin 4 in an autocrine mechanism resulting in the development of concanavalin A-induced hepatitis. *J Exp Med*. (2000) 191:105–14. doi: 10.1084/jem.191.1.105

58. Ito H, Ando K, Ishikawa T, Nakayama T, Taniguchi M, Saito K, et al. Role of Vα14+ NKT cells in the development of Hepatitis B virus-specific CTL: activation of Vα14+ NKT cells promotes the breakage of CTL tolerance. *Int Immunol*. (2008) 20:869–79. doi: 10.1093/intimm/dxn046

59. Raus S, Lopez-Scarim J, Luthy J, Billerbeck E. Hepatic iNKT cells produce type 2 cytokines and restrain antiviral T cells during acute hepacivirus infection. *Front Immunol*. (2022) 13:953151. doi: 10.3389/fimmu.2022.953151

60. Seino KI, Fukao K, Muramoto K, Yanagisawa K, Takada Y, Kakuta S, et al. Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. *Proc Natl Acad Sci U.S.A*. (2001) 98:2577–81. doi: 10.1073/pnas.041608298

61. Broxmeyer HE, Dent A, Cooper S, Hangoc G, Wang Z-Y, Du W, et al. A role for natural killer T cells and CD1d molecules in counteracting suppression of hematopoiesis in mice induced by infection with murine cytomegalovirus. *Exp Hematol*. (2007) 35:87–93. doi: 10.1016/j.exphem.2007.01.015

62. Grubor-Bauk B, Simmons A, Mayrhofer G, Speck PG. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant Vα14-Jα281 TCR1. *J Immunol*. (2003) 170:1430–4. doi: 10.4049/jimmunol.170.3.1430

63. Bedel R, Matsuda JL, Brigl M, White J, Kappler J, Marrack P, et al. Lower TCR repertoire diversity in Traj18-deficient mice. *Nat Immunol*. (2012) 13:705–6. doi: 10.1038/ni.2347

64. Rao P, Wen X, Lo JH, Kim S, Li X, Chen S, et al. Herpes simplex virus 1 specifically targets human CD1d antigen presentation to enhance its pathogenicity. *J Virol*. (2018) 92:e01490-18. doi: 10.1128/JVI.01490-18

65. Chiu Y-H, Park S-H, Benlagha K, Forestier C, Jayawardena-Wolf J, Savage PB, et al. Multiple defects in antigen presentation and T cell development by mice expressing cytoplasmic tail–truncated CD1d. *Nat Immunol*. (2002) 3:55–60. doi: 10.1038/ni740

66. Yang G, Artiaga BL, Hackmann TJ, Samuel MS, Walters EM, Salek-Ardakani S, et al. Targeted disruption of CD1d prevents NKT cell development in pigs. *Mamm Genome*. (2015) 26:264–70. doi: 10.1007/s00335-015-9564-0

67. Yang J, Chang T, Tang L, Deng H, Chen D, Luo J, et al. Increased expression of tim-3 is associated with depletion of NKT cells in SARS-CoV-2 infection. *Front Immunol*. (2022) 13:796682. doi: 10.3389/fimmu.2022.796682

68. Liu J, Yang X, Wang H, Li Z, Deng H, Liu J, et al. Analysis of the long-term impact on cellular immunity in COVID-19-recovered individuals reveals a profound NKT cell impairment. *mBio*. (2021) 12:e00085-21. doi: 10.1128/mBio.00085-21

69. Jouan Y, Guillon A, Gonzalez L, Perez Y, Boisseau C, Ehrmann S, et al. Phenotypical and functional alteration of unconventional T cells in severe COVID-19 patients. *J Exp Med*. (2020) 217:e20200872. doi: 10.1084/jem.20200872

70. Leite-de-Moraes MC, Hameg A, Arnould A, Machavoine FO, Koezuka Y, Schneider E, et al. A distinct IL-18-induced pathway to fully activate NK T lymphocytes independently from TCR engagement. *J Immunol*. (1999) 163:5871–6. doi: 10.4049/jimmunol.163.11.5871

71. Sobkowiak MJ, Paquin-Proulx D, Bosnjak L, Moll M, Sällberg Chen M, Sandberg JK. Dynamics of IL-15/IL-15R-α expression in response to HSV-1 infection reveal a novel mode of viral immune evasion counteracted by iNKT cells. *Eur J Immunol*. (2022) 52:462–71. doi: 10.1002/eji.202149287

72. Missiha SB, Ostrowski M, Heathcote EJ. Disease progression in chronic hepatitis C: modifiable and nonmodifiable factors. *Gastroenterology*. (2008) 134:1699–714. doi: 10.1053/j.gastro.2008.02.069

73. Senff T, Menne C, Cosmovici C, Lewis-Ximenez LL, Aneja J, Broering R, et al. Peripheral blood iNKT cell activation correlates with liver damage during acute hepatitis C. *JCI Insight*. (2022) 7:e155432. doi: 10.1172/jci.insight.155432

74. Durante-Mangoni E, Wang R, Shaulov A, He Q, Nasser I, Afdhal N, et al. Hepatic CD1d expression in hepatitis C virus infection and recognition by resident proinflammatory CD1d-reactive T cells. *J Immunol*. (2004) 173:2159–66. doi: 10.4049/jimmunol.173.3.2159

75. Chambers BS, Parkhouse K, Ross TM, Alby K, Hensley SE. Identification of hemagglutinin residues responsible for H3N2 antigenic drift during the 2014-2015 influenza season. *Cell Rep*. (2015) 12:1–6. doi: 10.1016/j.celrep.2015.06.005

76. de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJD, Chau TNB, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. *Nat Med*. (2006) 12:1203–7. doi: 10.1038/nm1477

77. Ho L-P, Denney L, Luhn K, Teoh D, Clelland C, Mcmichael AJ. Activation of invariant NKT cells enhances the innate immune response and improves the disease course in influenza A virus infection. *Eur J Immunol*. (2008) 38:1913–22. doi: 10.1002/eji.200738017

78. De Santo C, Salio M, Masri SH, Lee LY, Dong T, Speak AO, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. *J Clin Invest*. (2008) 118:4036–48. doi: 10.1172/JCI36264

79. Paget C, Ivanov S, Fontaine J, Renneson J, Blanc F, Pichavant M, et al. Interleukin-22 is produced by invariant natural killer T lymphocytes during influenza A virus infection: potential role in protection against lung epithelial damages *. *J Biol Chem*. (2012) 287:8816–29. doi: 10.1074/jbc.M111.304758

80. Schäfer A, Hühr J, Schwaiger T, Dorhoi A, Mettenleiter TC, Blome S, et al. Porcine invariant natural killer T cells: functional profiling and dynamics in steady state and viral infections. *Front Immunol*. (2019) 10. doi: 10.3389/fimmu.2019.01380

81. Abendroth A, Lin I, Slobedman B, Ploegh H, Arvin AM. Varicella-zoster virus retains major histocompatibility complex class I proteins in the Golgi compartment of infected cells. *J Virol*. (2001) 75:4878–88. doi: 10.1128/JVI.75.10.4878-4888.2001

82. Taefehshokr N, Lac A, Vrieze AM, Dickson BH, Guo PN, Jung C, et al. SARS-CoV-2 NSP5 antagonizes MHC II expression by subverting histone deacetylase 2. *J Cell Sci*. (2024) 137:jcs262172. doi: 10.1242/jcs.262172

83. Neumann JR, Eis-HüBinger AM, Koch N. Herpes simplex virus type 1 targets the MHC class II processing pathway for immune evasion. *J Immunol*. (2003) 171:3075–83. doi: 10.4049/jimmunol.171.6.3075

84. Abendroth A, Slobedman B, Lee E, Mellins E, Wallace M, Arvin AM. Modulation of major histocompatibility class II protein expression by varicella-zoster virus. *J Virol*. (2000) 74:1900–7. doi: 10.1128/JVI.74.4.1900-1907.2000

85. Motsinger A, Haas DW, Stanic AK, Van Kaer L, Joyce S, Unutmaz D. CD1d-restricted human natural killer T cells are highly susceptible to human immunodeficiency virus 1 infection. *J Exp Med*. (2002) 195:869–79. doi: 10.1084/jem.20011712

86. Chen N, Mccarthy C, Drakesmith H, Li D, Cerundolo V, Mcmichael AJ, et al. HIV-1 down-regulates the expression of CD1d via Nef. *Eur J Immunol*. (2006) 36:278–86. doi: 10.1002/eji.200535487

87. Moll M, Andersson SK, Smed-Sörensen A, Sandberg JK. Inhibition of lipid antigen presentation in dendritic cells by HIV-1 Vpu interference with CD1d recycling from endosomal compartments. *Blood*. (2010) 116:1876–84. doi: 10.1182/blood-2009-09-243667

88. Sandberg JK, Fast NM, Palacios EH, Fennelly G, Dobroszycki J, Palumbo P, et al. Selective loss of innate CD4(+) V alpha 24 natural killer T cells in human immunodeficiency virus infection. *J Virol*. (2002) 76:7528–34. doi: 10.1128/JVI.76.15.7528-7534.2002

89. Paquin-Proulx D, Lal KG, Phuang-Ngern Y, Creegan M, Tokarev A, Suhkumvittaya S, et al. Preferential and persistent impact of acute HIV-1 infection on CD4(+) iNKT cells in colonic mucosa. *Proc Natl Acad Sci U.S.A*. (2021) 118:e2104721118. doi: 10.1073/pnas.2104721118

90. Cohrs RJ, Gilden DH. Human herpesvirus latency. *Brain Pathol*. (2001) 11:465–74. doi: 10.1111/j.1750-3639.2001.tb00415.x

91. Han J, Rho SB, Lee JY, Bae J, Park SH, Lee SJ, et al. Human cytomegalovirus (HCMV) US2 protein interacts with human CD1d (hCD1d) and down-regulates invariant NKT (iNKT) cell activity. *Mol Cells*. (2013) 36:455–64. doi: 10.1007/s10059-013-0221-8

92. Sanchez DJ, Gumperz JE, Ganem D. Regulation of CD1d expression and function by a herpesvirus infection. *J Clin Invest*. (2005) 115:1369–78. doi: 10.1172/JCI200524041

93. Coscoy L, Ganem D. Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proc Natl Acad Sci U.S.A.* (2000) 97:8051–6. doi: 10.1073/pnas.140129797

94. van Gent M, Gram AM, Boer IGJ, Geerdink RJ, Lindenbergh MFS, Lebbink RJ, et al. Silencing the shutoff protein of Epstein–Barr virus in productively infected B cells points to (innate) targets for immune evasion. *J Gen Virol.* (2015) 96:858–65. doi: 10.1099/jgv.0.000021

95. Traves R, Opadchy T, Slobedman B, Abendroth A. Varicella zoster virus downregulates expression of the nonclassical antigen presentation molecule CD1d. *J Infect Dis.* (2023) 230:jiad512. doi: 10.1093/infdis/jiad512

96. Rao P, Pham HT, Kulkarni A, Yang Y, Liu X, Knipe DM, et al. Herpes simplex virus 1 glycoprotein B and US3 collaborate to inhibit CD1d antigen presentation and NKT cell function. *J Virol.* (2011) 85:8093–104. doi: 10.1128/JVI.02689-10

97. Yuan W, Dasgupta A, Cresswell P. Herpes simplex virus evades natural killer T cell recognition by suppressing CD1d recycling. *Nat Immunol.* (2006) 7:835–42. doi: 10.1038/ni1364

98. Raftery MJ, Winau F, Kaufmann SHE, Schaible UE, SchöNrich GN. CD1 antigen presentation by human dendritic cells as a target for herpes simplex virus immune evasion. *J Immunol.* (2006) 177:6207–14. doi: 10.4049/jimmunol.177.9.6207

99. Miura S, Kawana K, Schust DJ, Fujii T, Yokoyama T, Iwasawa Y, et al. CD1d, a sentinel molecule bridging innate and adaptive immunity, is downregulated by the human papillomavirus (HPV) E5 protein: a possible mechanism for immune evasion by HPV. *J Virol.* (2010) 84:11614–23. doi: 10.1128/JVI.01053-10

100. Renukaradhya GJ, Webb TJR, Khan MA, Lin YL, Du W, Gervay-Hague J, et al. Virus-induced inhibition of CD1d1-mediated antigen presentation: reciprocal regulation by p38 and ERK1. *J Immunol.* (2005) 175:4301–8. doi: 10.4049/jimmunol.175.7.4301

101. Webb TJR, Litavecz RA, Khan MA, Du W, Gervay-Hague J, Renukaradhya GJ, et al. Inhibition of CD1d1-mediated antigen presentation by the vaccinia virus B1R and H5R molecules. *Eur J Immunol.* (2006) 36:2595–600. doi: 10.1002/eji.200636024

102. Monson EA, Trenerry AM, Laws JL, Mackenzie JM, Helbig KJ. Lipid droplets and lipid mediators in viral infection and immunity. *FEMS Microbiol Rev.* (2021) 45: fuaa066. doi: 10.1093/femsre/fuaa066

103. Vogt DA, Camus G, Herker E, Webster BR, Tsou CL, Greene WC, et al. Lipid droplet-binding protein TIP47 regulates hepatitis C Virus RNA replication through interaction with the viral NS5A protein. *PloS Pathog.* (2013) 9:e1003302. doi: 10.1371/journal.ppat.1003302

104. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, et al. The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol.* (2007) 9:1089–97. doi: 10.1038/ncb1631

105. Galli A, Ramirez S, Bukh J. Lipid Droplets Accumulation during Hepatitis C Virus Infection in Cell-Culture Varies among Genotype 1-3 Strains and Does Not Correlate with Virus Replication. *Viruses.* (2021) 13:389. doi: 10.3390/v13030389

106. Dias SSG, Soares VC, Ferreira AC, Sacramento CQ, Fintelman-Rodrigues N, Temerozo JR, et al. Lipid droplets fuel SARS-CoV-2 replication and production of inflammatory mediators. *PloS Pathog.* (2020) 16:e1009127. doi: 10.1371/journal.ppat.1009127

107. Monson EA, Crosse KM, Duan M, Chen W, O'shea RD, Wakim LM, et al. Intracellular lipid droplet accumulation occurs early following viral infection and is required for an efficient interferon response. *Nat Commun.* (2021) 12:4303. doi: 10.1038/s41467-021-24632-5

108. Szatmari I, Pap A, Rühl R, Ma JX, Illarionov PA, Besra GS, et al. PPARgamma controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells. *J Exp Med.* (2006) 203:2351–62. doi: 10.1084/jem.20060141

109. Chen Q, Ross AC. Retinoic acid regulates CD1d gene expression at the transcriptional level in human and rodent monocytic cells. *Exp Biol Med.* (2007) 232:488–94. doi: 10.3181/00379727-232-232048

110. Li Y-R, Zhou Y, Kim YJ, Zhu Y, Ma F, Yu J, et al. Development of allogeneic HSC-engineered iNKT cells for off-the-shelf cancer immunotherapy. *Cell Rep Med.* (2021) 2:100449. doi: 10.1016/j.xcrm.2021.100449

111. Rubio MT, Bouillié M, Bouazza N, Coman T, Trebeden-Nègre H, Gomez A, et al. Pre-transplant donor CD4– invariant NKT cell expansion capacity predicts the occurrence of acute graft-versus-host disease. *Leukemia.* (2017) 31:903–12. doi: 10.1038/leu.2016.281

112. Li Y-R, Zhou Y, Yu J, Kim YJ, Li M, Lee D, et al. Generation of allogeneic CAR-NKT cells from hematopoietic stem and progenitor cells using a clinically guided culture method. *Nat Biotechnol.* (2024). doi: 10.1038/s41587-024-02226-y

113. Motohashi S, Ishikawa A, Ishikawa E, Otsuji M, Iizasa T, Hanaoka H, et al. A phase I study of *in vitro* expanded natural killer T cells in patients with advanced and recurrent non–small cell lung cancer. *Clin Cancer Res.* (2006) 12:6079–86. doi: 10.1158/1078-0432.CCR-06-0114

114. Aoki T, Motohashi S, Koseki H. Regeneration of invariant natural killer T (iNKT) cells: application of iPSC technology for iNKT cell-targeted tumor immunotherapy. *Inflammation Regener.* (2023) 43:27. doi: 10.1186/s41232-023-00275-5

115. Hammond TC, Purbhoo MA, Kadel S, Ritz J, Nikiforow S, Daley H, et al. A phase 1/2 clinical trial of invariant natural killer T cell therapy in moderate-severe acute respiratory distress syndrome. *Nat Commun.* (2024) 15:974. doi: 10.1038/s41467-024-44905-z

116. Li Y-Q, Yan C, Wang X-F, Xian M-Y, Zou G-Q, Gao X-F, et al. A new iNKT-cell agonist-adjuvanted SARS-CoV-2 subunit vaccine elicits robust neutralizing antibody responses. *ASC Infect Dis.* (2022) 8:2161–70. doi: 10.1021/acsinfecdis.2c00296

117. Tsuji M, Nair MS, Masuda K, Castagna C, Chong Z, Darling TL, et al. An immunostimulatory glycolipid that blocks SARS-CoV-2, RSV, and influenza infections *in vivo*. *Nat Commun.* (2023) 14:3959. doi: 10.1038/s41467-023-39738-1

118. Weinstein JS, Herman EI, Lainez B, Licona-Limón P, Esplugues E, Flavell R, et al. TFH cells progressively differentiate to regulate the germinal center response. *Nat Immunol.* (2016) 17:1197–205. doi: 10.1038/ni.3554

119. McGuire HM, Vogelzang A, Warren J, Loetsch C, Natividad KD, Chan TD, et al. IL-21 and IL-4 collaborate to shape T-dependent antibody responses. *J Immunol.* (2015) 195:5123–35. doi: 10.4049/jimmunol.1501463

120. Gaya M, Barral P, Burbage M, Aggarwal S, Montaner B, Warren Navia A, et al. Initiation of antiviral B cell immunity relies on innate signals from spatially positioned NKT cells. *Cell.* (2018) 172:517–533.e20. doi: 10.1016/j.cell.2017.11.036

121. Parekh VV, Wilson MT, Olivares-Villagómez D, Singh AK, Wu L, Wang CR, et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest.* (2005) 115:2572–83. doi: 10.1172/JCI24762

122. Li X, Fujio M, Imamura M, Wu D, Vasan S, Wong CH, et al. Design of a potent CD1d-binding NKT cell ligand as a vaccine adjuvant. *Proc Natl Acad Sci U.S.A.* (2010) 107:13010–5. doi: 10.1073/pnas.1006662107

123. Krammer F, Smith GJD, Fouchier RAM, Peiris M, Kedzierska K, Doherty PC, et al. Influenza. *Nat Rev Dis Primers.* (2018) 4:3. doi: 10.1038/s41572-018-0002-y

124. Artiaga BL, Yang G, Hutchinson TE, Loeb JC, Richt JA, Lednicky JA, et al. Rapid control of pandemic H1N1 influenza by targeting NKT-cells. *Sci Rep.* (2016) 6:37999. doi: 10.1038/srep37999

125. Gu W, Madrid DMD, Yang G, Artiaga BL, Loeb JC, Castleman WL, et al. Unaltered influenza disease outcomes in swine prophylactically treated with α-galactosylceramide. *Dev Comp Immunol.* (2021) 114:103843. doi: 10.1016/j.dci.2020.103843

126. Madrid DMDC, Gu W, Artiaga BL, Yang G, Loeb J, Hawkins IK, et al. Comparison of oseltamivir and α-galactosylceramide for reducing disease and transmission in pigs infected with 2009 H1N1 pandemic influenza virus. *Front Vet Sci.* (2022) 9. doi: 10.3389/fvets.2022.999507

127. Kamali A, Holodniy M. Influenza treatment and prophylaxis with neuraminidase inhibitors: a review. *Infect Drug Resist.* (2013) 6:187–98. doi: 10.2147/IDR.S36601

128. Ko S-Y, Ko H-J, Chang W-S, Park S-H, Kweon M-N, Kang C-Y. [amp]]alpha;-galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor. *J Immunol.* (2005) 175:3309–17. doi: 10.4049/jimmunol.175.5.3309

129. Tefit JN, Crabé S, Orlandini B, Nell H, Bendelac A, Deng S, et al. Efficacy of ABX196, a new NKT agonist, in prophylactic human vaccination. *Vaccine.* (2014) 32:6138–45. doi: 10.1016/j.vaccine.2014.08.070

130. Mooney AH, Draper SL, Burn OK, Anderson RJ, Compton BJ, Tang C, et al. Preclinical evaluation of therapeutic vaccines for chronic hepatitis B that stimulate antiviral activities of T cells and NKT cells. *JHEP Rep.* (2024) 6:101038. doi: 10.1016/j.jhepr.2024.101038

131. Woltman AM, Ter Borg MJ, Binda RS, Sprengers D, Von Blomberg BME, Scheper RJ, et al. [amp]]alpha;-galactosylceramide in chronic hepatitis B infection: results from a randomized placebo-controlled phase I/II trial. *Antivir Ther.* (2008) 14:809–18. doi: 10.3851/IMP1295

132. Canchis PW, Bhan AK, Landau SB, Yang L, Balk SP, Blumberg RS. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. *Immunology.* (1993) 80:561–5.

133. Meijlink MA, Chua YC, Chan STS, Anderson RJ, Rosenberg MW, Cozijnsen A, et al. 6″-Modifed α-GalCer-peptide conjugate vaccine candidates protect against liver-stage malaria. *RSC Chem Biol.* (2022) 3:551–60. doi: 10.1039/D1CB00251A

134. Sada-Ovalle I, Sköld M, Tian T, Besra GS, Behar SM. Alpha-galactosylceramide as a therapeutic agent for pulmonary Mycobacterium tuberculosis infection. *Am J Respir Crit Care Med.* (2010) 182:841–7. doi: 10.1164/rccm.200912-1921OC

135. Yanagisawa K, Yue S, van der Vliet HJ, Wang R, Alatrakchi N, Golden-Mason L, et al. Ex vivo analysis of resident hepatic pro-inflammatory CD1d-reactive T cells and hepatocyte surface CD1d expression in hepatitis C. *J Viral Hepat.* (2013) 208:556–65. doi: 10.1111/jvh.12081

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 17 September 2024
DOI 10.3389/fimmu.2024.1457690

OPEN ACCESS

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RECEIVED 01 July 2024
ACCEPTED 26 August 2024
PUBLISHED 17 September 2024

CITATION
Park YH, Lee SW, Kim T-C, Park HJ, Van Kaer L and Hong S (2024) The iNKT cell ligand α-GalCer prevents murine septic shock by inducing IL10-producing iNKT and B cells. *Front. Immunol.* 15:1457690. doi: 10.3389/fimmu.2024.1457690

The iNKT cell ligand α-GalCer prevents murine septic shock by inducing IL10-producing iNKT and B cells

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Introduction: α-galactosylceramide (α-GalCer), a prototypical agonist of invariant natural killer T (iNKT) cells, stimulates iNKT cells to produce various cytokines such as IFNγ and IL4. Moreover, repeated α-GalCer treatment can cause protective or pathogenic outcomes in various immune-mediated diseases. However, the precise role of α-GalCer-activated iNKT cells in sepsis development remains unclear. To address this issue, we employed a lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced murine sepsis model and two alternative models.

Methods: Sepsis was induced in wild-type (WT) C57BL/6 (B6) mice by three methods (LPS/D-GalN, α-GalCer/D-GalN, and cecal slurry), and these mice were monitored for survival rates. WT B6 mice were intraperitoneally injected with α-GalCer or OCH (an IL4-biased α-GalCer analog) one week prior to the induction of sepsis. To investigate the effects of α-GalCer-mediated iNKT cell activation on sepsis development, immune responses were analyzed by flow cytometry using splenocytes and liver-infiltrating leukocytes. In addition, a STAT6 inhibitor (AS1517499) and an IL10 inhibitor (AS101) were employed to evaluate the involvement of IL4 or IL10 signaling. Furthermore, we performed B cell adoptive transfers to examine the contribution of α-GalCer-induced regulatory B (Breg) cell populations in sepsis protection.

Results: *In vivo* α-GalCer pretreatment polarized iNKT cells towards IL4- and IL10-producing phenotypes, significantly attenuating LPS/D-GalN-induced septic lethality in WT B6 mice. Furthermore, α-GalCer pretreatment reduced the infiltration of immune cells to the liver and attenuated pro-inflammatory cytokine production. Treatment with a STAT6 inhibitor was unable to modulate disease progression, indicating that IL4 signaling did not significantly affect iNKT cell-mediated protection against sepsis. This finding was confirmed by pretreatment with OCH, which did not alter sepsis outcomes. However, interestingly, prophylactic effects of α-GalCer on sepsis were significantly suppressed by treatment with an IL10 antagonist, suggesting induction of IL10-dependent anti-inflammatory responses. In addition to IL10-producing iNKT cells, IL10-producing B cell populations were significantly increased after α-GalCer pretreatment.

10.3389/fimmu.2024.1457690

Conclusion: Overall, our results identify α-GalCer-mediated induction of IL10 by iNKT and B cells as a promising option for controlling the pathogenesis of postoperative sepsis.

KEYWORDS

α-GalCer, iNKT cells, sepsis, B cells, IL10

1 Introduction

Sepsis is a critical condition that poses a significant risk to one's life. It develops as an immune response to severe infection, causing a dysregulated hyperinflammatory reaction within the body. If multiple organs fail acutely, the condition progresses to septic shock. Unfortunately, sepsis is a frequent cause of death among critically ill patients, contributing to one in three deaths of hospitalized patients (1). During sepsis, various pathogen-derived molecules such as lipopolysaccharide (LPS) and lipoteichoic acid can induce pro-inflammatory cytokines such as IL12 and IL1β by hepatic macrophages that, in turn, can activate a variety of other cell types such as natural killer (NK) cells, ultimately leading to septic shock (2). Like Gram-negative bacteria-derived LPS, Gram-positive bacteria (e.g., group B *Streptococci*)-derived glycolipids can induce septic shock in a manner dependent on the T cell receptor (TCR)-mediated activation of CD1d-restricted invariant natural killer T (iNKT) cells (3). Activated iNKT cells can facilitate liver injury during septic shock by secreting type 1 cytokines such as IFNγ and TNFα (4, 5). While LPS plus 2-amino-2-deoxy-D-galactose (D-galactosamine [D-GalN]) co-injection mimics Gram-negative bacteria-induced sepsis, glycolipid alpha-galactosylceramide (α-GalCer) plus D-GalN can induce iNKT cell-mediated sepsis mimicking the sepsis elicited by Gram-positive bacteria-derived glycolipids (4, 6).

iNKT cells are a unique T cell subset recognizing glycolipids, presented by the non-classical MHC molecule CD1d. Moreover, iNKT cells can be divided into iNKT1 (PLZF^{low}T-bet^{hi} and IFNγ), iNKT2 (PLZF^{hi}GATA3^{hi} and IL4), or iNKT17 (PLZF^{int}RORγt^{hi} and IL17) cells based on their transcription factor and cytokine profiles (7). Upon stimulation, iNKT cells can release both pro-inflammatory cytokines (e.g., IFNγ, TNFα, and IL6) and anti-inflammatory cytokines (e.g., IL4, IL10, IL22, and TGFβ) (8, 9). As iNKT cells rapidly produce various cytokines following TCR stimulation, these cells can initiate acute immune responses and sustain immune responses, leading to chronic states of immune cell activation (4, 10, 11). In addition, another subset of iNKT cells, IL10-producing iNKT10 cells characterized by the expression of the E4BP4 transcription factor, is predominantly located in the white adipose tissue and displays anti-inflammatory functions (12).

IL10-producing B cells, also known as regulatory B (Breg) cells, release soluble immunomodulatory molecules (e.g., adenosine and indoleamine 2,3-dioxygenase [IDO]) as well as anti-inflammatory cytokines (e.g., IL35 and TGFβ). Thus, it has been suggested that these cells play a vital role in controlling autoimmunity (13, 14). Moreover, CD1d-expressing B cells can ameliorate arthritis by inducing the differentiation of iNKT cells with immune suppressive effector functions (15). In particular, it has been reported that IL10-producing B cells display notable therapeutic potential in the endotoxin-induced septic shock model (16).

α-GalCer, a glycolipid derived from the marine sponge *Agelas mauritianus*, has been identified as a potent ligand for both human and mouse iNKT cells. Due to its potent immune-modulating properties, α-GalCer shows therapeutic efficacy against immune-related disorders, including cancer, microbial infections, autoimmune diseases, and inflammatory conditions (17). Furthermore, repeated *in vivo* α-GalCer stimulation can induce profound iNKT cell anergy and differentiation into iNKT10 cells (18–20). Previous studies have reported that α-GalCer-mediated induction of iNKT10 cells can be sustained for a month, and these iNKT10 cells thereby significantly influenced immune responses such as tumor progression and experimental autoimmune encephalomyelitis (EAE) (18, 21). Although iNKT cells have been implicated in the severity of inflammatory diseases, the role of α-GalCer-activated iNKT cells in sepsis has yet to be fully elucidated. Therefore, in this study, we investigated the effects of α-GalCer-mediated pre-activation of iNKT cells on sepsis.

2 Materials and methods

2.1 Mice and reagents

Wild-type (WT) C57BL/6 (B6) mice were purchased from Jung Ang Lab Animal Inc. (Seoul, Republic of Korea). IL4/GFP cytokine reporter (4Get) mice were kindly provided by Dr. R. Locksley (University of California, San Francisco, CA, USA). All mice used in this study were on a B6 genetic background, maintained at Sejong University, and used for experiments at 6-12 weeks of age. Mice were maintained on a 12-hour light/12-hour dark cycle in a temperature-controlled barrier facility with free access to food and water. Mice were fed a γ-irradiated sterile diet and provided with autoclaved tap water. Age- and sex-matched mice were used for all experiments in this study. The animal experiments were

approved by the Institutional Animal Care and Use Committee of Sejong University (SJ-20210704E1). Alpha-GalCer was purchased from Enzo Life Sciences (Farmingdale, NY, USA). LPS derived from *Escherichia coli* (serotype 0111:B4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). STAT6 inhibitor (AS1517499, Cat. #HY-100614) and IL10 inhibitor (AS101, Cat. #2446/10) were purchased from MedChemExpress (Monmouth Junction, NJ, USA) and Tocris Bioscience (Bristol, UK), respectively.

2.2 *In vivo* treatment of mice with α-GalCer or OCH

For activation of iNKT cells with α-GalCer or OCH, mice were injected intraperitoneally (i.p.) with α-GalCer (2 μg/mouse) or OCH (2 μg/mouse) dissolved in phosphate-buffered saline (PBS). Littermates injected with PBS only were used as a negative control.

2.3 Induction of septic shock

Mice were injected i.p. with α-GalCer (2 μg/mouse) or Vehicle (Veh). Seven days later, septic shock was induced by three different methods, as follows: First, for LPS/D-GalN-induced septic shock, mice were injected i.p. with LPS (2 μg/mouse) plus D-GalN (25 mg/mouse). Second, for α-GalCer/D-GalN-induced septic shock, mice were injected i.p. with α-GalCer (2 μg/mouse) plus D-GalN (10 mg/mouse) (4). After the challenge, all animals were monitored to evaluate sepsis-induced lethality for 72 hours. Third, for cecal slurry (CS)-induced sepsis, CS was prepared from the cecal feces of WT B6 male mice, as described by Starr et al. (22). Briefly, cecal feces was diluted with water and subsequently filtered with the first 860 μm and second 190 μm screen mesh (Bellco Glass, Inc., Vineland, NJ, USA) to remove fecal debris, then resuspended in 15% glycerol at 100 mg/ml. Aliquots were stored at -80°C until ready for use. Mice were injected i.p. with CS (1.3 mg feces/g body weight) to induce sepsis. All animals were monitored to evaluate sepsis-induced lethality for 48 hours after the challenge.

2.4 Injection of STAT6 or IL10 inhibitors

To evaluate whether IL4 or IL10 signaling are involved in α-GalCer-mediated preventive effects on sepsis, mice were injected with either a STAT6 inhibitor (AS1517499, 10 mg/kg body weight) or an IL10 inhibitor (AS101, 10 μg/mouse) every other day or daily for one week, respectively, starting from α-GalCer (2 μg/mouse) pretreatment until sepsis induction.

2.5 Isolation of liver leukocytes

Mice were anesthetized using a combination of ketamine (40 mg/kg) and xylazine (4 mg/kg). They were perfused via the left heart ventricle with cold and sterile PBS for 3 min to remove peripheral blood mononuclear cells (PBMCs) from the blood vessels. The liver was removed after perfusion, cut into small pieces by scissors and a scalpel, and digested with collagenase type IV (Sigma, St. Louis, MO, USA; 2.5 mg/ml) and DNase I (Promega, Madison, WI, USA; 1 mg/ml) for 15 min at 37°C. Subsequently, the digested tissues were dissociated into single-cell suspensions using a combination of C Tubes and a gentleMACS™ dissociator (Miltenyi, Bergisch Gladbach, Germany). The single-cell suspensions were filtered using a 70-μm-pore cell strainer, and subsequently, the cells were washed once with PBS (10% FBS). Mononuclear cells were collected from the 40/70% Percoll interphase after discontinuous Percoll gradient centrifugation. After washing with PBS, the number of total mononuclear cells was determined using 0.4% trypan blue (Welgene, Gyeongsan-si, Republic of Korea) and a hemocytometer before antibody staining (23).

2.6 Flow cytometry

The following monoclonal antibodies (mAbs) were obtained from BD Biosciences (San Jose, CA, USA): Fluorescein isothiocyanate (FITC)-conjugated anti-CD3ε (clone 145-2C11); FITC-, phycoerythrin (PE)- or PE/Cy7-conjugated anti-CD4 (clone RM4-5); FITC- or PE-conjugated anti-IL10 (clone JES5-16E3); PE- or PE/Cy7-conjugated anti-CD11b (clone M1/70); PE-conjugated anti-CD1d (clone 1B1); PE-conjugated anti-RORγt (clone Q31-378); PE-conjugated anti-NK1.1 (clone PK-136); PE-conjugated anti-TNFα (clone MP6-XT220); PE/Cy7-conjugated anti-CD69 (clone H1.2F3); PE/Cy7-conjugated anti-CD11c (clone HL3); PE/Cy7-conjugated anti-CD45 (clone 30-F11). The following mAbs from Thermo Fisher Scientific (Waltham, MA, USA) were used: FITC-, PE- or allophycocyanin (APC)-conjugated anti-CD19 (clone 1D3); FITC-conjugated anti-F4/80 (clone BM8); FITC-conjugated anti-NK1.1 (clone PK-136); PE-conjugated anti-FcεRI (clone MAR-1); PE-conjugated anti-T-bet (clone 4B10); PE-conjugated anti-E4BP4 (clone S2M-E19); PE-conjugated anti-Nur77 (clone 12.14); PE-conjugated anti-PLZF (clone Mags.21F7); PE-conjugated anti-IL17A (clone eBio17B7); PE-conjugated anti-IL1β (clone NJTEN3); PE/Cy7-conjugated anti-CD23 (clone B3B4); PE/Cy7-conjugated anti-CD3ε (clone 145-2C11); FITC- or APC-conjugated anti-Ly6G (Gr-1) (clone 1A8-Ly6g); APC-conjugated anti-CD200R3 (clone Ba13). The following mAbs from BioLegend (San Diego, CA, USA) were used: FITC- or APC-conjugated anti-CD45 (clone 30-F11); PE-conjugated anti-CD21/CD35 (clone 7E9); PE/Cy7-conjugated anti-CD5 (clone 53-7.3); PE/Cy7-conjugated anti-CD1d (clone 1B1); PE/Cy7-conjugated anti-PLZF (clone 9E12); PE/Cy7-conjugated anti-CD19 (clone 6D5); APC-conjugated anti-CD3ε (clone 17A2). Flow cytometric data were acquired using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using FlowJo software (version 8.7; Tree Star, Ashland, OR, USA). Cells were harvested and washed twice with cold 0.5% BSA-containing PBS (FACS buffer) to perform surface staining. The cells were incubated with anti-CD16/CD32 mAbs (clone 2.4G2) on ice for 10 min and stained with fluorescence-labeled mAbs to block the Fc receptor (24).

2.7 Intracellular cytokine/transcription factor staining

To perform intracellular cytokine/transcription factor staining, single-cell suspensions from the spleen were incubated for two hours at 37°C with intracellular protein transport inhibitor (brefeldin A, 10 μg/ml) in RPMI complete medium consisting of RPMI 1640 (Gibco BRL, Gaithersburg, MD, USA) medium supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin, and 5 mM 2-mercaptoethanol. The cells were stained for cell-specific surface markers, then fixed with 1% paraformaldehyde in PBS, washed with cold (4°C) FACS buffer, and permeabilized with 0.5% saponin in PBS for 10 min. Subsequently, the permeabilized cells were stained for 30 min at room temperature (RT) with the indicated anti-cytokine mAbs (FITC-conjugated anti-IL10, PE-conjugated anti-IL10, anti-TNFα, anti-IL17A, anti-IL1β, and FITC- or PE-conjugated rat IgG isotype control mAbs). Intracellular staining for transcription factors was performed using the Foxp3 staining buffer set kit (Thermo Fisher Scientific, Waltham, MA, USA) with the indicated mAbs (PE-conjugated anti-T-bet, anti-RORγt, anti-E4BP4, anti-PLZF, anti-Nur77 and PE/Cy7-conjugated anti-PLZF; PE or PE/Cy7-conjugated rat IgG isotype control mAbs). Samples (more than 5,000 cells) were acquired using a FACSCalibur, and the data were analyzed using the FlowJo software package (version 8.7; Tree Star, Ashland, OR, USA) (23, 24).

2.8 CD1d/α-GalCer dimer staining for iNKT cells

To stain iNKT cells specifically, mCD1d/Ig fusion proteins (CD1d dimer; mouse CD1d DimerX, BD Biosciences, San Jose, CA, USA) were incubated overnight at 37°C with a 40-fold molar excess of α-GalCer (in PBS containing 0.5% Tween 20). The staining cocktail was prepared by mixing α-GalCer-loaded mCD1d/Ig proteins with FITC-, PE- or APC-conjugated anti-mouse IgG1 Ab (clone A85-1, BD Biosciences, San Jose, CA, USA) at a 1:2 ratio of dimer to anti-mouse IgG1 Ab. Subsequently, the mixture was incubated for two hours at RT.

2.9 B cell enrichment by magnetically activated cell sorting

WT B6 mice were i.p. injected with α-GalCer (2 μg/mouse). Seven days later, splenocytes were harvested and stained with PE-conjugated anti-CD19 mAb. A single-cell suspension of splenocytes was prepared and resuspended in RPMI complete medium. $CD19^+$ B cells from B6 mice were enriched by positive selection with anti-PE MACS and LD columns (Miltenyi Biotech, Bergisch Gladbach, Germany), following the manufacturer's instructions. The MACS-purified $CD19^+$ B cell population was >98% pure.

2.10 Analysis of liver sections

The livers were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 6 μm sections using a microtome (RM 2235, Leica, Wetzlar, Germany). The sections were stained with hematoxylin and eosin (H&E) to analyze histological changes.

2.11 Gene set enrichment analysis

GSEA was conducted using GSEA software (GSEA 4.2.1). Gene expression was analyzed in 14 patients using RNA-seq data. Significantly enriched gene sets were identified using p-value ≤0.05 and q-value ≤0.25 as a cutoff. Data were deposited in the NCBI's Gene Expression Omnibus database (GEO GSE197775). GSEA from the Broad Institute (http://www.broad.mit.edu/gsea) was used to calculate the enrichment of genes in each set (25).

2.12 Statistical analysis

Statistical significance was determined using Excel (Microsoft, Redmond, WA, USA). Student's *t*-test was performed for the comparison of two groups (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered significant in the Student's *t*-test). Kaplan-Meier plots with a log-rank test was carried out using Statistics Kingdom (https://www.statskingdom.com/kaplan-meier.html) (accessed on 17 January 2023) (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered to be significant in the log-rank test).

3 Results

3.1 A single pre-administration of α-GalCer alters iNKT cell subsets and suppresses sepsis severity

To investigate whether α-GalCer administration alters the iNKT cell subset distribution, WT B6 mice were injected i.p. with either α-GalCer or Veh and, seven days later, splenic iNKT cells were analyzed by flow cytometry. We found that spleen weights and total cell numbers were increased in α-GalCer-pretreated mice compared to Veh-injected controls (Figure 1A), indicating that α-GalCer pretreatment induces immune cell activation and proliferation. However, there were no significant changes in the total iNKT cell numbers and the prevalence of the $CD4^+$ iNKT cell subset (Figures 1B, C). Next, we examined the profile of iNKT cell subsets based on the expression of subset-specific transcription factors for iNKT1 (T-bet^+), iNKT2 ($PLZF^+$), iNKT17 ($ROR\gamma t^+$), and iNKT10 ($E4BP4^+$) cells. We found that the frequencies of iNKT1 and iNKT17 cell subsets were decreased whereas those of the iNKT2 and iNKT10 cell subsets were increased in α-GalCer-pretreated

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mice (Figure 1D). Thus, our results revealed that a single administration of α-GalCer is sufficient to induce alterations in iNKT cell subsets in WT B6 mice. Because iNKT cells can modulate immune responses, we tested whether α-GalCer-induced alterations of iNKT cell subsets can affect the development of sepsis. To address this issue, we employed two different sepsis models, induced by LPS/D-GalN or α-GalCer/D-GalN in WT B6 mice. WT B6 mice pretreated with α-GalCer displayed a significantly higher survival rate than mice pretreated with Veh (Figures 1E, F; Supplementary Figure S1A). In addition, α-GalCer-pretreated mice showed significant decreases in liver immune cell infiltration and liver enzymes (aspartate transaminase [AST] and alanine transaminase [ALT]), which are common indicators of liver inflammation (Figures 1G, H; Supplementary Figures S1B, C). To support our findings in a more physiological manner, we employed the CS injection model, an alternative method to induce

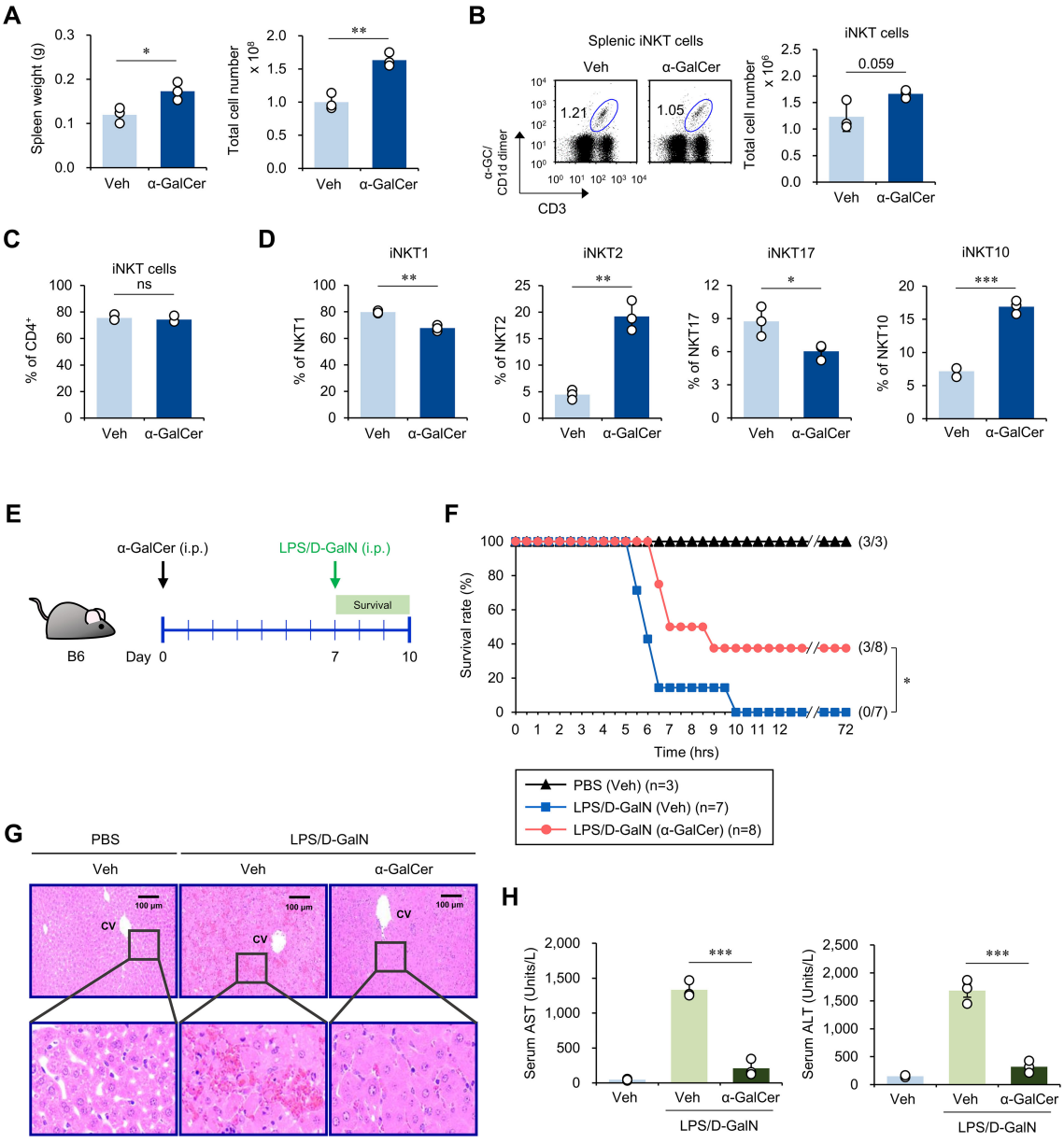

FIGURE 1
α-GalCer pretreatment alters iNKT cell subsets and attenuates sepsis severity. WT B6 mice were injected i.p. with α-GalCer (2 μg/mouse) and, seven days later, the spleens were harvested for the following analyses. **(A)** Weight and total cell number of spleens. **(B)** The frequency and cell number of splenic iNKT cells (α-GalCer/CD1d-dimer$^+$CD3$^+$). **(C)** The frequency of CD4$^+$ iNKT cells in the spleen. **(D)** The relative frequencies of iNKT cell subsets (i.e., T-bet$^+$ iNKT1, PLZF$^+$ iNKT2, RORγt$^+$ iNKT17, and E4BP4$^+$ iNKT10 cells) were determined by flow cytometry. **(E)** Experimental outline: WT B6 mice were injected i.p. with α-GalCer (2 μg/mouse) and, seven days later, these mice were injected i.p. with LPS (2 μg/mouse) plus D-GalN (25 mg/mouse) for induction of sepsis. **(F)** Subsequently, these mice were monitored to evaluate their survival for three days. **(G, H)** H&E staining of liver sections (CV, central vein) **(G)** and serum levels of AST and ALT **(H)** were analyzed five hours after LPS/D-GalN injection. The mean values ± SD (n = 3 in **(A–D, H)**; per group in the experiment; Student's t-test; *p < 0.05, **p < 0.01, and ***p < 0.001) are shown. The survival rate was analyzed by Kaplan-Meier plots with a log-rank test (*p < 0.05). One representative experiment of two experiments is shown. ns, not significant.

polymicrobial sepsis by i.p. injection of cecal contents (22). As expected, the survival rate of α-GalCer-pretreated mice was significantly increased in CS-induced sepsis (Supplementary Figure S1D). Therefore, we conclude that pre-administration of α-GalCer can attenuate sepsis severity.

3.2 α-GalCer pretreatment attenuates inflammatory responses in sepsis

Sepsis is accompanied by excessive inflammatory responses. For example, septic patients display increased serum levels of inflammatory cytokines (e.g., IL1, IL17A, and TNFα) and inflammatory immune cells (e.g., lymphocytes and neutrophils) than healthy individuals (26). To examine the immune cellularity in α-GalCer-pretreated mice, the spleen and liver were harvested and analyzed for tissue weights and total cell numbers. We found that α-GalCer pretreatment increased the splenic cell numbers in both untreated and LPS/D-GalN-treated mice (Supplementary Figure S2A). However, α-GalCer pretreatment dramatically reduced hepatic cell numbers in LPS/D-GalN-treated mice (Supplementary Figure S2B), suggesting inhibition of immune cell infiltration.

Next, we examined the absolute cell numbers of immune cells (i.e., neutrophils, T cells, NK cells, and NKT cells) known to participate in inflammatory immune responses caused by infection (27–29). Despite the immune-activating effects of α-GalCer (expansion of immune cells such as neutrophils), treatment with α-GalCer prior to LPS/D-GalN administration did not modify the total cell numbers of T cells, NK cells, and NKT cells in the spleen (Supplementary Figure S2C). However, interestingly, α-GalCer pretreatment significantly decreased the infiltration of immune cells to the liver following LPS/D-GalN injection (Supplementary Figure S2D). Collectively, our results suggest that the protective effects of α-GalCer pretreatment on LPS/D-GalN-induced sepsis are associated with reduced immune cell expansion and decreased infiltration of the liver.

In addition, we examined whether α-GalCer pretreatment affects the production of pro-inflammatory cytokines involved in the development of various inflammatory diseases (2, 30). For this purpose, we analyzed the expression of inflammatory cytokines, including IL1β, IL17A, and TNFα, by neutrophils, T cells, NK cells, and NKT cells. We found that α-GalCer-pretreatment decreased IL1β expression by splenic neutrophils and NKT cells both with or without LPS/D-GalN administration (Figure 2A). Similar effects of α-GalCer pretreatment on IL1β expression were observed in the liver (Figure 2B). For IL17A, α-GalCer pretreatment affected its production by neutrophils in spleen in the absence but not presence of LPS/D-GalN administration (Figure 2C). However, α-GalCer pretreatment decreased IL17A production by immune cells, including neutrophils, T cells, NK cells, and NKT cells in the liver (Figure 2D). Furthermore, TNFα expression was also suppressed by α-GalCer pretreatment in the spleen (Figure 2E) and liver (Figure 2F) during LPS/D-GalN-induced sepsis. Thus, our results demonstrated that α-GalCer pretreatment reduces the production of inflammatory cytokines more prominently in the liver than in the spleen.

3.3 The protective effects of α-GalCer pretreatment do not depend on IL4-STAT6 signaling

It has been previously reported that OCH, an analog of α-GalCer, can activate and polarize iNKT cells towards an IL4-biased type 2 cytokine production phenotype (31, 32). Since α-GalCer pretreatment increased the IL4-producing iNKT2 cell subset (Figure 1D), we reasoned that OCH pretreatment may confer similar or more enhanced protective effects against sepsis than α-GalCer. First, we confirmed that OCH treatment activated iNKT cells (Supplementary Figure S3A). Next, we confirmed the effects of OCH pretreatment on iNKT cells. Similar to α-GalCer treatment, OCH treatment did not affect the total cell numbers of iNKT cells (Figure 3A). However, unlike α-GalCer treatment, OCH treatment also did not affect the iNKT cell subset composition (Figure 3B). To investigate the impact of OCH on sepsis, we treated WT B6 mice with α-GalCer or OCH one week prior to induction of sepsis by LPS/D-GalN or α-GalCer/D-GalN injection. Unexpectedly, we found a dramatic difference in the mortality rate between the α-GalCer and OCH treatment groups (Figure 3C; Supplementary Figure S3B), indicating the absence of prophylactic effects of OCH on sepsis development.

To investigate the effect of α-GalCer on IL4 production, we analyzed splenic and hepatic IL4-producing $CD4^+$ T cells in α-GalCer-pretreated mice. We found reduced splenic $IL4^+$ cells in α-GalCer-treated compared to Veh-treated mice, while no significant difference was observed for hepatic $IL4^+$ cells (Supplementary Figure S4A). We found reduced frequency of IL4-producing $CD4^+$ T cells in the spleen, but no difference in the liver (Supplementary Figure S4B). In addition, since basophil-derived IL4 can promote tissue repair following liver damage induced by bacterial infections (33), we examined whether α-GalCer pretreatment induces IL4 expression by basophils. We found that neither the frequency nor the number of basophils producing IL4 in the spleen and liver were increased (Supplementary Figures S4C, D), implying that IL4-producing immune cells such as $CD4^+$ T cells and basophils are not essential for mediating the protective effects of α-GalCer pretreatment against sepsis.

Next, to confirm that the IL4 signaling pathway does not play a substantial role in the protection conferred by α-GalCer against sepsis, we employed AS1517499, an inhibitor of the STAT6 transcription factor that is critical for IL4 signaling (34). Alpha-GalCer-treated WT B6 mice were injected 4 times with AS1517499 every other day prior to sepsis induction by LPS/D-GalN (Figure 3D) or α-GalCer/D-GalN (Supplementary Figure S3C). We found that the STAT6 inhibitor did not cause any significant differences in mortality rates in α-GalCer-treated mice compared to PBS-injected controls (Figure 3E; Supplementary Figure S3D), suggesting that α-GalCer pretreatment confers its protective effects independently of IL4-STAT6 signaling.

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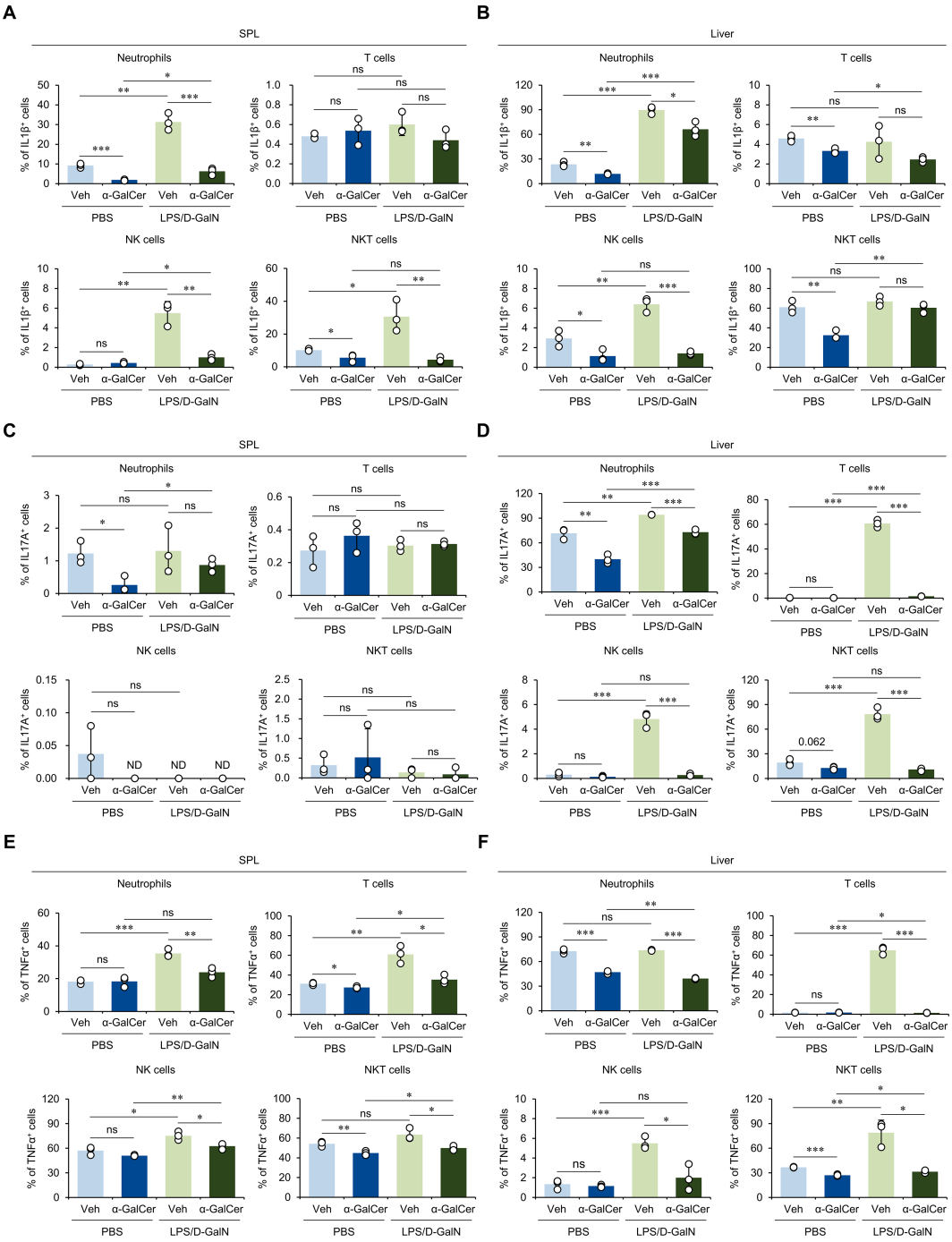

FIGURE 2
α-GalCer pretreatment attenuates the pro-inflammatory cytokine production by immune cells. WT B6 mice were injected i.p. with α-GalCer (2 μg/mouse) and, seven days later, these mice were injected i.p. with LPS (2 μg/mouse) plus D-GalN (25 mg/mouse) for induction of sepsis. Five hours later, the spleens and livers from these mice were harvested for the following analyses. **(A–F)** The frequencies of IL1β- **(A, B)**, IL17A- **(C, D)**, and TNFα-expressing populations **(E, F)** among neutrophils ($Gr1^+$ $CD11b^+$), T cells ($CD3^+$ $NK1.1^-$), NK cells ($CD3^-$ $NK1.1^+$), and NKT cells ($CD3^+$ $NK1.1^+$) in the spleen and liver. The mean values ± SD (n = 3 in **(A–F)**; per group in the experiment; Student's t-test; $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$) are shown. One representative experiment of two experiments is shown. ns, not significant.

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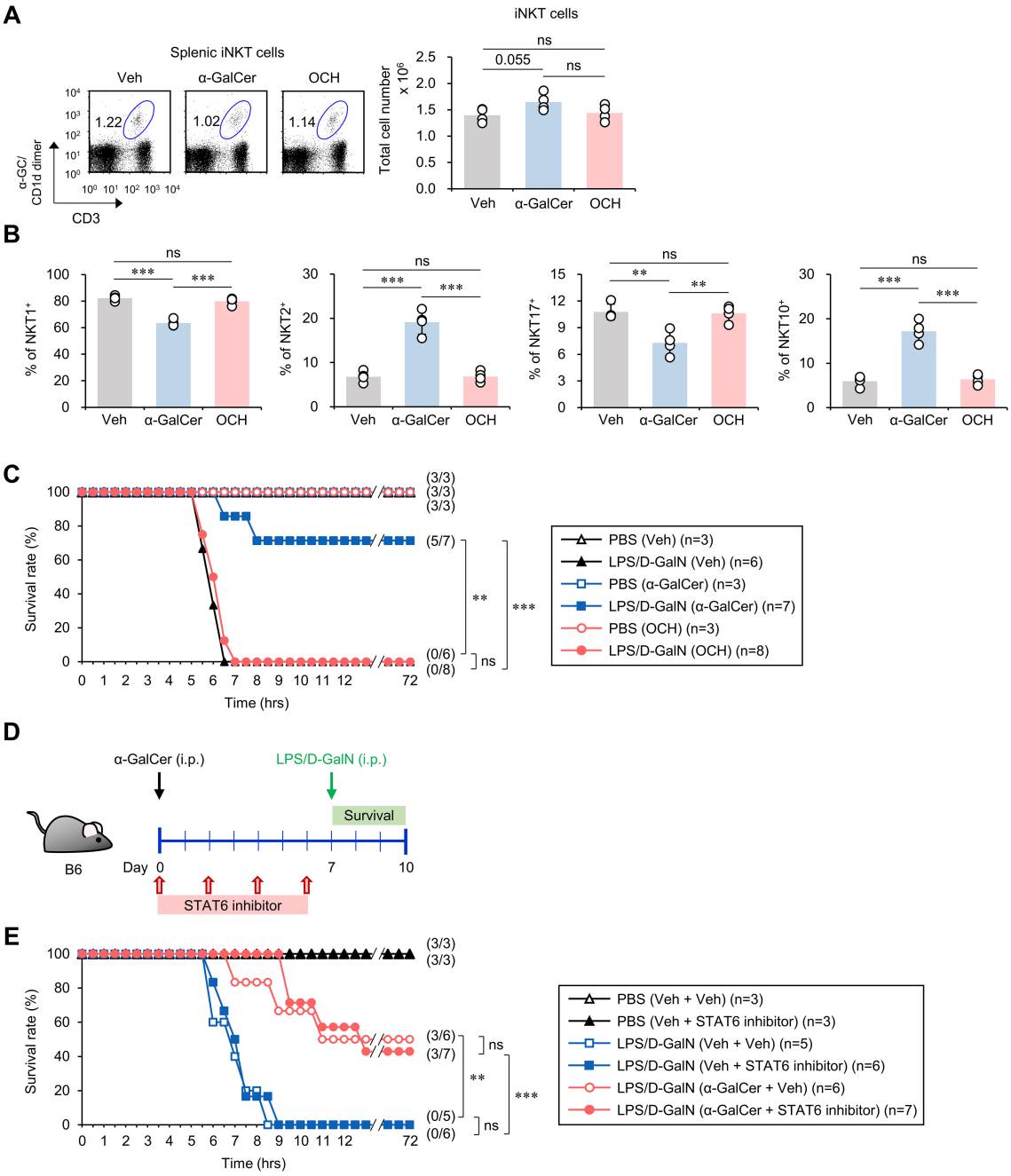

FIGURE 3
The preventive effects of α-GalCer pretreatment on sepsis do not correlate with the IL4-STAT6 signaling pathway. WT B6 mice were injected i.p. with α-GalCer (2 μg/mouse) or OCH (2 μg/mouse) and, seven days later, the spleens were harvested. **(A)** The frequency and absolute cell number of splenic iNKT cells were determined by flow cytometry. **(B)** Flow cytometric analysis for the expression of transcription factors (T-bet, PLZF, RORγt, and E4BP4) by splenic iNKT cells. **(C)** WT B6 mice were injected i.p. with α-GalCer (2 μg/mouse) or OCH (2 μg/mouse) and, seven days later, mice were injected i.p. with LPS (2 μg/mouse) plus D-GalN (25 mg/mouse) for induction of sepsis. Subsequently, these mice were monitored to evaluate their survival for three days after LPS/D-GalN injection. **(D)** Experimental outline: WT B6 mice were injected i.p. with α-GalCer (2 μg/mouse) on day 0 and, seven days later, mice were injected i.p. with LPS (2 μg/mouse) plus D-GalN (25 mg/mouse) for induction of sepsis. To evaluate the effect of IL4 signaling on α-GalCer-mediated attenuation of sepsis, WT B6 mice were injected i.p. four times with a STAT6 inhibitor (AS1517499, 10mg/kg) every other day starting from day 0. **(E)** Subsequently, these mice were monitored to evaluate their survival for three days after LPS/D-GalN injection. The mean values ± SD (n = 4 in **(A, B)**; per group in the experiment; Student's *t*-test; $^{**}p < 0.01$ and $^{***}p < 0.001$) are shown. The survival rate was analyzed by Kaplan-Meier plots with a log-rank test ($^{**}p < 0.01$ and $^{***}p < 0.001$). One representative experiment of two experiments is shown. ns, not significant.

3.4 The anti-inflammatory effects of α-GalCer pretreatment on septic shock correlate with the expansion of IL10-producing immune cells

To elucidate the mechanism by which α-GalCer pretreatment protects against sepsis, we considered our finding that α-GalCer pretreatment augments the iNKT10 cell subset (Figure 1D), as prior studies have demonstrated protective effects of the anti-inflammatory cytokine IL10 in sepsis (35, 36). We found that α-GalCer pretreatment increased the frequency of IL10-producing immune cells in the spleen and liver (Figure 4A). In addition, we tested whether α-GalCer pretreatment alters the composition of IL10-producing immune cells (i.e., T cells, B cells, DCs, macrophages, iNKT cells, and others) in the spleen and liver. We found that α-GalCer pretreatment significantly expanded the numbers of IL10-producing iNKT cells, B cells, and macrophages in the spleen and liver (Figure 4B). Next, we examined whether α-GalCer-induced IL10 can affect the outcome of septic shock by employing an IL10 inhibitor (AS101) (Figure 4C; Supplementary Figure S5A). While the survival rate of B6 mice treated with α-GalCer was significantly improved, administration of the IL10 inhibitor diminished the protective effects of α-GalCer against septic shock (Figure 4D). Moreover, we found that IL10 inhibition more profoundly affected the severity of iNKT-mediated sepsis (Supplementary Figure S5B). These results provide strong support for the critical role of IL10 in mediating the capacity of α-GalCer to protect against sepsis.

To provide evidence that IL10-mediated immune responses correlate with protection against sepsis pathogenesis in human, we performed GSEA on PBMCs of groups of sepsis survivors and non-survivors. We found that survivors downregulated inflammatory response genes (Figure 4E) but upregulated the IL10RA gene (Figure 4F), implying that α-GalCer-induced, IL10-associated phenotypic changes could be utilized as a therapeutic approach in humans.

3.5 α-GalCer pretreatment expands Breg cells that partially contribute to sepsis protection upon adoptive transfer

It has been reported that IL10-producing Breg cells can suppress exacerbated inflammatory immune responses (13). Moreover, iNKT cells can assist in the expansion of IL10-producing Breg cells (37). α-GalCer treatment increased both the frequency and absolute cell number of IL10-producing B cells in the spleen and liver, although no significant changes in total B cell numbers were observed (Figures 5A, B). Next, to identify the B cell subset producing IL10 upon α-GalCer pre-stimulation, we analyzed B cell subsets based on the expression of CD1d and CD21 molecules, to distinguish between follicular B (FOB) cells, marginal zone B (MZB) cells, and B1 cells (Figure 5C). Although α-GalCer pretreatment did not influence the cell number of FOB and B1a cells, intriguingly, the number of MZB cells increased in the spleen but decreased in the liver (Supplementary Figures S6A, B). Although there were no significant alterations in cell numbers, α-GalCer pretreatment dramatically increased the frequency and absolute cell numbers of IL10-producing B cells, except for hepatic MZB and B1 cell populations (Figures 5D, E). Thus, our results demonstrate that α-GalCer pretreatment induces IL10-producing Breg cell populations, strongly suggesting that $IL10^+$ FOB cells might be prominent contributors to IL10 production.

Since it has been reported that B cell-derived IL10 is critical in resolving LPS-induced acute lung injury (38), we next investigated whether Breg cells induced by α-GalCer can protect against sepsis. We isolated splenic $CD19^+$ B cells from WT B6 mice pretreated with either Veh or α-GalCer, adoptively transferred these B cells to WT B6 mice, and induced sepsis in the recipient animals by injection with LPS/D-GalN (Figure 5F). We found that adoptive transfer of B cells from α-GalCer-pretreated mice slowed the progression of sepsis development compared with B cells from Veh-pretreated mice. This protection was only partial (Figure 5G), suggesting that α-GalCer-mediated expansion of IL10-producing B cells partially contributes to alleviating septic shock.

4 Discussion

In summary, the present study demonstrates that α-GalCer pretreatment can regulate inflammatory immune responses such as septic shock by shifting iNKT and B cells towards anti-inflammatory phenotypes, particularly expression of the anti-inflammatory cytokine IL10.

It has been reported that IL10 levels are elevated in the blood of patients with sepsis (39). Such IL10 increases might reflect mechanisms in patients with systemic inflammatory response syndrome that counteract organ failure (35). It has been reported that IL10 inhibits the differentiation of naive CD4 T cells into effector Th cells by inhibiting IL12/IL4 production and DC function, thereby modulating both Th1-type and Th2-type responses (40). Our results in Supplementary Figure S4B imply that IL10 (from iNKT10 cells and Breg cells) produced by α-GalCer-pretreatment (Supplementary Figures S7A, B) may contribute to downregulating IL4-mediated immune responses.

A recent study demonstrated that $IL10^+$ macrophages expanded during LPS-induced sepsis promote homeostasis and survival (41). Consistent with this report, our results show that α-GalCer pretreatment increases IL10-producing macrophages in the liver (Figure 4B), which might be associated with counteracting inflammatory immune responses. Thus, it will be interesting to further investigate the phenotype of $IL10^+$ macrophages induced by α-GalCer pretreatment.

Inadequate migration of neutrophils to injured target organs contributes to the systemic inflammatory response associated with high sepsis mortality rates (27). In addition, severe sepsis is associated with a decrease in circulating neutrophils due to increased apoptosis (42). Interestingly, we found that α-GalCer

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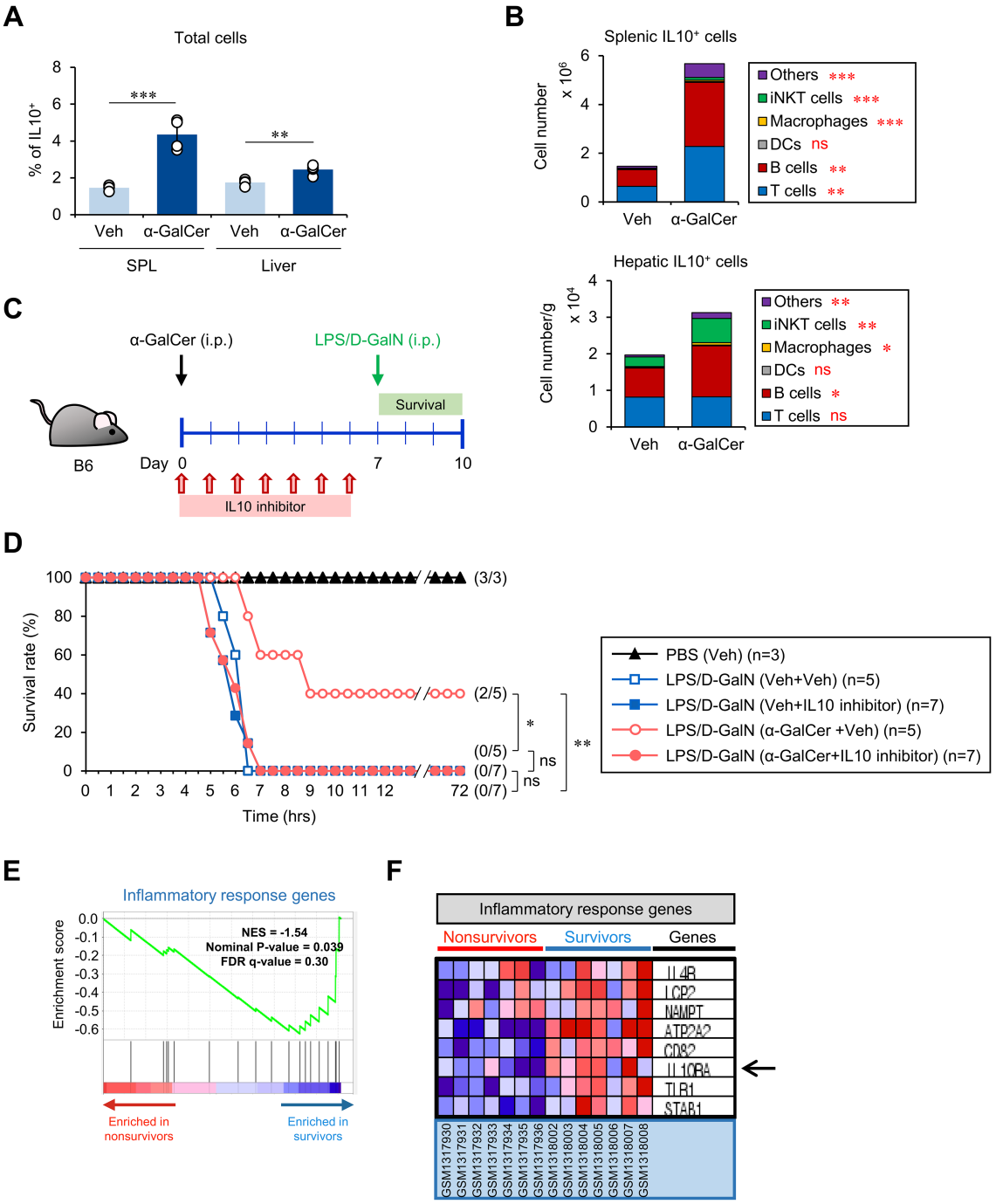

FIGURE 4
The preventive effects of α-GalCer pretreatment correlate with the expansion of IL10$^+$ immune cells. WT B6 mice were injected i.p. with α-GalCer (2 μg/mouse) and, seven days later, the spleens and livers were harvested for the following analyses. **(A)** The frequency of IL10$^+$ cells in the spleen and liver. **(B)** The absolute cell numbers of IL10-producing cells (i.e., T cells, B cells, DCs, macrophages, iNKT cells, and others) in the spleen and liver were determined by flow cytometry. **(C)** Experimental outline: WT B6 mice were injected i.p. with α-GalCer (2 μg/mouse) on day 0. Subsequently, these mice were injected i.p. with an IL10 inhibitor (AS101, 10 μg/mouse) daily for one week starting from day 0. On day 7, these mice were injected i.p. with LPS (2 μg/mouse) plus D-GalN (25 mg/mouse) for induction of sepsis. **(D)** Subsequently, these mice were monitored to evaluate their survival for three days after LPS/D-GalN injection. **(E, F)** Comparison of transcriptional profiles of inflammatory response genes in PBMCs of either non-survivors (red) or survivors (blue) of sepsis in human subjects by relevant gene set enrichment plots from GSEA (NES, normalized enrichment score; FDR, false discovery rate). The mean values ± SD (n = 4 in **(A, B)**; per group in the experiment; Student's *t*-test; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$) are shown. The survival rate was analyzed by Kaplan-Meier plots with a log-rank test ($*p < 0.05$ and $**p < 0.01$). One representative experiment of two experiments is shown. ns, not significant.

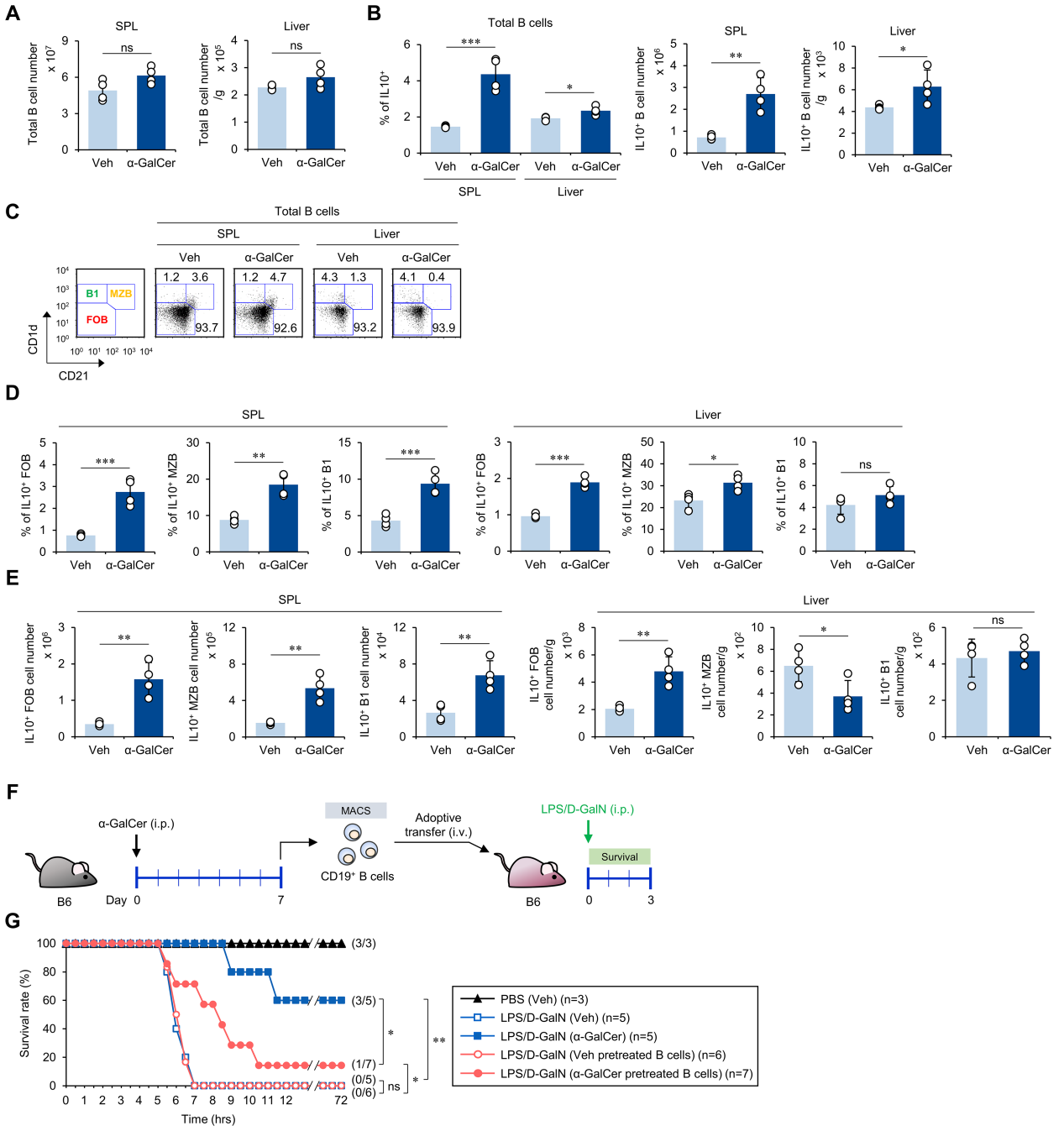

FIGURE 5
α-GalCer pretreatment expands IL10-producing B cells. WT B6 mice were injected i.p. with α-GalCer (2 μg/mouse) and, seven days later, the spleens and livers were harvested for the following analyses. **(A, B)** The total CD19$^+$ B cell numbers **(A)** and the frequency and absolute cell numbers **(B)** of IL10-producing B cells in the spleen and liver. **(C)** The frequencies of B cell subsets (FOB, MZB, and B1 cells) in the spleen and liver. **(D, E)** The frequencies **(D)** and absolute cell numbers **(E)** of IL10-producing B cell subsets (FOB, MZB, and B1 cells) in the spleen and liver. **(F)** Experimental outline: WT B6 mice were injected i.p. with α-GalCer (2 μg/mouse) and, seven days later, splenic CD19$^+$ B cells were isolated by the MACS system. Subsequently, MACS-purified CD19$^+$ B cells (5 x 10^6 cells/mouse) were adoptively transferred to recipient mice. The recipient mice were injected i.p. with LPS (2 μg/mouse) plus D-GalN (25 mg/mouse) for induction of sepsis. **(G)** Subsequently, these mice were monitored to evaluate their survival for 3 days after LPS/D-GalN. The mean values ± SD (n = 4 in **(A, B, D, E)**; per group in the experiment; Student's *t*-test; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$) are shown. The survival rate was analyzed by Kaplan-Meier plots with a log-rank test ($*p < 0.05$ and $**p < 0.01$). One representative experiment of two experiments is shown. ns, not significant.

pretreatment increased splenic neutrophil numbers in LPS/D-GalN-injected mice, and opposite findings were observed in the liver (Supplementary Figures S2C, D), suggesting that α-GalCer pretreatment may affect neutrophil apoptosis and migration. Furthermore, our data in Figure 2 demonstrated that α-GalCer treatment suppresses production of IL1β and TNFα by N1-type neutrophils (43). In addition, it has been reported that anti-inflammatory N2-type neutrophils display increased viability as

compared with pro-inflammatory N1-type neutrophils (44). Together, our results suggest that α-GalCer pretreatment can alter the phenotype of neutrophils from a pathogenic N1-type towards a protective N2-type.

Our data demonstrated that α-GalCer pretreatment reduces the prevalence of splenic iNKT17 cells (Figure 1D). Furthermore, IL17A production by hepatic NKT cells was significantly decreased in α-GalCer-pretreated mice during LPS/D-GalN-induced sepsis (Figure 2D). Since IL17 signaling is crucial in recruiting neutrophils (45), it is likely that α-GalCer pretreatment modulates IL17A production by iNKT cells, which, in turn, overcomes the defect in liver neutrophil recruitment. This possibility will be explored in future studies.

Although much progress has been made in the medical field, postoperative sepsis remains a significant contributor to in-hospital mortality (46, 47). From this point of view, our results suggest a novel approach to preventing postoperative sepsis, by promoting anti-inflammatory conditions prior to surgery and potential exposures to microbial infection. This scenario was tested in Supplementary Figure S1D by employing a CS-induced sepsis model mimicking post-operative sepsis. Moreover, from GSEA of patient PBMCs of both survivor and non-survivor groups (Figures 4E, F), we found that the survivor group downregulates inflammatory response genes and upregulates IL10 responsiveness by increasing IL10RA gene expression (Figure 4F). Therefore, our study suggests α-GalCer treatment as a prophylactic option to prevent sepsis in humans.

5 Conclusions

Here, we demonstrated that α-GalCer pretreatment polarizes iNKT cells from a pro-inflammatory phenotype (i.e., iNKT1 cells and iNKT17 cells) towards an anti-inflammatory phenotype (i.e., iNKT2 cells and iNKT10 cells) that attenuates sepsis severity. Such preventive effects of α-GalCer pretreatment on sepsis development were attributed to reduced pro-inflammatory cytokine production (i.e., TNFα and IL1β) and immune cell infiltration to the liver. Our results identify iNKT cells as critical players in controlling type 1 inflammatory responses during sepsis, with potential prophylactic implications.

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 animal study was approved by The Institutional Animal Care and Use Committee of Sejong University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YP: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. T-CK: Data curation, Formal analysis, Writing – original draft. HP: Data curation, Formal analysis, Funding acquisition, Writing – original draft. LVK: Formal analysis, Writing – original draft, Writing – review & editing. SH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (NRF-2021R1I1A1A01054418 to SL; NRF-2021R1I1A1A01051465 to HP; NRF-2019R1A2C1009926 and NRF-2022R1A2C1009590 to SH).

Conflict of interest

LVK is a scientific advisory board member of Isu Abxis Co., Ltd. Republic of 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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1457690/full#supplementary-material

References

1. Liu V, Escobar GJ, Greene JD, Soule J, Whippy A, Angus DC, et al. Hospital deaths in patients with sepsis from 2 independent cohorts. *JAMA*. (2014) 312:90–2. doi: 10.1001/jama.2014.5804

2. Hotchkiss RS, Moldawer LL, Opal SM, Reinhart K, Turnbull IR, Vincent JL. Sepsis and septic shock. *Nat Rev Dis Primers*. (2016) 2:16045. doi: 10.1038/nrdp.2016.45

3. Kinjo Y, Illarionov P, Vela JL, Pei B, Girardi E, Li X, et al. Invariant natural killer T cells recognize glycolipids from pathogenic gram-positive bacteria. *Nat Immunol*. (2011) 12:966–74. doi: 10.1038/ni.2096

4. Lee SW, Park HJ, Van Kaer L, Hong S, Hong S. Graphene oxide polarizes inkt cells for production of tgfβ and attenuates inflammation in an inkt cell-mediated sepsis model. *Sci Rep*. (2018) 8:10081. doi: 10.1038/s41598-018-28396-9

5. Leung B, Harris HW. Nkt cells in sepsis. *Clin Dev Immunol*. (2010) 2010:414650. doi: 10.1155/2010/414650

6. Fujii H, Seki S, Kobayashi S, Kitada T, Kawakita N, Adachi K, et al. A murine model of nkt cell-mediated liver injury induced by alpha-galactosylceramide/D-galactosamine. *Virchows Arch*. (2005) 446:663–73. doi: 10.1007/s00428-005-1265-8

7. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of il-4 modulates immunity in mouse strains and is determined by lineage diversity of inkt cells. *Nat Immunol*. (2013) 14:1146–54. doi: 10.1038/ni.2731

8. Lee SW, Park HJ, Van Kaer L, Hong S. Roles and therapeutic potential of cd1d-restricted nkt cells in inflammatory skin diseases. *Front Immunol*. (2022) 13:979370. doi: 10.3389/fimmu.2022.979370

9. Van Kaer L, Postoak JL, Song W, Wu L. Innate and innate-like effector lymphocytes in health and disease. *J Immunol*. (2022) 209:199–207. doi: 10.4049/jimmunol.2200074

10. Van Kaer L, Postoak JL, Wang C, Yang G, Wu L. Innate, innate-like and adaptive lymphocytes in the pathogenesis of ms and eae. *Cell Mol Immunol*. (2019) 16:531–9. doi: 10.1038/s41423-019-0221-5

11. Lee SW, Park HJ, Van Kaer L, Hong S. Opposing roles of dcs and inkt cells in the induction of foxp3 expression by mln cd25(+)Cd4(+) T cells during ifngamma-driven colitis. *Int J Mol Sci*. (2022) 23:15316. doi: 10.3390/ijms232315316

12. Chen D, Zhao H, Gao X, Chen S, Liu H, Zhang J, et al. Subcutaneous administration of alpha-galcer activates inkt10 cells to promote M2 macrophage polarization and ameliorates chronic inflammation of obese adipose tissue. *Int Immunopharmacol*. (2019) 77:105948. doi: 10.1016/j.intimp.2019.105948

13. Catalan D, Mansilla MA, Ferrier A, Soto L, Oleinika K, Aguillon JC, et al. Immunosuppressive mechanisms of regulatory B cells. *Front Immunol*. (2021) 12:611795. doi: 10.3389/fimmu.2021.611795

14. Carter NA, Rosser EC, Mauri C. Interleukin-10 produced by B cells is crucial for the suppression of th17/th1 responses, induction of T regulatory type 1 cells and reduction of collagen-induced arthritis. *Arthritis Res Ther*. (2012) 14:R32. doi: 10.1186/ar3736

15. Oleinika K, Rosser EC, Matei DE, Nistala K, Bosma A, Drozdov I, et al. Cd1d-dependent immune suppression mediated by regulatory B cells through modulations of inkt cells. *Nat Commun*. (2018) 9:684. doi: 10.1038/s41467-018-02911-y

16. Tao L, Wang Y, Xu J, Su J, Yang Q, Deng W, et al. Il-10-producing regulatory B cells exhibit functional defects and play a protective role in severe endotoxic shock. *Pharmacol Res*. (2019) 148:104457. doi: 10.1016/j.phrs.2019.104457

17. Van Kaer L. Alpha-galactosylceramide therapy for autoimmune diseases: prospects and obstacles. *Nat Rev Immunol*. (2005) 5:31–42. doi: 10.1038/nri1531

18. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. Il-10-producing nkt10 cells are a distinct regulatory invariant nkt cell subset. *J Clin Invest*. (2014) 124:3725–40. doi: 10.1172/JCI72308

19. Uchida T, Nakashima H, Yamagata A, Ito S, Ishikiriyama T, Nakashima M, et al. Repeated administration of alpha-galactosylceramide ameliorates experimental lupus nephritis in mice. *Sci Rep*. (2018) 8:8225. doi: 10.1038/s41598-018-26470-w

20. Park HJ, Kim TC, Park YH, Lee SW, Jeon J, Park SH, et al. Repeated alpha-galcer administration induces a type 2 cytokine-biased inkt cell response and exacerbates atopic skin inflammation in valpha14(Tg) nc/nga mice. *Biomedicines*. (2021) 9:1619. doi: 10.3390/biomedicines9111619

21. Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR, et al. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest*. (2005) 115:2572–83. doi: 10.1172/JCI24762

22. Starr ME, Steele AM, Saito M, Hacker BJ, Evers BM, Saito H. A new cecal slurry preparation protocol with improved long-term reproducibility for animal models of sepsis. *PLoS One*. (2014) 9:e115705. doi: 10.1371/journal.pone.0115705

23. Park HJ, Lee SW, Van Kaer L, Lee MS, Hong S. Il-7 deficiency exacerbates atopic dermatitis in nc/nga mice. *Int J Mol Sci*. (2023) 24:9956. doi: 10.3390/ijms24129956

24. Lee SW, Oh SY, Park HJ, Kim TC, Park YH, Van Kaer L, et al. Phosphorothioate-linked guanine/cytosine-based stem-loop oligonucleotides induce the extracellular release of mitochondrial DNA from peritoneal B1a cells. *Int J Biol Macromol*. (2022) 223:252–62. doi: 10.1016/j.ijbiomac.2022.10.280

25. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U.S.A*. (2005) 102:15545–50. doi: 10.1073/pnas.0506580102

26. Wang L, Zhao H, Wang D. Inflammatory cytokine expression in patients with sepsis at an intensive care unit. *Exp Ther Med*. (2018) 16:2126–31. doi: 10.3892/etm.2018.6376

27. Sonego F, Castanheira FV, Ferreira RG, Kanashiro A, Leite CA, Nascimento DC, et al. Paradoxical roles of the neutrophil in sepsis: protective and deleterious. *Front Immunol*. (2016) 7:155. doi: 10.3389/fimmu.2016.00155

28. Souza-Fonseca-Guimaraes F, Adib-Conquy M, Cavaillon JM. Natural killer (Nk) cells in antibacterial innate immunity: angels or devils? *Mol Med*. (2012) 18:270–85. doi: 10.2119/molmed.2011.00201

29. Vogt S, Mattner J. Nkt cells contribute to the control of microbial infections. *Front Cell Infect Microbiol*. (2021) 11:718350. doi: 10.3389/fcimb.2021.718350

30. Xu W, Wang Y, Jin C, Zhang W, Chen J, Chen X, et al. Il-17 imbalance promotes the pyroptosis in immune-mediated liver injury through stat3-ifi16 axis. *Immune Netw*. (2023) 23:e46. doi: 10.4110/in.2023.23.e46

31. Wun KS, Cameron G, Patel O, Pang SS, Pellicci DG, Sullivan LC, et al. A molecular basis for the exquisite cd1d-restricted antigen specificity and functional responses of natural killer T cells. *Immunity*. (2011) 34:327–39. doi: 10.1016/j.immuni.2011.02.001

32. Yu KO, Im JS, Molano A, Dutronc Y, Illarionov PA, Forestier C, et al. Modulation of cd1d-restricted nkt cell responses by using N-acyl variants of alpha-galactosylceramides. *Proc Natl Acad Sci U.S.A*. (2005) 102:3383–8. doi: 10.1073/pnas.0407488102

33. Blériot C, Dupuis T, Jouvion G, Eberl G, Disson O, Lecuit M. Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection. *Immunity*. (2015) 42:145–58. doi: 10.1016/j.immuni.2014.12.020

34. Pesu M, Takaluoma K, Aittomaki S, Lagerstedt A, Saksela K, Kovanen PE, et al. Interleukin-4-induced transcriptional activation by stat6 involves multiple serine/threonine kinase pathways and serine phosphorylation of stat6. *Blood*. (2000) 95:494–502. doi: 10.1182/blood.V95.2.494

35. Latifi SQ, O'Riordan MA, Levine AD. Interleukin-10 controls the onset of irreversible septic shock. *Infect Immun*. (2002) 70:4441–6. doi: 10.1128/IAI.70.8.4441-4446.2002

36. Howard M, Muchamuel T, Andrade S, Menon S. Interleukin 10 protects mice from lethal endotoxemia. *J Exp Med*. (1993) 177:1205–8. doi: 10.1084/jem.177.4.1205

37. Vomhof-DeKrey EE, Yates J, Hagglof T, Lanthier P, Amiel E, Veerapen N, et al. Cognate interaction with inkt cells expands il-10-producing B regulatory cells. *Proc Natl Acad Sci U.S.A*. (2015) 112:12474–9. doi: 10.1073/pnas.1504790112

38. Sun Z, Chen A, Fang H, Sun D, Huang M, Cheng E, et al. B cell-derived il-10 promotes the resolution of lipopolysaccharide-induced acute lung injury. *Cell Death Dis*. (2023) 14:418. doi: 10.1038/s41419-023-05954-2

39. Friedman G, Jankowski S, Marchant A, Goldman M, Kahn RJ, Vincent JL. Blood interleukin 10 levels parallel the severity of septic shock. *J Crit Care*. (1997) 12:183–7. doi: 10.1016/s0883-9441(97)90030-7

40. Ng TH, Britton GJ, Hill EV, Verhagen J, Burton BR, Wraith DC. Regulation of adaptive immunity; the role of interleukin-10. *Front Immunol*. (2013) 4:129. doi: 10.3389/fimmu.2013.00129

41. Yeung ST, Ovando LJ, Russo AJ, Rathinam VA, Khanna KM. Cd169+ Macrophage intrinsic il-10 production regulates immune homeostasis during sepsis. *Cell Rep*. (2023) 42:112171. doi: 10.1016/j.celrep.2023.112171

42. Kumar V. Immunometabolism: another road to sepsis and its therapeutic targeting. *Inflammation*. (2019) 42:765–88. doi: 10.1007/s10753-018-0939-8

43. Wang X, Qiu L, Li Z, Wang XY, Yi H. Understanding the multifaceted role of neutrophils in cancer and autoimmune diseases. *Front Immunol*. (2018) 9:2456. doi: 10.3389/fimmu.2018.02456

44. Ohms M, Moller S, Laskay T. An attempt to polarize human neutrophils toward N1 and N2 phenotypes in vitro. *Front Immunol*. (2020) 11:532. doi: 10.3389/fimmu.2020.00532

45. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. Requirement of interleukin 17 receptor signaling for lung cxc chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med*. (2001) 194:519–27. doi: 10.1084/jem.194.4.519

46. Vogel TR, Dombrovskiy VY, Carson JL, Graham AM, Lowry SF. Postoperative sepsis in the United States. *Ann Surg*. (2010) 252:1065–71. doi: 10.1097/SLA.0b013e3181dcf36e

47. Ou L, Chen J, Hillman K, Flabouris A, Parr M, Assareh H, et al. The Impact of Post-Operative Sepsis on Mortality after Hospital Discharge among Elective Surgical Patients: A Population-Based Cohort Study. *Crit Care*. (2017) 21:34. doi: 10.1186/s13054-016-1596-7

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 20 September 2024
DOI 10.3389/fimmu.2024.1467774

OPEN ACCESS

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RECEIVED 20 July 2024
ACCEPTED 30 August 2024
PUBLISHED 20 September 2024

CITATION
Guo Y, Ohki S, Kawano Y, Kong WS, Ohno Y, Honda H, Kanno M and Yasuda T (2024) Eed-dependent histone modification orchestrates the iNKT cell developmental program alleviating liver injury.
Front. Immunol. 15:1467774.
doi: 10.3389/fimmu.2024.1467774

Eed-dependent histone modification orchestrates the iNKT cell developmental program alleviating liver injury

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Polycomb repressive complex 2 (PRC2) is an evolutionarily conserved epigenetic modifier responsible for tri-methylation of lysine 27 on histone H3 (H3K27me3). Previous studies have linked PRC2 to invariant natural killer T (iNKT) cell development, but its physiological and precise role remained unclear. To address this, we conditionally deleted Eed, a core subunit of PRC2, in mouse T cells. The results showed that Eed-deficient mice exhibited a severe reduction in iNKT cell numbers, particularly NKT1 and NKT17 cells, while conventional T cells and NKT2 cells remained intact. Deletion of Eed disrupted iNKT cell differentiation, leading to increased cell death, which was accompanied by a severe reduction in H3K27me3 levels and abnormal expression of *Zbtb16*, *Cdkn2a*, and *Cdkn1a*. Interestingly, Eed-deficient mice were highly susceptible to acetaminophen-induced liver injury and inflammation in an iNKT cell-dependent manner, highlighting the critical role of Eed-mediated H3K27me3 marks in liver-resident iNKT cells. These findings provide further insight into the epigenetic orchestration of iNKT cell-specific transcriptional programs.

KEYWORDS

iNKT, Eed, H3K27me3, PRC2, liver injury

1 Introduction

The functional repertoire of T cells is shaped by highly ordered development and selection processes in the thymus, driven by T-cell receptor (TCR) rearrangement and signal strength, which enables the thymus to contribute to host immunity (1–3). Immature CD4/CD8 double-positive (DP) progenitors expressing distinct αβ TCRs undergo positive

selection in the thymus. Those with lower-affinity TCRs are selected for weak reactivity to MHC, eventually becoming naive $CD4^+$ and $CD8^+$ conventional T cells, while agonist-selected those with higher TCR affinity become regulatory T cells (Tregs) or invariant natural killer T cells (iNKTs) (4, 5). Although Tregs continue to perceive higher TCR signals in the periphery, iNKT cells stop receiving TCR signals as they mature and migrate (6).

iNKT cells, an innate-like unconventional T-cell subset, exhibit unique effector and memory phenotypes, promptly secrete multiple cytokines upon activation (IFN-γ, GM-CSF, IL-2, IL-17, and TNF-α), and are involved in various diseases, including inflammatory conditions (7–9). The invariant TCRs (Vα14-Jα18 in mice; Vα24-Jα18 in humans) expressed in iNKT cells recognize lipid antigens presented by the MHC class I-like molecule CD1d on other DP thymocytes, along with SLAM family receptor co-stimulation (7, 10–12). Upon commitment to the iNKT cell lineage, they undergo sequential differentiation stages: S0 ($CD24^+CD44^-NK1.1^-$), S1 ($CD24^-CD44^-NK1.1^-$), S2 ($CD24^-CD44^+NK1.1^-$), and S3 ($CD24^-CD44^+NK1.1^+$) mature iNKT cells (7, 13). PLZF, encoded by *Zbtb16*, is induced by TCR signaling and is required for the development and functionality of iNKT cells (14, 15). Expressions of T-bet, GATA3, or RORγt transcription factors instruct NKT progenitors (NKTp) to IFN-γ-secreting NKT1, IL-4-secreting NKT2, or IL-17-secreting NKT17 effector cells, respectively (16).

Cell lineage commitment and specification rely on coordinated gene expression and epigenetic regulation (17). Histone H3 lysine 27 trimethylation (H3K27me3), associated with transcriptional repression, is facilitated by the enzymatic activity of polycomb repressive complex 2 (PRC2), comprising Eed, Suz12, and Ezh1/Ezh2 subunits and maintains specific gene expression patterns during development. Ezh1 and Ezh2 are catalytic subunits of the PRC2 methyltransferase for H3K27me3, which requires Eed and Suz12 for catalytic activation (18, 19). Eed recognizes H3K27me3, inducing a conformational change in PRC2 and activating the Ezh2 enzyme. The proposed positive feedback loop model involves initial H3K27me3 deposition by PRC2. Subsequently, PRC2 is further recruited through the binding of its Eed subunit to H3K27me3, which allosterically activates PRC2. This leads to additional H3K27me3 deposition, enabling stable chromatin domains (20, 21). Thus, Eed is essential for both initial activation and amplifying PRC2 activity for H3K27me3. Deficiency of Ezh2 in mouse T cells partially reduces H3K27me3 levels in iNKT cells and accumulates Ezh2-deficient iNKT cells, suggesting that incomplete loss of PRC2 activity perturbs iNKT cell development (22, 23). Conversely, removing Eed or Suz12 from T cells severely inhibits iNKT cell development, highlighting the essential role of PRC2 activity (23). However, the underlying epigenetic mechanisms directing iNKT cells from DP thymocytes and those required for iNKT effector subset differentiation remain unknown. Therefore, we conditionally deleted Eed from DP thymocytes in mice to investigate its role in iNKT cell lineage commitment and effector subset differentiation. We further analyzed the physiological effect of Eed-dependent iNKT cells on liver homeostasis in response to hepatocyte death and associated inflammation.

2 Materials and methods

2.1 Mice

Eed-flox mice were backcrossed to C57BL/6 for more than 7 generations (24). CD4-Cre transgenic mice were crossed with Eed-flox mice to generate Eed conditional knockout (cKO) mice (25). C57BL/6 (Ly5.2) mice were obtained from CLEA Japan and C57BL/6 background Ly5.1 mice were obtained from the Jackson Laboratory. Mice were bred and maintained under the specific pathogen-free conditions.

2.2 Flow cytometry

Single-cell suspensions from the spleen and bone marrow were resuspended in Gey's or ACK solutions for red blood cell lysis. Cells were treated with Fc Block (2.4G2, BD Biosciences) or TruStain FcX (93, BioLegend) followed by staining with fluorochrome-conjugated antibodies or biotinylated antibodies. Anti-CD24 (M1/69), CD44 (IM7), TCRβ (H57-597), NK1.1 (PK136), CD4 (RM4-5), CD8 (53-6.7), PLZF (R17-809), RORγt (Q31-378), Ly49A (JR9-318), Ly49C and Ly49I (5E6), Ly49G2 (4D11), IL-4 (11B11), IL-17A (TC11-18H10), IFN-γ (XMG1.2) were purchased from BD Biosciences; Anti-T-bet (4B10) was purchased from BioLegend. The cells stained with biotinylated antibodies were detected by fluorochrome-conjugated streptavidin. Dead Cells were excluded using propidium iodide (Sigma-Aldrich). To detect iNKT cells, the cells were labeled with α-GalCer (Funakoshi)-loaded CD1d-tetramer PE (MBL) for 30 min at room temperature. For intracellular staining, the cells were fixed and permeabilized using a Foxp3 staining kit (Thermo Fisher Scientific). For cytokine staining, the cells were treated with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml of ionomycin with BD GolgiStop containing monensin for 6 hours (h) followed by staining using Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences). For H3K27me3 and Eed staining, anti-trimethyl-Histone H3 (Lys27) polyclonal antibody (07-449, Upstate), anti-Eed (E4L5E) XP rabbit monoclonal antibody (85322, CST), and normal rabbit IgG (2729; CST) as isotype control were labeled using Zenon Alexa Fluor 647 rabbit IgG Labeling Kit (Z25308; Invitrogen) followed by intracellular staining procedure. Stained cells were analyzed using FACSCanto II (BD Biosciences) or CytoFLEX S (Beckman Coulter). Cell sorting was performed using FACSAria II (BD Biosciences). Data were analyzed on FlowJo software (BD Biosciences).

2.3 Isolation of lymphocytes from the liver and lung

To isolate lymphocytes from the liver and lung, mice were anesthetized and perfused with PBS. For liver lymphocytes, the liver cells were filtered through 100-μm nylon mesh, washed with 2% FBS/PBS, and resuspended in 33.75% Percoll PLUS (GE Healthcare

Life Sciences) followed by centrifugation at 2,300 rpm for 25 min at room temperature. For lung lymphocytes, the lung was cut into small pieces and digested with 1 mg/ml collagenase D (Roche) and 0.5 mg/ml DNase I (Roche) for 1 h at 37°C with shaking. The cells were filtered through 70-μm nylon mesh and then treated with Gey's solution to remove red blood cells.

2.4 Fetal thymus organ culture

Fetal thymus organ culture (FTOC) was performed as described previously (26). For timed pregnancies, the day of the vaginal plug was designated E0.5. The thymic lobes were isolated from control or Eed cKO E15.5 fetuses, which were then transferred to standard FTOC conditions and analyzed for iNKT cell development on the indicated day.

2.5 Generation of BM mixed chimeras

Bone marrow (BM) cells ($2x10^6$) obtained from control or Eed cKO mice (Ly5.2) were mixed with C57BL/6 (Ly5.1/5.2) BM cells at a 1:1 ratio and transferred into 9-Gy irradiated C57BL/6 (Ly5.1) recipient mice. Recipient mice were analyzed after 4 months (Mon).

2.6 Acetaminophen (N-acetyl-para-aminophenol, APAP)-induced liver injury

APAP (00204-82, Nacalai Tesque) was dissolved at 15 mg/ml in PBS at 37°C. Seven- to eleven-week-old control and Eed cKO mice were fasted for 16 h before APAP treatment. For liver histology, female mice were administrated with 360 mg/kg APAP and sacrificed mice at 48 or 72 h after treatment. Hematoxylin and eosin (H&E) staining was performed with frozen liver tissues, firstly stained with Mayer's Hematoxylin Solution (FUJIFILM) and 1% Eosin Y Solution (FUJIFILM), then followed by ethanol and xylene dehydration steps. The images were obtained from BZ-X800 microscope (Keyence). The percentage of necrosis area was measured by Keyence analysis software (Keyence). For the inflammation score (27), severity was categorized into four levels: 0, no inflammation; 1, moderate and inflammatory cells were scattered; 2, marked and inflammatory cells formed foci; 3, severe and cells were inflammatory diffuse. For iNKT transfer experiments, liver iNKT cells sorted from wild-type Ly5.1 mice were injected intravenously into control or Eed cKO mice at $3\text{-}5x10^5$ cells per mouse, then fasted mice were treated with APAP after 3 days. To analyze the survival, male mice were injected 375 mg/kg APAP by intraperitoneal and checked mice survival every 12 h.

2.7 RNA-seq

$CD4^+CD8^+TCR\beta^+CD69^+$ thymocytes ($1x10^5$) were sorted from 3 pairs of each control or Eed cKO mice, then kept in RNA later solution (Thermo Fisher Scientific). Total RNA was isolated by using QIAzol and miRNeasy Micro kit (QIAGEN). cDNA synthesis (1x105) was used SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing Kit (Takara). Libraries were prepared by Nextera XT v2 Kit (Illumina) and sequenced on an Illumina HiSeq 2500. Quality control and adapter trimming for raw reads were performed by fastp (v0.21.0). Trimmed read mapping and quantification were performed with RSEM (v1.3.1) using STAR (v2.7.10) as an aligner. Differential expressions were identified by using DEseq2 (v1.34.0). RNA-seq data were used for GSEA analysis with gene sets obtained from publicly available databases (28, 29).

2.8 ChIP-seq

H3K27me3 ChIP-seq dataset was obtained from GSE84238 (30). ChIP-seq raw reads were mapped onto reference mouse genome mm39 by using HISAT2 and peaks were called using MACS2. SparK and IGV were used to visualize bedGraph coverage tracks.

2.9 ChIP-qPCR

DP and iNKT cells sorted from control and Eed cKO mice were fixed with 1% formaldehyde for 10 min at room temperature and sonicated with Bioruptor, and then sonicated chromatins were immunoprecipitated by control rabbit IgG antibody (2729, CST) or anti-trimethylated H3K27 antibody (07-449, Upstate) coupled with SureBeads Protein A/G Magnet Beads (Bio-Rad) overnight. After washing and reverse crosslinking, DNA was purified using Phenol: Chloroform: Isoamyl alcohol (Nacalai Tesque). qPCR was performed using Thunderbird SYBR qPCR mix (TOYOBO) or TB Green Premix Ex Taq II (Takara). Primers are listed in Supplementary Table 1.

2.10 Statistical analysis

All data analyses were performed with Prism 7 software (GraphPad). The results were presented as mean ± SD. *p* values were calculated by unpaired t-test, multiple t-tests with the Holm-Sidak method at $\alpha = 0.05$, or one-way ANOVA. Statistical significance was determined with alpha<0.05 and presented as *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001.

3 Results

3.1 Eed is essential for iNKT cell development

We examined H3K27me3 levels during iNKT cell development. H3K27me3 signals were detected in developing thymic iNKT cells from DP to the S3 stage, indicating that H3K27me3 histone modification occurs during the early development of iNKT cells. Notably, H3K27me3 levels significantly increased during the transition from DP/S0 to the S2 stage, suggesting that additional

activation of the PRC2 complex leads to *de novo* deposition of H3K27me3 with stage progression (Figure 1A). We compared the mRNA expression of PRC2 core subunits among the T-cell subsets. Similar to the H3K27me3 levels, *Eed* mRNA expression levels were increased in thymic and splenic iNKT cells compared to thymic DP cells, whereas *Ezh2* and *Suz12* mRNAs were equal or downregulated (Figure 1B). These results prompted us to investigate the specific function of Eed in iNKT cell development.

We bred Eed-flox mice with CD4-Cre transgenic mice expressing Cre recombinase after the DP stage in the T-cell lineage (named Eed cKO mice). We first examined the deletion of Eed in different T-cell populations in Eed cKO mice. The expression of Eed protein remained intact in double-negative (DN) cells but was partially abrogated in DP cells and S0 iNKT cells, with nearly complete loss of Eed in CD4 or CD8 single-positive (SP) cells, S1/3 iNKT cells, and splenic T cells (Figure 1C; Supplementary Figure 1A). Additionally, *Eed* mRNA was severely decreased in the S1 to S3 stages, but only partially decreased in the S0 stages of iNKT cells from Eed cKO mice (Supplementary Figure 1B). These results confirmed Eed protein expression in conventional T cells and iNKT cells, consistent with the mRNA expression profile, indicating that the deletion of Eed using CD4-Cre resulted in a delay beyond the thymic DP cell stage, similar to previous findings (31).

In Eed cKO mice, we observed a significant reduction in iNKT cells characterized by TCRβ$^+$CD1d-tetramer (Tet)$^+$ cells in various tissues, including the thymus, spleen, liver, and lung (Figure 1D, E). To further examine the early developmental stages of impaired thymic iNKT cells in Eed cKO mice, we utilized the surface markers CD24, CD44, and NK1.1, to distinguish the developmental stages of iNKT cells. Compared to control mice, Eed cKO mice showed a significant increase in the percentage of iNKT cells from the S0 to S2 stage, followed by a substantial decline at the S3 stage (Figures 1F, G). Furthermore, the absolute number of S3 iNKT cells in Eed cKO mice was severely reduced compared to that of control mice, indicating a critical role of Eed in the S2 to S3 transition during iNKT cell development, and the increased proportions of S0, S1, and S2 iNKT cells were mainly due to the reduction of the S3 iNKT cells. Conversely, the numbers of DN, DP, CD4 SP, CD8 SP, and Foxp3$^+$ Tregs T-cell subsets were not affected by Eed deficiency in thymus (Supplementary Figure 2). These results suggest that Eed plays an indispensable and specific role in the development of iNKT cells.

3.2 Age-dependent and cell-intrinsic role of Eed in iNKT cell development

The differentiation of iNKT cells in mice begins after birth (32). To investigate potential age-related differences in iNKT cell development, we examined iNKT cell development in neonatal mice. TCRβ$^+$CD1d-Tet$^+$ iNKT cells were first detected at postnatal day 4 (P4), which was confirmed by the detection of NK1.1$^+$ iNKT cells in P4 control mice (Figure 2A; Supplementary Figure 3). While the total numbers of thymocytes increased with postnatal days in both control and Eed cKO mice, a marked reduction in the iNKT cell fraction was observed in the Eed cKO thymus at P8 (Figure 2B). In control mice, the majority of iNKT cells were S2 cells; however, in Eed cKO mice, most iNKT cells were identified as S0 cells, with few S1, S2, and S3 cells (Figures 2C, D). We further utilized fetal thymic organ cultures to analyze iNKT cell development from embryonic progenitors. In FTOC, progenitors from the E15.5 fetal thymic lobe revealed a significant reduction in Eed-deficient iNKT cell numbers after day 11 of culture compared to that of controls, primarily due to a severe reduction in S2/3 cells, consistent with the neonatal results (Figures 2E, F).

CD1d and SLAM family receptors play critical roles in initial iNKT cell development (11, 33). To assess whether Eed affects the expression of these molecules, we measured the expression of CD1d, CD150 (Slamf1), and Ly108 (Slamf6) in DP thymocytes, and found no obvious differences between control and Eed cKO mice (Supplementary Figure 4A). In mice, over 80% of CD1d-restricted TCRαβ$^+$ iNKT cells express Vα14-Jα18 chain combined with Vβ8, Vβ7, or Vβ2 chain (7). Therefore, we determined TCR Vβ usage on thymic iNKT cells in Eed cKO mice, finding no abnormalities in the distribution of TCRVβ2, Vβ5, Vβ7, Vβ8.1/8.2, and Vβ8.3 (Supplementary Figures 4B, C). Additionally, mRNA expression level of the Vα14-Jα18 chain was similar between control and Eed cKO DP cells (Supplementary Figure 4D). Thus, the abnormal development of iNKT cells in Eed cKO mice was not due to dysregulated expression of CD1d, SLAM family, Vα14-Jα18 chain, or Vβ usage on DP thymocytes.

Considering that the cellular interaction between DP thymocytes in the thymic microenvironment is crucial for the initial development of iNKT cells (4, 34), we aimed to discriminate the environmental influences caused by Eed deletion in other cell types from cell-intrinsic defects in iNKT cells. Therefore, we isolated BM cells from Ly5.2 control and Eed cKO mice and transferred them together with Ly5.1/5.2 wild-type BM cells into congenic Ly5.1 mice. The results showed a significant reduction in the percentage of iNKT cells developed from Eed cKO BM cells compared to that from control BM cells (Figures 2G, H). In summary, Eed is critically required for iNKT progenitors to differentiate into mature iNKT cells in a cell-intrinsic manner, particularly at the transition from the S0/S1 to S2 stage in embryos/neonates and from S2 to S3 in adults.

3.3 Eed is indispensable for NKT1 and NKT17 cell development

Shortly after the initial PLZF expression, iNKT cells transition to a progenitor state known as NKTp, which further differentiates into three mature NKT subsets exhibiting distinct effector functions and cytokine secretion (16). We examined the effect on the terminal maturation of iNKT subsets in Eed cKO mice based on the cytokine profiles of thymic iNKT cells, observing a significant reduction in both the percentage and cell number of IFN-γ-producing NKT1 cells and IL-17-producing NKT17 cells in Eed cKO mice compared to that of control mice, while those of IL-4-producing NKT2 cells were comparable with control mice (Figures 3A, B).

10.3389/fimmu.2024.1467774

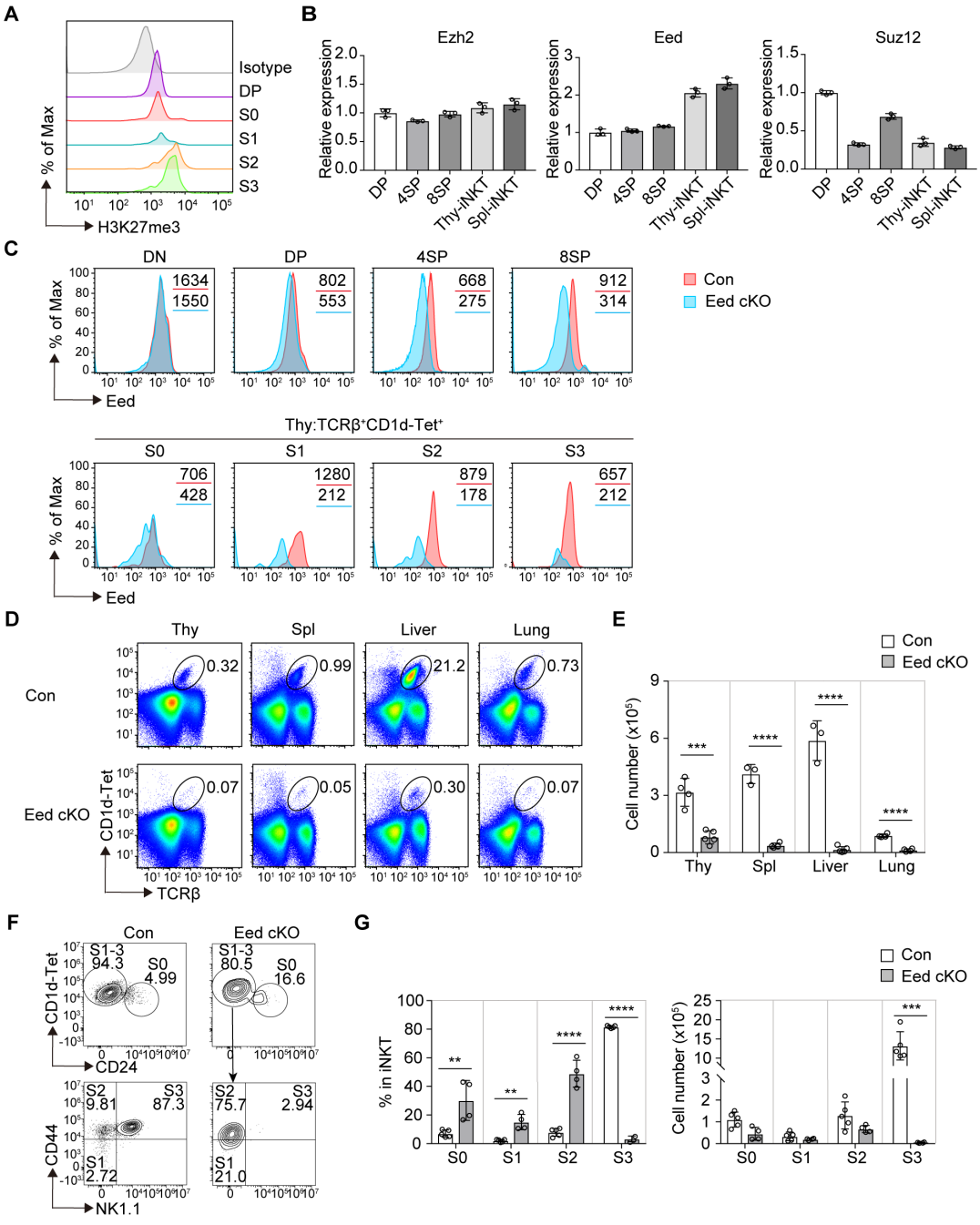

FIGURE 1
Eed is essential for iNKT cell development. **(A)** FACS analysis of H3K27me3 levels in the indicated thymic iNKT cell populations. The gray histogram indicates isotype control. S0, $CD24^+$; S1, $CD24^-CD44^-NK1.1^-$; S2, $CD24^-CD44^+NK1.1^-$; and S3, $CD24^-CD44^+NK1.1^+$ cells are gated on $TCR\beta^+CD1d\text{-}Tet^+$. DP, $CD4^+CD8^+$. **(B)** Real-time PCR analysis of the expression of the PRC2 core members Ezh2, Eed, and Suz12 in the indicated T-cell subsets. Expression values were normalized to *Ywhaz*. DP, $CD4^+CD8^+$; 4SP, $CD4^+CD8^-$; 8SP, $CD4^-CD8^+$ from thymus. iNKT, $TCR\beta^+CD1d\text{-}Tet^+$cells from thymus (Thy) and spleen (Spl). **(C)** Eed expression in the indicated cell populations of control (*red*) and Eed cKO (*blue*) mice. The values in each panel show the mean fluorescence intensity (MFI). DN, $CD4^-CD8^-$; DP, $CD4^+CD8^+$; 4SP, $CD4^+CD8^-$; and 8SP, $CD4^-CD8^+$ cells from thymus. **(D, E)** Representative FACS plots and absolute number of $TCR\beta^+CD1d\text{-}Tet^+$ iNKT cells in the thymus, spleen, liver, and lung from control (n=3-4) and Eed cKO mice (n=4-5). **(F)** Representative FACS plots of the thymic iNKT populations from control and Eed cKO mice. Expression of CD44 and NK1.1 are analyzed on $CD1d\text{-}Tet^+CD24^-$ fraction corresponding to S1-3 stages of iNKT cells. **(G)** Percentage and absolute cell number of the indicated cell populations from **(F)** of control (n=5) and Eed cKO mice (n=4). Data are mean ± SD with statistical significance determined by unpaired t-tests **(E)** or multiple t-test **(G)**. *p* values are represented as **, <0.01; ***, <0.001; ****, <0.0001. Data are representative of at least two independent experiments.

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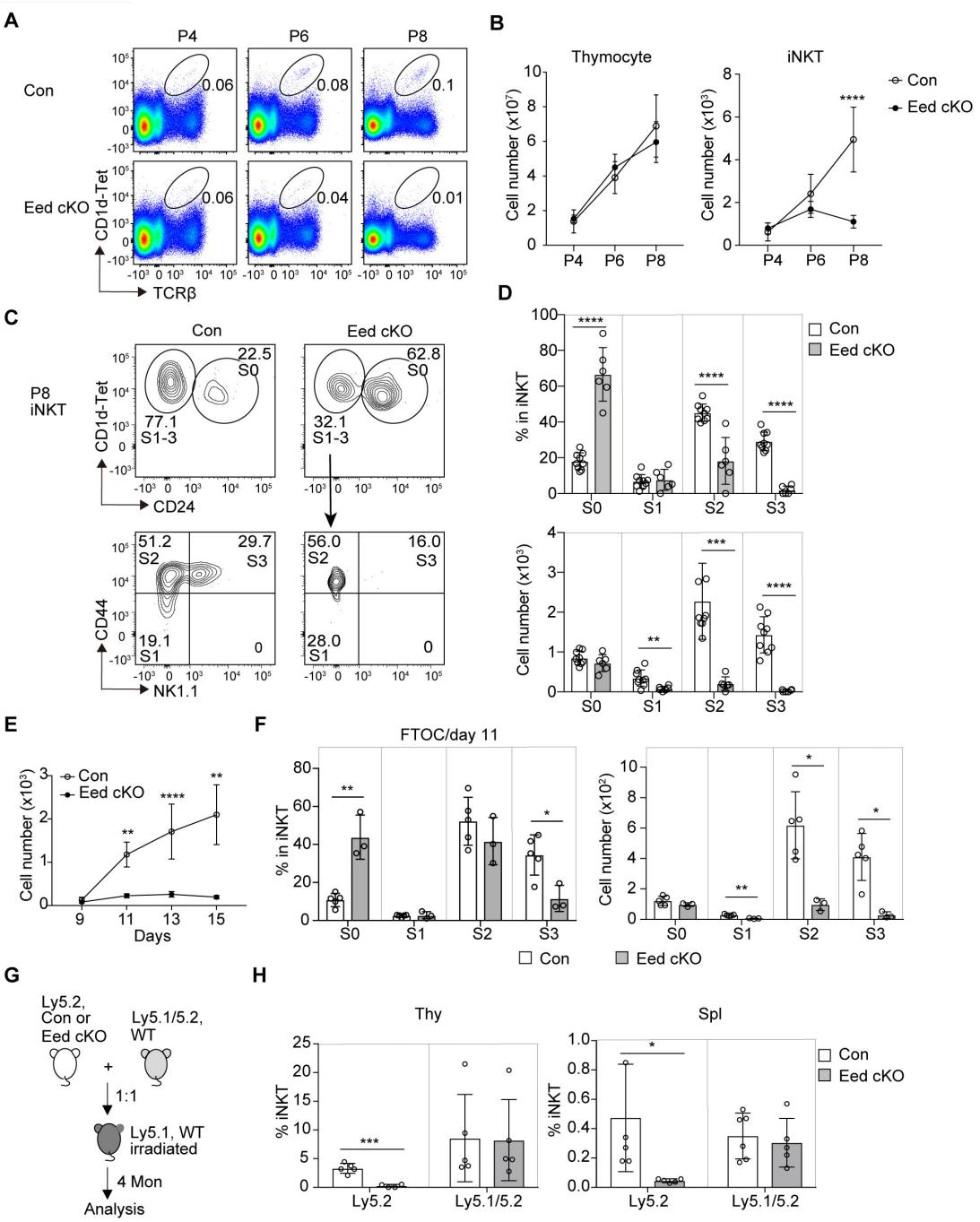

FIGURE 2
Age-dependent and cell-intrinsic role of Eed in the development of iNKT cells. **(A)** Representative FACS plots of TCRβ$^+$CD1d-Tet$^+$ iNKT cells in the thymus of control and Eed cKO mice at postnatal day 4 (P4), 6 (P6), and 8 (P8). **(B)** Absolute numbers of total thymocytes and TCRβ$^+$CD1d-Tet$^+$ iNKT cells in the thymus of control and Eed cKO mice at P4 (n>3), P6 (n>5), and P8 (n>5). **(C, D)** Representative FACS plots, percentage, and absolute cell number of the indicated thymic iNKT populations from control and Eed cKO mice at P8. **(E, F)** E15.5 fetal thymic lobes were isolated from control or Eed cKO mice and cultured on FTOC condition. **(E)** Absolute number of TCRβ$^+$CD1d-Tet$^+$ iNKT cells in control and Eed cKO fetal thymus on days 9, 11, 13, and 15 of culture. The number of lobes in FTOC cultures: day 9 (n=3-4), day 11 (n=3-5), day 13 (n=6-10), and day 15 (n=3-5). **(F)** Percentage and absolute number of S0 to S3 iNKT cells in control and Eed cKO fetal thymus after day 11 of FTOC culture (n=3-5). **(G)** Scheme for the generation of BM chimeras. **(H)** Percentage of TCRβ$^+$CD1d-Tet$^+$ iNKT cells in CD45.1$^-$CD45.2$^+$(Ly5.2) and CD45.1$^+$CD45.2$^+$ (Ly5.1/5.2) cells in the indicated organs from BM chimeras (n=5-6). Data are mean ± SD with statistical significance determined by unpaired t-test **(B, E, H)** or multiple t-tests **(D, F)**. *p* values are represented as *, <0.05; **, <0.01; ***, <0.001; ****, <0.0001. Data are representative of at least two independent experiments.

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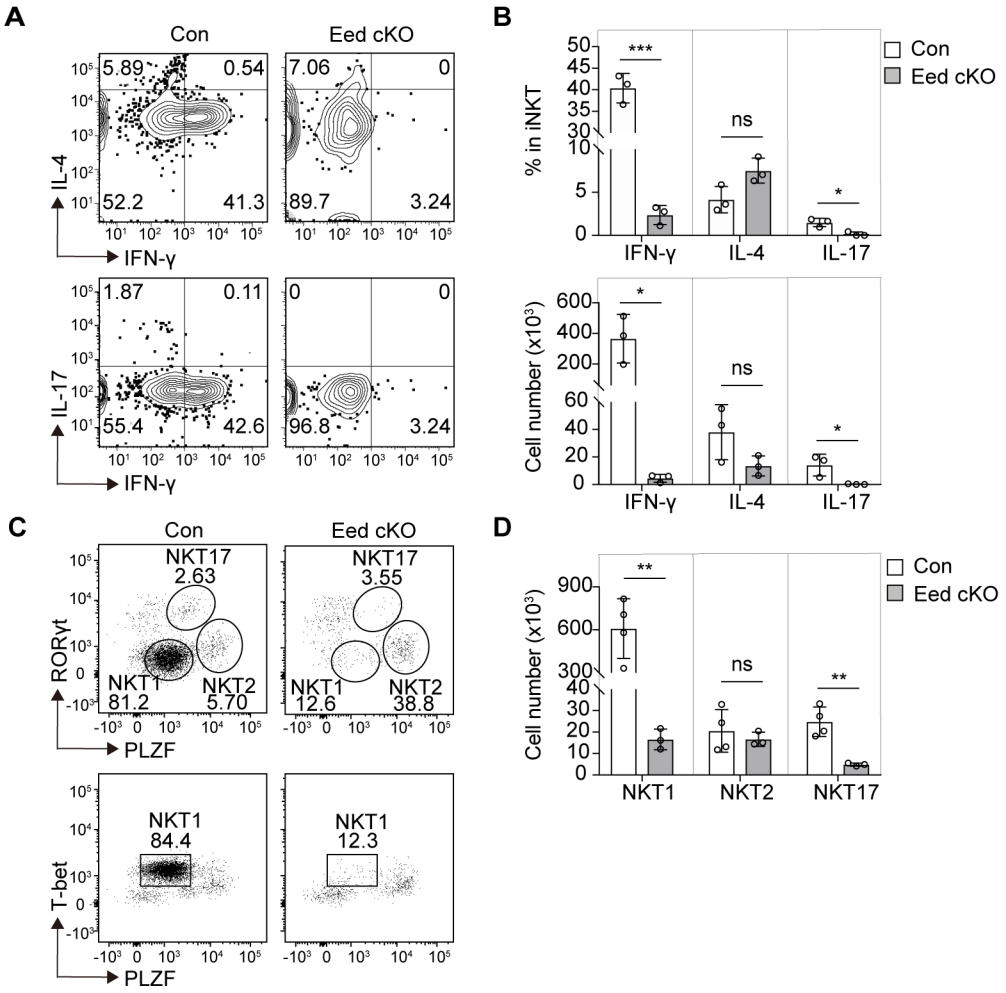

FIGURE 3
Eed is indispensable for the development of NKT1 and NKT17 cells. **(A, B)** Representative FACS plots, percentage, and absolute number of thymic TCRβ$^+$CD1d-Tet$^+$ iNKT cells with IFN-γ, IL-4, or IL-17 expression from control and Eed cKO mice (n=3 each). **(C, D)** Representative FACS plots and absolute number of thymic TCRβ$^+$CD1d-Tet$^+$ iNKT cells with T-bet, PLZF, and/or RORγt expression, determined as NKT1 (T-bet$^+$PLZF^{low}), NKT2 (RoRγt$^-$PLZF^{hi}) and NKT17 (RORγt$^+$PLZF^{med}) from control (n=4) and Eed cKO (n=3) mice. Data are mean ± SD with statistical significance determined by unpaired t-test. *p* values are represented as *, <0.05; **, <0.01; ***, <0.001. ns, not significant. Data are representative of at least two independent experiments.

Different NKT subsets are characterized by specific transcription factors, including NKT1 (RORγt^{lo}PLZF^{lo}T-bet$^+$), NKT2 (RORγt^{lo}PLZF^{hi}), and NKT17 (RORγt^{hi}PLZF^{int}) (16). Further analysis based on transcription factor expression revealed a significant reduction in NKT1 and NKT17 cells in Eed cKO mice, whereas NKT2 cells remained comparable to those in the control mice (Figures 3C, D). Moreover, distinct iNKT subsets defined by cell-surface markers demonstrated that CD122$^+$ NKT1 and CD138$^+$ NKT17 cells were significantly decreased in Eed cKO mice, whereas the absolute number of CCR7$^-$PD-1$^+$ NKT2 cells in Eed cKO mice was comparable to that in control mice (Supplementary Figure 5). Since we detected Eed expression in NKT1, NKT2, and NKT17 cells, a different requirement of Eed in NKT subsets is not due to different expression levels of Eed (Supplementary Figure 6). Overall, our findings suggest that Eed is indispensable for the development of both NKT1 and NKT17 cells, but not NKT2 cells.

3.4 Eed regulates H3K27me3 markers and iNKT cell transcriptional program

To understand how Eed deficiency inhibits iNKT cell development at the transcriptional level, we performed RNA-sequencing (RNA-seq) analysis on CD4$^+$CD8$^+$TCRβ$^+$CD69$^+$ thymocytes from control and Eed cKO mice. We identified 83 upregulated and 125 downregulated genes (fold change >2 or <0.5, p <0.05) in Eed cKO mice compared to the control (Figure 4A). These differentially expressed genes (DEGs) were compared with previously reported iNKT cell stage-specific datasets (29), revealing that 11 of the 125 downregulated genes in Eed cKO thymocytes. *St8sia6*, *S100a6*, *Csf1*, *Eed*, *Gpr68*, *Il18rap*, *Cxcr3*, and *Arsb* were downregulated during the DP to S1 transition, whereas *Il18rap*, *Cxcr3*, *Arsb*, *Klra9*, *Klrb1c*, and *Samd3* were downregulated during the S1 to S2 transition in an Eed-dependent manner, suggesting the

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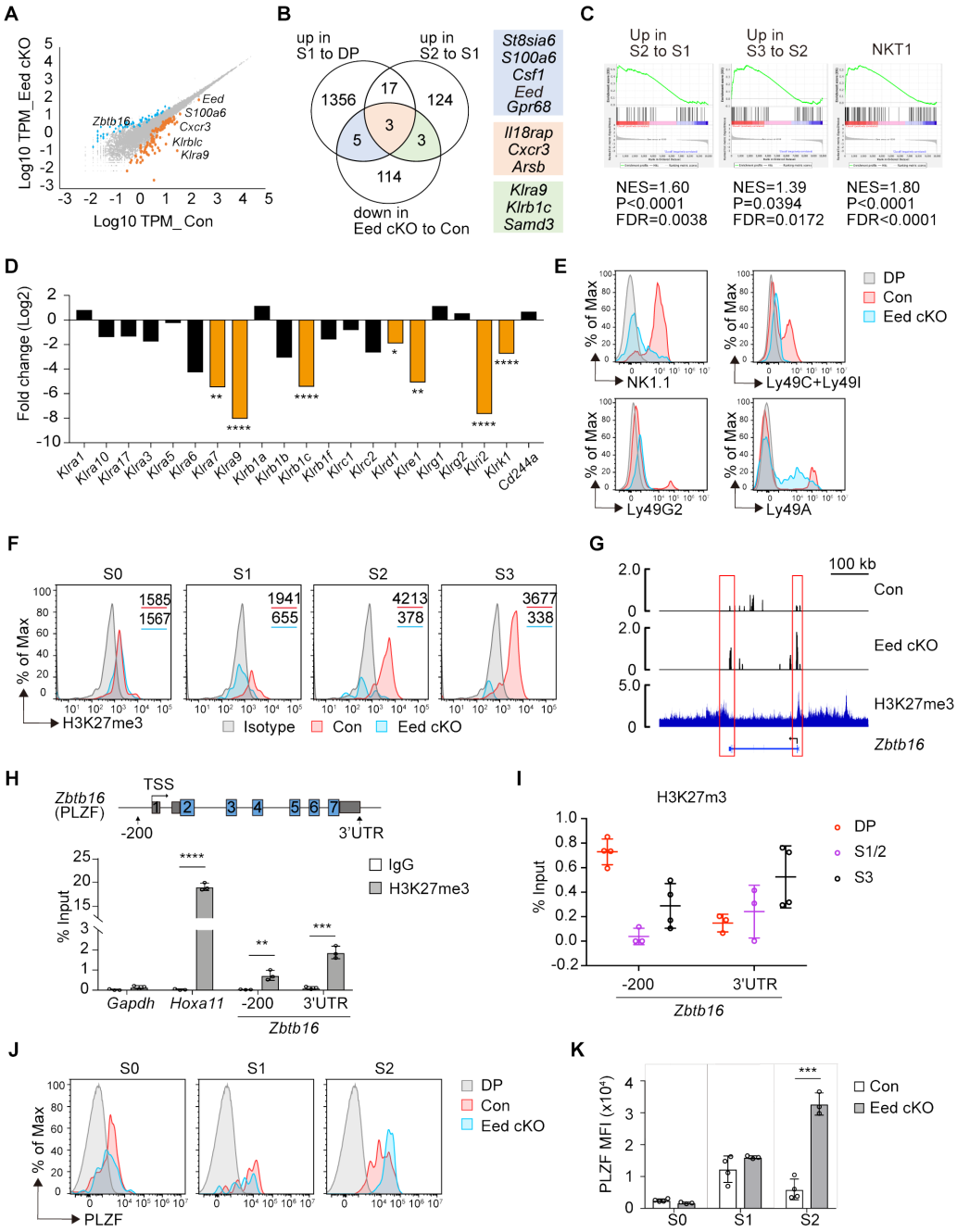

FIGURE 4
Eed is responsible for H3K27me3 marks and transcriptional programming of iNKT cells. **(A)** RNA-seq analysis on $CD4^+CD8^+TCR\beta^+CD69^+$ thymocytes from control and Eed cKO mice (n=3 each). The scatter plot shows genes that were differentially expressed with p<0.05 and fold change >2 (*orange*) or <0.5 (*blue*) in control compared to Eed cKO mice. **(B)** Venn diagram of downregulated DEGs in Eed cKO mice to control with the gene datasets from the publicly available database (29) that upregulated in S1 to DP or S2 to S1. The number of genes is indicated in each compartment. **(C)** DEGs from control to Eed cKO DP cells are used for GSEA analysis with gene sets obtained from publicly available databases (28). Upregulated in S2 to S1 iNKT cells (*left*), upregulated in S3 to S2 iNKT cells (*middle*), and NKT1 cells (*right*). **(D)** The relative expression of NKRs in DP thymocytes. Fold changes of mRNA expression in Eed cKO mice to control mice are shown. **(E)** Surface expression of NK1.1(*Klrb1c*), Ly49C/Ly49I (*Klra3/Klra7*), Ly49G2 (*Klra9*), and Ly49A(*Klra1*) on S2/3 ($CD24^-CD44^+$) iNKT cells from control (*red*) and Eed cKO (*blue*) mice, with DP cells from control mice (*gray*). **(F)** H3K27me3 level in the indicated thymic iNKT cells from control (*red*) and Eed cKO (*blue*) mice. The gray histogram indicates isotype control. The values in each panel show the MFI. **(G)** H3K27me3 ChIP-seq peaks at the *Zbtb16* locus in iNKT cells (*bottom*) are shown with RNA-seq tracks in control (*top*) and Eed cKO (*middle*) DP cells. Representative data from triplicated pairs of control and Eed cKO mice. H3K27me3 ChIP-seq dataset is from GSE84238. **(H)** ChIP-qPCR analysis of H3K27me3 (*filled bars*) and control IgG (*open bars*) enrichments at the proximal promoter and 3'UTR region of *Zbtb16* locus in sorted control thymic iNKT cells (n=3). *Gapdh* and *Hoxa11* were used as the negative and positive controls, respectively. **(I)** ChIP-qPCR analysis of H3K27me3 enrichment at the *Zbtb16* locus in DP, S1/2, and S3 cells (n=3-4). **(J, K)** Representative FACS histograms and the MFI of PLZF in the indicated iNKT cells from control (*red*, n=4) and Eed cKO (*blue*, n=3) mice. The gray histogram indicates DP cells from control mice. Data are mean ± SD with statistical significance determined by unpaired t-test **(D, H)** or multiple t-tests **(K)**. *p* values are represented as *, <0.05, **, <0.01; ***, <0.001; ****, <0.0001. Data are representative of at least two independent experiments.

importance of Eed in the developmental transition of iNKT cells (Figure 4B). Furthermore, S1 to S2, S2 to S3, and NKT1 transition datasets positively correlated with DEGs in control to Eed cKO DP cells (Figure 4C).

We then analyzed genes associated with natural cell function. Among the NK receptors (NKRs) normally expressed at the S2/S3 stage, the expression of *Klra7*, *Klra9*, *Klrb1c*, *Klrd1*, *Klre1*, *Klri2*, and *Klrk1* were significantly downregulated in Eed cKO cells (Figure 4D). We confirmed reduced protein levels of NK1.1 (*Klrb1c*), Ly49C/Ly49I (*Klra3*/*Klra7*), and Ly49G2 (*Klra9*) in S2/3 ($CD44^+CD24^-$) iNKT cells in Eed cKO mice (Figure 4E). These results indicate that Eed participates in the transcriptional regulation of various genes related to the effector function of iNKT cells.

We observed upregulation of *Zbtb16*, encoding the zinc-finger transcription factor PLZF, in Eed cKO DP cells (Figure 4A), whose expression is normally upregulated in S1/2 and then downregulated in S3 during iNKT cell differentiation (29). This suggests that the failure of iNKT cell development in Eed cKO mice may be associated with abnormal expression of PLZF due to a lack of H3K27me3-mediated inactivation of *Zbtb16*. Consequently, we first assessed the impact of Eed deletion on H3K27me3 levels in iNKT cells. Intracellular staining of H3K27me3 in thymocytes revealed a significant reduction of H3K27me3 levels in Eed cKO iNKT cells compared to that of controls at the S1, S2, and S3 stages by 3.0-, 11.1-, and 10.9-fold, respectively (Figure 4F). To further understand the relationship between H3K27me3 status and *Zbtb16* transcription, we compared the peaks from RNA-seq and H3K27me3 Chromatin immunoprecipitation sequencing (ChIP-seq) obtained from iNKT cells around the *Zbtb16* locus (30). H3K27me3 signals were detected at the transcriptional start and end regions of *Zbtb16*, corresponding to elevated RNA-seq peaks in Eed cKO cells (Figure 4G; Supplementary Table 2). Additionally, we detected H3K27me3 signals at the proximal promoter and 3′ untranslated region (3′ UTR) of *Zbtb16* in iNKT cells, along with a known Eed target *Hoxa11* (Figure 4H) (35). Notably, H3K27me3 enrichment was higher at the proximal promoter region of *Zbtb16* in the DP stage, followed by a decline in the S1/S2 stage and subsequent recovery in the S3 stage, inversely correlating with the PLZF expression pattern (Figure 4I). As expected, PLZF protein expression significantly increased in Eed cKO iNKT cells at the S2 stage (Figures 4J, K). Thus, Eed likely negatively regulates PLZF expression through H3K27me3 at the proximal promoter and 3′ UTR region of *Zbtb16* during iNKT cell development.

3.5 Increased cell death in Eed-deficient iNKT cells

The proliferation and survival of iNKT cells significantly influence their development and population size (7). We evaluated these aspects in Eed cKO iNKT cells by assessing BrdU incorporation for proliferation and Annexin V staining for survival. While BrdU incorporation rates in Eed cKO iNKT cells were similar to or higher than those in control mice, a significant increase in the percentage of Annexin V^+ iNKT cells was observed at the S2 stage in Eed cKO mice (Figures 5A-D). This indicates that impaired cell survival, particularly at the S2 developmental stage, contributes to developmental defects in Eed-deficient iNKT cells.

Tumor suppressor *Cdkn2a*, a target of PRC2 (36), encodes two proteins (p16Ink4a and p19Arf) that promote cell cycle arrest and apoptosis by regulating RB and p53 proteins, respectively (37). Additionally, *Cdkn1a*, encoding a p21 CDK inhibitor, is involved in the p53-RB signaling pathway that regulates the cell cycle (38). Both *Cdkn2a* and *Cdkn1a* are implicated in apoptosis induction in hematopoietic stem cells following Eed deficiency (36). We hypothesized that the abnormal expression of *Cdkn2a* and *Cdkn1a* contributes to the impaired survival and development of Eed-deficient iNKT cells. H3K27me3 ChIP-seq analysis using iNKT cells revealed H3K27me3 signals around the transcriptional promoter and 3′ UTR region of *Cdkn2a* and *Cdkn1a* in iNKT cells (Figure 5E). Moreover, proximal promoter regions of *Cdkn2a* and *Cdkn1a* were significantly enriched with H3K27me3 in control S1/2 cells in an Eed-dependent manner (Figure 5F). Furthermore, mRNA expression of *p16Ink4a*, *p19Arf*, and *p21* was significantly increased in S2 cells from Eed cKO mice compared to that in the control (Figure 5G). These results strongly support that *Cdkn2a* and *Cdkn1a* are direct targets of Eed for H3K27me3, regulating cell death and promoting iNKT cell development.

3.6 Increased susceptibility to liver injury and inflammation in Eed cKO mice

As Eed deficiency leads to a reduction in thymic NKT1 cells, we examined NKT1 cells in peripheral tissues and observed a substantial proportion residing in the liver. However, the numbers of NKT1 cells in the spleen, liver, and lung of Eed cKO mice were significantly diminished (Figure 6A). CD1d- and Jα18-deficient mice lacking iNKT cells are highly susceptible to acetaminophen (APAP)-induced liver injury (AILI) (39, 40), a severe consequence of sudden hepatocyte injury, often induced by APAP overdose in developed countries (41), but the critical iNKT cell subset involved and the role of epigenetic modification remain unknown. To investigate the physiological significance of Eed-dependent iNKT cell development, we used an AILI mouse model. Compared with control mice, Eed cKO mice exhibited a lower survival rate after APAP challenge, with mortality being dose-dependent (Supplementary Figure 7A). Histological analysis of the livers collected 48h or 72h post-APAP treatment revealed significantly enlarged necrotic areas accompanied by severe inflammation in the liver of Eed cKO mice compared to controls (Figures 6B, C). In APAP-treated mice, iNKT cells were significantly reduced in Eed cKO mice, whereas the numbers of $CD4^+$ and $CD8^+$ T cells were comparable to those in control mice (Supplementary Figures 7B, C). To determine whether the defect induced in APAP-treated Eed cKO mice resulted from a lack of iNKT cells, we transferred iNKT cells from the wild-type liver (mostly NKT1 cells) into Eed cKO mice. The results showed that iNKT cell transfer significantly increased survival and reduced necrosis in Eed cKO mice 24 h after APAP treatment (Figures 6D–F). Collectively, Eed-dependent liver resident NKT

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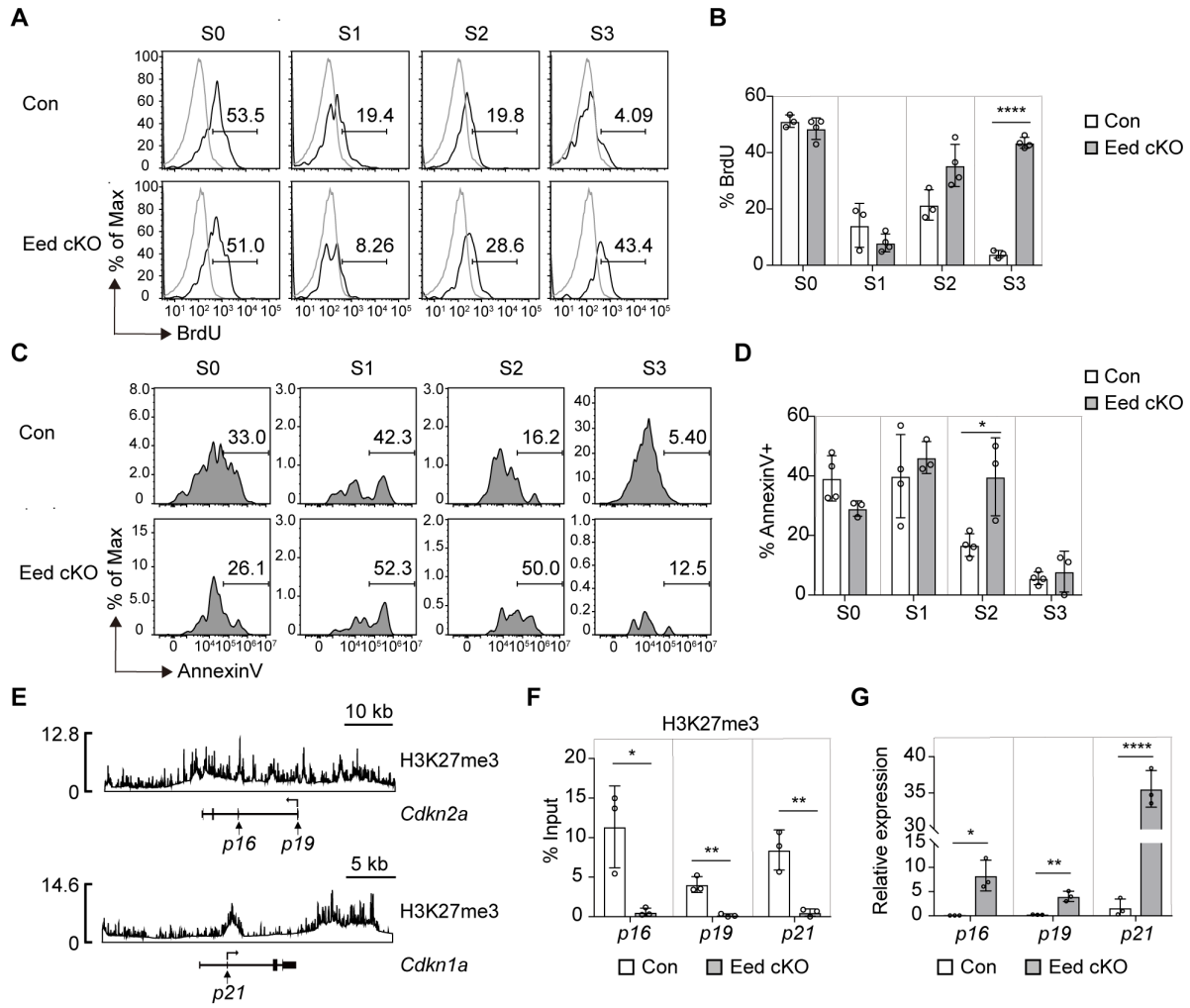

FIGURE 5
Increased cell death in Eed-deficient iNKT cells is associated with abnormal expression of *Cdkn2a* and *Cdkn1a*. **(A, B)** Representative FACS histograms and percentage of BrdU$^+$ iNKT cells in the thymus from BrdU-pulsed control (n=3) and Eed cKO (n=4) mice. The gray line indicates CD4$^+$CD8$^-$SP cells. **(C, D)** Representative FACS histograms and percentage of Annexin V$^+$-dead cells in the indicated iNKT cells from control (n=4) and Eed cKO (n=3) mice. **(E)** ChIP-seq analysis of H3K27me3 in iNKT cells at the *Cdkn2a* locus (*upper*) and *Cdkn1a* locus (*lower*). **(F)** ChIP-qPCR analysis of H3K27me3 enrichment at the proximal promoter region of *p16*, *p19*, and *p21* in sorted thymic CD24$^-$NK1.1$^-$ S1/2 iNKT cells from control and Eed cKO mice (n=3 each). **(G)** Real-time PCR analysis of the expression of *p16*, *p19*, and *p21* in sorted thymic S2 iNKT cells from control and Eed cKO mice (n=3 each). These expressions were normalized to *Ywhaz*. Data are mean ± SD with statistical significance determined by unpaired t-test **(F, G)** or multiple t-tests **(B, D)**. *p* values are represented as *, <0.05; **, <0.01; ****, <0.0001. Data are representative of at least two independent experiments.

cells, particularly NKT1 cells, play a critical role in protecting hepatocytes from APAP-mediated necrosis and liver inflammation.

4 Discussion

Eed plays a unique role in initiating Ezh1/2 activation and amplifying PRC2 activity through its interaction with H3K27me3, essential for H3K27me3 deposition (20, 21). Here, we demonstrated that H3K27me3 levels significantly increase with iNKT cell differentiation, and Eed is crucial for maintaining these marks and facilitating cell differentiation. While both Ezh2 and Eed are integral to PRC2, the diametrically opposite iNKT cell phenotypes between Ezh2 cKO and Eed cKO mice indicate distinct roles of these proteins in iNKT cell development (22, 23). In contrast to the significant reduction of H3K27me3 due to Eed deficiency, H3K27me3 levels were not impacted by Ezh2 deficiency in iNKT cells (23). Therefore, H3K27me3 in iNKT cells may be primarily catalyzed by the PRC2 complex consisting of Ezh1 and Eed. H3K27me3 level is higher in S2 and S3 iNKT cells, while Eed expression is higher in S1 and S2 iNKT cells. This discrepancy could be explained by the PRC2-mediated amplification of H3K27me3 to neighboring sites of the initial trimethylations. After the initial H3K27me3 deposition at S1 stage, PRC2 is recruited by binding of Eed to H3K27me3, leading to further H3K27me3 deposition in a positive feedback loop, which could cumulatively increase H3K27me3 levels in S2 and S3 iNKT cells. However, sustained H3K27me3 levels caused by the loss of the H3K27me3

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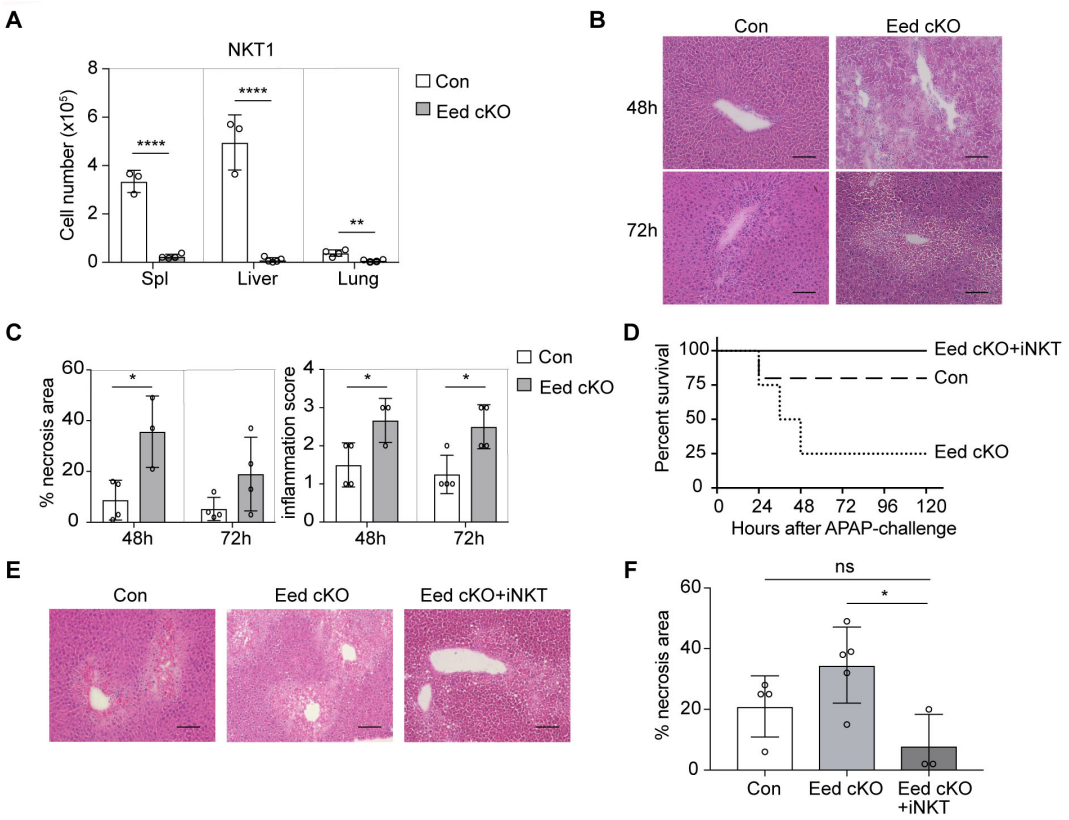

FIGURE 6
Increased susceptibility to liver injury in Eed-deficient mice. **(A)** Absolute cell number of NKT1 in the spleen, liver, and lung from control (n=3-4) and Eed cKO (n=4) mice. **(B)** Representative images of H&E staining of the liver from control and Eed cKO mice at 48 h and 72 h after APAP treatment. Image scale, 100 μm. **(C)** Percentage of necrosis area and inflammation score in the livers of B (n=3-4 each). **(D)** Survival curve after APAP treatment in control (*dotted line*), Eed cKO (*dashed line*) mice, and Eed cKO mice transferred with iNKT cells from wild-type liver (Eed cKO+iNKT, *solid line*) (n=3-5 each). **(E)** Representative images of H&E staining of the liver at 24 h after APAP treatment of the indicated mice. Image scale, 100 μm. **(F)** Percentage of necrosis area in the livers of **(E)** Data are mean ± SD with statistical significance determined by unpaired t-test **(A, C)**, or one-way ANOVA **(F)**. *p* values are represented as *, <0.05; **, <0.01; ****, <0.0001. n.s., not significant. Data are representative of at least two independent experiments.

demethylases Utx and Jmjd3 also affect iNKT cell development. UTX-deficient iNKT cells exhibit impaired expression of iNKT-cell signature genes due to decreased activation-associated H3K4me3 markers and increased repressive H3K27me3 markers within the promoters occupied by UTX (30). Utx- or Jmjd3-deficient iNKT cells fail to activate PLZF and its target genes, resulting in reduced iNKT cells (22, 30). Therefore, the Eed-PRC2 histone methyltransferase and Utx/Jmjd3 histone demethylases may differentially regulate H3K27me3 target genes, leading to similar knockout phenotypes.

PLZF, exclusively expressed in iNKT cells, is induced immediately after positive selection of iNKT cell precursors. During iNKT cell development, PLZF expression reaches a plateau at the S1 stage and then declines markedly at the S3 stage (14). In PLZF-deficient mice, iNKT cells showed impaired intra-thymic expansion accompanied by a significant reduction in the thymus, spleen, and liver, lacked NK markers and failed to secrete effector cytokines after activation (14, 15). Therefore, properly organized PLZF expression directs innate-like effector differentiation from T-cell progenitors. Our data indicated that Eed-mediated upregulation of H3K27me3 at S2/3 silences the *Zbtb16* locus, promoting NKT1 and NKT17 differentiation. This model is supported by observations in PLZF-active mutant mice, where constitutively active PLZF inhibits the progression of NKT precursors to subsequent differentiation into NKT1 and NKT17, but not NKT2 cells (42). Moreover, the absence of the PRC2 components, Ezh2 or Jarid2, upregulates the PLZF protein by modulating the stability of PLZF without altering H3K27me3, thereby expanding iNKT cells with the IL-4 secreting NKT2 phenotype, indicating the significance of PLZF downregulation at the S2 stage (23, 43). Eed deletion resulted in an elevation of the PLZF protein at S2, as observed in Ezh2- or Jarid2-deficient mice; however, there was a significant reduction in thymic and peripheral iNKT cells without NKT2 expansion, suggesting additional roles of Eed.

iNKT cells migrate to the peripheral tissues after development; however, the physiological significance of Eed-dependent iNKT cells remains unclear. Here, we demonstrated that T cell-specific Eed-deficient mice are highly susceptible to AILI and associated inflammation due to a lack of iNKT cells. In AILI, metabolized APAP forms cytotoxic mitochondrial adducts that cause hepatocyte death and the release of danger-associated molecules, thereby promoting the release of pro-inflammatory cytokines (44).

Although how iNKT cells protect hepatocytes from death remains unclear, liver-resident iNKT cells may provide anti-apoptotic signals to hepatocytes through effector molecules like IFN-γ and IL-17 or CD1d, which is constitutively expressed on hepatocytes (45, 46).

Eed-deficient iNKT cells show increased induction of senescence-associated genes such as *p16Ink4a*, *p19Arf*, and *p21*, promoting apoptosis, cell cycle arrest, senescence, and inflammation (47). This enhanced apoptosis could be attributed to the induction of p19Arf expression via p53-dependent or -independent pathways (48). However, $CD8^+$ T cells can eliminate p16Ink4a-positive senescent cells, which ameliorates various age-related mouse phenotypes (49). Therefore, the enhanced apoptosis observed in Eed-deficient iNKT cells may be the result of cytotoxic $CD8^+$ T cells targeting senescent iNKT cells.

NKT1 and NKT17 cells differentiate from NKTp cells at different rates, with NKT17 cells emerging within 3–5 days and NKT1 cells requiring 14–20 days, contrasting with NKT2 cells remaining $PLZF^{hi}$ similar to NKTp (50). Thus, NKT1 development involves more complex processes, including the IL-15 pathway and T-bet-mediated regulation. Our findings shed light on the role of Eed-mediated epigenetic modifications in determining iNKT cell expansion and differentiation through the orchestration of iNKT cell-specific transcriptional programs.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: PRJNA1054693 (SRA).

Ethics statement

The animal studies were approved by the Ethics Review Committee for Animal Experimentation of Hiroshima University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YG: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing, Validation, Visualization. SO: Data curation, Formal analysis, Writing – review & editing. YK: Writing – original draft, Writing – review & editing, Methodology. WK: Investigation, Writing – review & editing. YO: Investigation, Writing – review & editing. HH: Resources, Writing – review & editing. MK: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. TY: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the JSPS KAKENHI under Grant Numbers JP17K17916 to YG, JP18H02669, JP19K22538, and JP21H02751 to TY, by AMED under Grant Numbers 17hm0102012h0005, 18gm0710009h0205, 19gm0710009h0206 to MK.

Acknowledgments

We thank Dr. Akihiko Yoshimura for providing CD4-Cre transgenic mice, T. Kawaguchi and Y. Hayashi for technical assistance, N. Kikkawa and M. Tawa for administrative assistance. All lab members for useful discussion and comments. This work was supported in part by the Natural Science Center for Basic Research and Development (N-BARD-00134) and the Radiation Research Center for Frontier Science at Hiroshima University.

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.2024.1467774/full#supplementary-material

References

1. Anderson G, Moore NC, Owen JJ, Jenkinson EJ. Cellular interactions in thymocyte development. *Annu Rev Immunol.* (1996) 14:73–99. doi: 10.1146/annurev.immunol.14.1.73

2. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annu Rev Immunol.* (2003) 21:139–76. doi: 10.1146/annurev.immunol.21.120601.141107

3. Werlen G, Hausmann B, Naeher D, Palmer E. Signaling life and death in the thymus: timing is everything. *Science.* (2003) 299:1859–63. doi: 10.1126/science.1067833

4. Stritesky GL, Jameson SC, Hogquist KA. Selection of self-reactive T cells in the thymus. *Annu Rev Immunol.* (2012) 30:95–114. doi: 10.1146/annurev-immunol-020711-075035

5. Daniels MA, Teixeiro E, Gill J, Hausmann B, Roubaty D, Holmberg K, et al. Thymic selection threshold defined by compartmentalization of ras/mapk signalling. *Nature.* (2006) 444:724–9. doi: 10.1038/nature05269

6. Moran AE, Holzapfel KL, Xing Y, Cunningham NR, Maltzman JS, Punt J, et al. T cell receptor signal strength in treg and inkt cell development demonstrated by a novel fluorescent reporter mouse. *J Exp Med.* (2011) 208:1279–89. doi: 10.1084/jem.20110308

7. Bendelac A, Savage PB, Teyton L. The biology of nkt cells. *Annu Rev Immunol.* (2007) 25:297–336. doi: 10.1146/annurev.immunol.25.022106.141711

8. Kronenberg M. Toward an understanding of nkt cell biology: progress and paradoxes. *Annu Rev Immunol.* (2005) 23:877–900. doi: 10.1146/annurev.immunol.23.021704.115742

9. Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of valpha14 nkt cells in innate and acquired immune response. *Annu Rev Immunol.* (2003) 21:483–513. doi: 10.1146/annurev.immunol.21.120601.141057

10. Dashtsoodol N, Bortoluzzi S, Schmidt-Supprian M. T cell receptor expression timing and signal strength in the functional differentiation of invariant natural killer T cells. *Front Immunol.* (2019) 10:841. doi: 10.3389/fimmu.2019.00841

11. Griewank K, Borowski C, Rietdijk S, Wang N, Julien A, Wei DG, et al. Homotypic interactions mediated by slamf1 and slamf6 receptors control nkt cell lineage development. *Immunity.* (2007) 27:751–62. doi: 10.1016/j.immuni.2007.08.020

12. Hogquist K, Georgiev H. Recent advances in inkt cell development. *F1000Res.* (2020) 9(F1000 Faculty Rev):127. doi: 10.12688/f1000research.21378.1

13. Benlagha K, Wei DG, Veiga J, Teyton L, Bendelac A. Characterization of the early stages of thymic nkt cell development. *J Exp Med.* (2005) 202:485–92. doi: 10.1084/jem.20050456

14. Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, et al. The transcription factor plzf directs the effector program of the nkt cell lineage. *Immunity.* (2008) 29:391–403. doi: 10.1016/j.immuni.2008.07.011

15. Kovalovsky D, Uche OU, Eladad S, Hobbs RM, Yi W, Alonzo E, et al. The btb-zinc finger transcriptional regulator plzf controls the development of invariant natural killer T cell effector functions. *Nat Immunol.* (2008) 9:1055–64. doi: 10.1038/ni.1641

16. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of il-4 modulates immunity in mouse strains and is determined by lineage diversity of inkt cells. *Nat Immunol.* (2013) 14:1146–54. doi: 10.1038/ni.2731

17. Vigano MA, Ivanek R, Balwierz P, Berninger P, van Nimwegen E, Karjalainen K, et al. An epigenetic profile of early T-cell development from multipotent progenitors to committed T-cell descendants. *Eur J Immunol.* (2014) 44:1181–93. doi: 10.1002/eji.201344022

18. Montgomery ND, Yee D, Montgomery SA, Magnuson T. Molecular and functional mapping of eed motifs required for prc2-dependent histone methylation. *J Mol Biol.* (2007) 374:1145–57. doi: 10.1016/j.jmb.2007.10.040

19. Jiao L, Liu X. Structural basis of histone H3k27 trimethylation by an active polycomb repressive complex 2. *Science.* (2015) 350:aac4383. doi: 10.1126/science.aac4383

20. Lee CH, Holder M, Grau D, Saldana-Meyer R, Yu JR, Ganai RA, et al. Distinct stimulatory mechanisms regulate the catalytic activity of polycomb repressive complex 2. *Mol Cell.* (2018) 70:435–48 e5. doi: 10.1016/j.molcel.2018.03.019

21. Margueron R, Justin N, Ohno K, Sharpe ML, Son J, Drury WJ 3rd, et al. Role of the polycomb protein eed in the propagation of repressive histone marks. *Nature.* (2009) 461:762–7. doi: 10.1038/nature08398

22. Dobenecker MW, Kim JK, Marcello J, Fang TC, Prinjha R, Bosselut R, et al. Coupling of T cell receptor specificity to natural killer T cell development by bivalent histone H3 methylation. *J Exp Med.* (2015) 212:297–306. doi: 10.1084/jem.20141499

23. Vasanthakumar A, Xu D, Lun AT, Kueh AJ, van Gisbergen KP, Iannarella N, et al. A non-canonical function of ezh2 preserves immune homeostasis. *EMBO Rep.* (2017) 18:619–31. doi: 10.15252/embr.201643237

24. Ikeda K, Ueda T, Yamasaki N, Nakata Y, Sera Y, Nagamachi A, et al. Maintenance of the functional integrity of mouse hematopoiesis by eed and promotion of leukemogenesis by eed haploinsufficiency. *Sci Rep.* (2016) 6:29454. doi: 10.1038/srep29454

25. Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, et al. A critical role for dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity.* (2001) 15:763–74. doi: 10.1016/s1074-7613(01)00227-8

26. Miyazaki M, Miyazaki K, Itoi M, Katoh Y, Guo Y, Kanno R, et al. Thymocyte proliferation induced by pre-T cell receptor signaling is maintained through polycomb gene product bmi-1-mediated cdkn2a repression. *Immunity.* (2008) 28:231–45. doi: 10.1016/j.immuni.2007.12.013

27. Vetelainen RL, Bennink RJ, de Bruin K, van Vliet A, van Gulik TM. Hepatobiliary function assessed by 99mtc-mebrofenin cholescintigraphy in the evaluation of severity of steatosis in a rat model. *Eur J Nucl Med Mol Imaging.* (2006) 33:1107–14. doi: 10.1007/s00259-006-0125-3

28. Engel I, Seumois G, Chavez L, Samaniego-Castruita D, White B, Chawla A, et al. Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat Immunol.* (2016) 17:728–39. doi: 10.1038/ni.3437

29. Cohen NR, Brennan PJ, Shay T, Watts GF, Brigl M, Kang J, et al. Shared and distinct transcriptional programs underlie the hybrid nature of inkt cells. *Nat Immunol.* (2013) 14:90–9. doi: 10.1038/ni.2490

30. Beyaz S, Kim JH, Pinello L, Xifaras ME, Hu Y, Huang J, et al. The histone demethylase utx regulates the lineage-specific epigenetic program of invariant natural killer T cells. *Nat Immunol.* (2017) 18:184–95. doi: 10.1038/ni.3644

31. Chen P, Wang S, Janardhan KS, Zemans RL, Deng W, Karmaus P, et al. Efficient cd4cre-mediated conditional kras expression in alveolar macrophages and alveolar epithelial cells causes fatal hyperproliferative pneumonitis. *J Immunol.* (2019) 203:1208–17. doi: 10.4049/jimmunol.1900566

32. Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DIA, et al. (Nkt) cell developmental pathway iinvolving a thymus-dependent nk1.1(-)Cd4(+) cd1d-dependent precursor stage. *J Exp Med.* (2002) 195:835–44. doi: 10.1084/jem.20011544

33. Nunez-Cruz S, Yeo WC, Rothman J, Ojha P, Bassiri H, Juntilla M, et al. Differential requirement for the sap-fyn interaction during nk T cell development and function. *J Immunol.* (2008) 181:2311–20. doi: 10.4049/jimmunol.181.4.2311

34. Anderson G, Jenkinson EJ. Lymphostromal interactions in thymic development and function. *Nat Rev Immunol.* (2001) 1:31–40. doi: 10.1038/35095500

35. Sun B, Chang E, Gerhartl A, Szele FG. Polycomb protein eed is required for neurogenesis and cortical injury activation in the subventricular zone. *Cereb Cortex.* (2018) 28:1369–82. doi: 10.1093/cercor/bhx289

36. Xie H, Xu J, Hsu JH, Nguyen M, Fujiwara Y, Peng C, et al. Polycomb repressive complex 2 regulates normal hematopoietic stem cell function in a developmental-stage-specific manner. *Cell Stem Cell.* (2014) 14:68–80. doi: 10.1016/j.stem.2013.10.001

37. Sherr CJ. The ink4a/arf network in tumour suppression. *Nat Rev Mol Cell Biol.* (2001) 2:731–7. doi: 10.1038/35096061

38. Engeland K. Cell cycle regulation: P53-P21-rb signaling. *Cell Death Differ.* (2022) 29:946–60. doi: 10.1038/s41418-022-00988-z

39. Martin-Murphy BV, Kominsky DJ, Orlicky DJ, Donohue TM Jr., Ju C. Increased susceptibility of natural killer T-cell-deficient mice to acetaminophen-induced liver injury. *Hepatology.* (2013) 57:1575–84. doi: 10.1002/hep.26134

40. Bandyopadhyay K, Marrero I, Kumar V. Nkt cell subsets as key participants in liver physiology and pathology. *Cell Mol Immunol.* (2016) 13:337–46. doi: 10.1038/cmi.2015.115

41. Stravitz RT, Lee WM. Acute liver failure. *Lancet.* (2019) 394:869–81. doi: 10.1016/s0140-6736(19)31894-x

42. Klibi J, Joseph C, Delord M, Teissandier A, Lucas B, Chomienne C, et al. Plzf acetylation levels regulate nkt cell differentiation. *J Immunol.* (2021) 207:809–23. doi: 10.4049/jimmunol.2001444

43. Pereira RM, Martinez GJ, Engel I, Cruz-Guilloty F, Barboza BA, Tsagaratou A, et al. Jarid2 is induced by tcr signalling and controls inkt cell maturation. *Nat Commun.* (2014) 5:4540. doi: 10.1038/ncomms5540

44. Krenkel O, Mossanen JC, Tacke F. Immune mechanisms in acetaminophen-induced acute liver failure. *Hepatobiliary Surg Nutr.* (2014) 3:331–43. doi: 10.3978/j.issn.2304-3881.2014.11.01

45. Lei Z, Yu J, Wu Y, Shen J, Lin S, Xue W, et al. Cd1d protects against hepatocyte apoptosis in non-alcoholic steatohepatitis. *J Hepatol.* (2024) 80:194–208. doi: 10.1016/j.jhep.2023.10.025

46. Sekiya M, Adachi M, Takayama S, Reed JC, Imai K. Ifn-gamma upregulates anti-apoptotic gene expression and inhibits apoptosis in il-3-dependent hematopoietic cells. *Biochem Biophys Res Commun.* (1997) 239:401–6. doi: 10.1006/bbrc.1997.7478

47. Huang W, Hickson LJ, Eirin A, Kirkland JL, Lerman LO. Cellular senescence: the good, the bad and the unknown. *Nat Rev Nephrol.* (2022) 18:611–27. doi: 10.1038/s41581-022-00601-z

48. Kung CP, Weber JD. It's getting complicated-a fresh look at P53-mdm2-arf triangle in tumorigenesis and cancer therapy. *Front Cell Dev Biol.* (2022) 10:818744. doi: 10.3389/fcell.2022.818744

49. Wang TW, Johmura Y, Suzuki N, Omori S, Migita T, Yamaguchi K, et al. Blocking pd-L1-pd-1 improves senescence surveillance and ageing phenotypes. *Nature.* (2022) 611:358–64. doi: 10.1038/s41586-022-05388-4

50. Bortoluzzi S, Dashtsoodol N, Engleitner T, Drees C, Helmrath S, Mir J, et al. Brief homogeneous tcr signals instruct common inkt progenitors whose effector diversification is characterized by subsequent cytokine signaling. *Immunity.* (2021) 54:2497–513 e9. doi: 10.1016/j.immuni.2021.09.003

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