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Rôle des lymphocytes T non conventionnels dans la défense de l'hôte

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Liste de publications et/ou brevets et participation aux conférences

1) Liste des publications et/ou brevet [1] réalisées dans le cadre du projet de thèse :

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    2. 4th Germinal center workshop, Marseille, (Poster presentation)
    3. 3rd Joint Innate T cell French Symposium, Montpellier, November 9-10 of 2023 (Oral presentation)
    4. 6th edition of Immunity and Cancer Immunotherapy by Institute Curie, June 20-23 of 2023, Marseille

[1] Cette liste comprend les articles publiés, les articles soumis à publication et les articles en préparation ainsi que les livres, chapitres de livre et/ou toutes formes de valorisation des résultats des travaux propres à la discipline du projet de thèse. La référence aux publications doit suivre les règles standards de bibliographie et doit être conforme à la charte des publications d'AMU.

[2] Le terme « conférence » est générique. Il désigne à la fois « conférence », « congrès », « workshop », « colloques », « rencontres nationales et/ou internationales » … etc.

Indiquer si vous avez fait une présentation orale ou sous forme de poster.

5. CD1-MR1 scientific meeting, 26-29 February 2024, Hobart, Australia (Poster presentation)
6. 4th Joint Innate T cell French Symposium, Paris, November 7-8 of 2024

RESUME EN FRANCAISE

Les lymphocytes T non conventionnels (UTC) sont essentiels au maintien de l'homéostasie tissulaire et à la régulation de la réponse immunitaire lors des infections. La migration lymphatique des tissus vers les ganglions lymphatiques drainants (LN) crée un centre de régulation immunitaire qui atténue la pathogénèse systémique. Par conséquent, l'étude de la réponse tissulaire en parallèle avec la réponse LN est essentielle pour comprendre le rôle des UTC dans le système immunitaire. Dans la première partie, j'explore les fonctions des cellules NKT lors des infections où les cellules NKT se sont révélées importantes pour le changement de classe des cellules B du centre germinatif produisant IgG1 en fournissant de l'IL-4 dans les LN drainant les poumons (mLN) lors d'une infection respiratoire. Cependant, les cellules NKT ne sont pas essentielles aux mêmes processus lors de l'infection sous-cutanée, révélant une régulation spécifique du site de la réponse immunitaire par ces cellules. La deuxième partie se concentre sur la dynamique des cellules MAIT dans le poumon et les mLN. Alors que de précédentes études ont décrit les fonctions antimicrobiennes exercées par les cellules MAIT pulmonaires, j'illustre comment ces cellules se positionnent dans le mLN en tant que producteurs majeurs d'IL-17 pour limiter l'infection, prévenant ainsi le sepsis. De plus, les cellules MAIT soutiennent les réponses des cellules B plus tard dans l'infection, facilitant la différenciation des plasmoblastes et la sécrétion d'IgA. Ces résultats montrent que les cellules MAIT contribuent aux réponses immunitaires innées et adaptatives lors d'une infection pulmonaire d'une manière dépendante de l'IL-17.

Mots-clés : Cellules T non conventionnelles, Immunité innée, Immunité adaptative, infection respiratoire

ABSTRACT IN ENGLISH

Unconventional T (UTC) cells are crucial for maintaining tissue homeostasis and regulating immune responses during infections. Immune cells migrate from tissues to draining lymph nodes (LN), creating a hub for immune regulation that mitigates systemic pathogenesis. Hence, studying the tissue response alongside the LN response is essential for understanding UTCs' roles in the immune system. In the first part of this thesis, I explore the functions of NKT cells during infections where NKT cells were found to be important for IgG1-producing germinal center B cell class-switching by providing IL-4 in lung-draining LN during respiratory infection. However, NKT cells were not critical for similar processes during subcutaneous infection, revealing a site-specific immune response regulation by NKT cells. The second part focuses on the dynamics of MAIT cells in the lung and lung-draining LN. While previous studies have described antimicrobial functions exerted by pulmonary MAIT cells, I illustrate how MAIT cells position in the LN as major IL-17 producers to limit infection in the LN, thereby preventing sepsis. Moreover, MAIT cells support the B-cell responses later in infection, aiding plasmablast differentiation and IgA secretion. These findings show that MAIT cells contribute to innate and adaptive immune responses generated during pulmonary infection in an IL-17-dependent manner.

Keywords: Unconventional T cells, Innate Immunity, Adaptive immunity, respiratory infection

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LIST OF ABBREVIATIONS

Abbreviations and meaning

- **5-A-RU -** 5-Amino-6-D-Ribitylaminouracil
- **5-OE-RU -** 5-(2-Oxoethylideneamino)-6-D-Ribitylaminouracil
- **5-OP-RU -** 5-(2-Oxopropylideneamino)-6-D-Ribitylaminouracil
- **α-GalCer -** Alpha-Galactosylceramide
- **ADCC -** Antibody-Dependent Cellular Cytotoxicity
- **APC -** Antigen Presenting Cell
- **APRIL -** A Proliferation-Inducing Ligand
- **ASC -** Antibody-Secreting Cell
- **BALF -** Bronchoalveolar Lavage Fluid
- **BATF -** Basic Leucine Zipper ATF-Like Transcription Factor
- **BAFF -** B-Cell Activating Factor
- **BCL -** B-Cell Lymphoma
- **CAR T -** Chimeric Antigen Receptor T Cell
- **CCL -** Chemokine (C-C Motif) Ligand
- **CCR -** Chemokine (C-C Motif) Receptor
- **CD -** Cluster of Differentiation
- **CDR -** Complementarity-Determining Regions
- **CFU -** Colony-Forming Unit
- **CTL -** Cytotoxic T Lymphocyte
- **CTLA -** Cytotoxic T-Lymphocyte Antigen
- **CXCL -** C-X-C Motif Chemokine Ligand

- **CXCR -** C-X-C Motif Chemokine Receptor
- **DC -** Dendritic Cell
- **DN -** Double Negative
- **DPI -** Days Post-Infection
- **DP -** Double Positive
- **ELISA -** Enzyme-Linked Immunosorbent Assay
- **FOXP3 -** Forkhead Box P3
- **GC -** Germinal Center
- **GFP -** Green Fluorescent Protein
- **GM-CSF -** Granulocyte-Macrophage Colony-Stimulating Factor
- **GTP -** Guanosine Triphosphate
- **HA -** Hemagglutinin
- **HIF -** Hypoxia-Inducible Factor
- **IBD -** Inflammatory Bowel Disease
- **ICOS -** Inducible T-Cell Costimulator
- **ICOSL -** Inducible T-Cell Costimulatory Ligand
- **IFN -** Interferon
- **IL -** Interleukin
- **ILC -** Innate Lymphoid Cells
- **IRF -** Interferon Regulatory Factor
- **KP -** *Klebsiella pneumoniae*
- **LDTF -** Lineage-Defining Transcription Factor
- **LN -** Lymph Node
- **LPS -** Lipopolysaccharide

- **MAIT -** Mucosal-Associated Invariant T Cell
- **MAPK -** Mitogen-Activated Protein Kinase
- **MDCK -** Madin-Darby Canine Kidney (Cell Line)
- **MDSC -** Myeloid-Derived Suppressor Cells
- **MHC -** Major Histocompatibility Complex
- **miR -** MicroRNA
- **mLN –** Mediastinal Lymph Node
- **MR1 -** MHC Class I–Related Protein 1
- **NK -** Natural Killer
- **NP -** Nucleoprotein
- **Nr4a1 -** Nuclear Receptor Subfamily 4 Group A Member 1
- **NKT -** Natural Killer T Cell
- **PBS -** Phosphate-Buffered Saline
- **PD -** Programmed Death
- **PDL -** Programmed Death Ligand
- **PFU -** Plaque-Forming Unit
- **PLZF -** Promyelocytic Leukemia Zinc Finger Protein
- **PR8 -** Influenza A Virus Strain A/Puerto Rico/8/1934 (H1N1)
- **RA –** Rheumatoid Arthritis
- **RAG -** Recombination Activating Gene
- **RORγt -** Retinoic Acid-Related Orphan Receptor Gamma t
- **RUNX1 -** Runt-Related Transcription Factor 1
- **scRNA-seq -** Single-Cell RNA Sequencing
- **SCENITH -** Single-Cell Energetic Metabolism by Profiling Translation Inhibition

- **SLAM -** Signaling Lymphocytic Activation Molecule
- **SNP -** Single Nucleotide Polymorphism
- **STAT -** Signal Transducer and Activator of Transcription
- **TAP -** Transporter Associated with Antigen Processing
- **Tbet -** T-box Transcription Factor Expressed in T Cells
- **T_{CM} -** Central Memory T Cell
- **TCR -** T Cell Receptor
- **T_{EM} -** Effector Memory T Cell
- **T_{FH} -** T Follicular Helper (Cell)
- **Th -** T helper (Cells)
- **TGFβ -** Transforming Growth Factor Beta
- **T_{RM} -** Tissue-Resident Memory T Cell
- **T_{SCM} -** T Stem Cell Memory
- **Treg -** Regulatory T Cell
- **Tyr -** Tyrosine
- **UTC -** Unconventional T Cells
- **UMAP -** Uniform Manifold Approximation and Projection
- **VV -** Vaccinia Virus
- **WT -** Wild Type
- **XCL -** Chemokine (C Motif) Ligand
- **Zbtb -** Zinc Finger and BTB Domain-Containing Protein

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INTRODUCTION

1. T cells

1.1 T cells: T cell receptor (TCR) structure

T lymphocytes were first identified in the 1960s by immunologist Jacques Millers as essential components in regulating the immune system while investigating the importance of the thymus as an organ (Miller, 1959). As we know today, T lymphocytes are major orchestrators of immune responses whose diverse functions have been unveiled throughout the years. In this thesis, I describe T cells' development and functional properties, attributing to their paramount importance in the field of immunology.

A typical T cell bears a specific T-cell receptor (TCR) for antigens on its plasma membrane surface. TCRs are expressed by all T cells except their earliest precursors and therefore, all mature T cells bear TCRs. TCR is a heterodimeric molecule with a single antigen binding site. The vast majority of T cells in the body are alpha-beta (αβ) T cells, termed so because their receptor site is composed of two parts: an alpha polypeptide chain (49 kDa) and a beta polypeptide chain (43 kDa). Disulfide bonds stabilize each chain, the receptor is anchored in the plasma membrane, and parts of the α and β chains extend into the cytoplasm **(Figure 1)**. TCRs possess V (Variable) and C (Constant) regions, and the TCR genes undergo somatic DNA rearrangements by recombination of V, D, and J gene segments to produce a repertoire of receptors with considerable sequence diversity in the V region. This gene shuffling is discussed in the ‘T cell development’ section. A TCR has a cytoplasmic tail of minimal length and it is not involved in signaling directly (signaling is carried out by CD3). The variable (V) regions of the α and β chains each contain three highly variable loops called complementarity-determining regions (CDRs), which are crucial for antigen recognition. The CDR1 and CDR2 are located on both the α and β chains, these regions primarily interact with specific protein molecules displayed on the surfaces of antigen-presenting cells (APCs) called the major histocompatibility complex (MHC). The CDR3 region is found at the junction between the V, D, and J gene segments of the TCR is the most variable and plays a direct role in recognizing the specific antigenic peptide presentation. It is primarily responsible for determining the specificity of the TCR for its cognate antigen.

The diversity in CDR3 sequences is generated during the process of V(D)J recombination, contributing to the vast repertoire of TCRs capable of recognizing a wide array of antigens.

There are approximately 4 x 10^{11} T cells in a healthy adult human (Jenkins et al., 2010). The responding TCRs are highly diverse, with theoretical diversity up to 1×10^{20} different αβ TCRs (Lieber, 1991), although practical diversity in humans is around 5×10^{6} αβ TCRs (Arstila et al., 1999). This diversity means any specific T cell precursor is rare (about one to ten per 1×10^{6} T cells), limiting immediate protection. However, upon encountering an antigen, T cells undergo clonal expansion, producing a large effector population within 7–10 days. These αβ TCRs expressing T cells are broadly classified as conventional T cells due to their significantly high abundance in the body.

The MHC-encoded proteins that are involved in most instances of antigen recognition by T cells are the **MHC class I** and **MHC class II** molecules **(Figure 2)**. MHC class I molecules consist of a heavy transmembrane alpha (α) chain non-covalently linked to a small non-transmembrane chain called β2-microglobulin chain (β2m). The α chain is encoded within the MHC, but β2m is not. They are expressed on nearly all nucleated cells in the body. They present intracellular (endogenous) antigens from viruses or other intracellular pathogens. For the antigen presentation, the proteins inside the cell are degraded by the proteasome into peptides, which are then transported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP). These peptides are loaded onto MHC I molecules and displayed on the cell surface for recognition.

MHC II molecules are made up of two similar-sized chains, alpha, and beta, both of which are transmembrane proteins encoded by genes in the MHC. MHC II presents extracellular (exogenous) antigens, usually derived from pathogens like bacteria. They are primarily expressed on APCs, including dendritic cells, macrophages, and B cells. Antigens are taken up by APCs through phagocytosis or endocytosis, processed into peptides within endosomes, and loaded onto MHC II molecules. These complexes are then presented on the cell surface for recognition.

Certain APCs, especially DCs, capture extracellular antigens and present them via MHC class I molecules, rather than the usual MHC class II pathway. This enables DCs to initiate a cytotoxic T cell response against pathogens and cancerous cells (Bevan, 1976).

Together, MHC I and MHC II coordinate to detect and respond to both intracellular and extracellular threats, ensuring a broad and effective immune response.

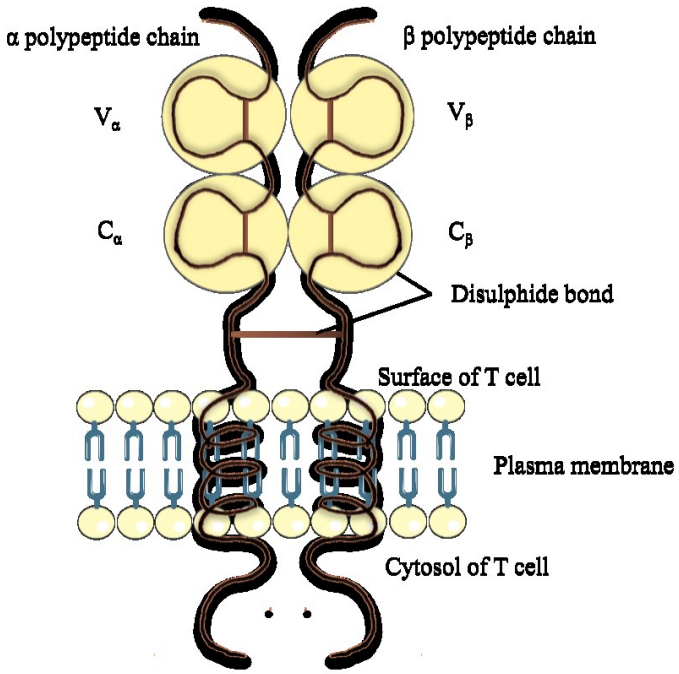


Figure 1. Schematic representation of the overall structure of the T cell receptor (TCR) site on a T cell plasma membrane. The receptor site is an alpha polypeptide and a beta polypeptide chain stabilized by a disulfide bond. Each chain has its own Variable (V_α and V_β) and constant (C_α and C_β) region.

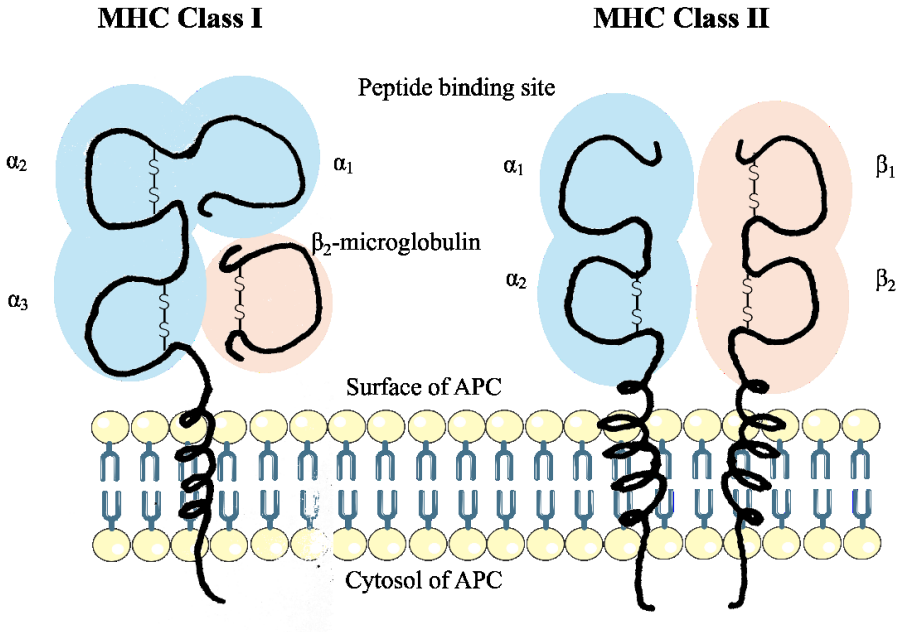


Figure 2. The membrane-bound Class I (left) and Class II (right) Major Histocompatibility Complex molecules. MHC Class I is a heterodimer composed of alpha protein, which is divided into three domains: α_1, α_2, and α_3 and the protein β_2 microglobulin. MHC Class II heterodimer is composed of two distinct proteins called alpha and beta. Each is divided into two domains, α_1, α_2, and β_1, β_2 respectively.

1.2 Development of T cells

The thymus secretes cytokines such as thymosin, thymotaxin, thymopoetin, and thymic-specific factors that draw T cells from bone marrow (in precursor form) to the thymus via the bloodstream (Savino & Dardenne, 2000; Shoham & Eshel, 1980). In the thymus, these cells lose the potential to become B cells and NK cells (Michie et al., 2000; A. Wilson et al., 2001). These cells are dedicated T cell precursors in the 'double negative' (DN) stage identified by the lack of surface CD4 and CD8. DN thymocytes can be subdivided further into four sequential stages of differentiation based on their surface expression of CD44 and CD25: DN1, $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$; and DN4, $CD44^{-}CD25^{-}$ (Michie et al., 2000). DN T cells can develop into either γδ or αβ TCR-expressing cells (Robey & Fowlkes, 1994). For those following the αβ TCR pathway, cells at the DN3 stage initially express pre-TCR-α, encoded by a non-rearranging gene locus. Pre-TCR-α pairs with the TCR β-chain, generated through somatic DNA rearrangements that depend on the expression of Recombination-activating gene 1 (RAG1) and RAG2 proteins (Braams et al., 2023; Yannoutsos et al., 2001). RAG1 and RAG2 expression in T cells is stimulated by thymic epithelial cells. These genes shuffle DNA to produce different proteins (TCR) for various antigens. They also activate other genes that are responsible for CD4 and CD8 proteins. At this phase, the T cells are double positive (DP) for CD8 and CD4 and thus, this stage is called 'double positive' selection. These DP T cells are then exposed to the thymic MHC-I and MHC-II. Through FAS mediated pathway (that recruits protease caspase-8 to induce apoptosis), thymic cells induce apoptosis of the cells that do not bind to MHC-I and MHC-II. If the TCR strongly recognizes the self-peptide of MHC-I or MHC-II, they undergo apoptosis and this process is called 'Negative selection'. If a cell interacts with MHC-II peptide with lesser strength, then CD4 genes are upregulated, and they turn into CD4+ T helper cells. If MHC-I interaction occurs, then CD8 genes are upregulated and the cells turn into CD8+ cytotoxic T cells.

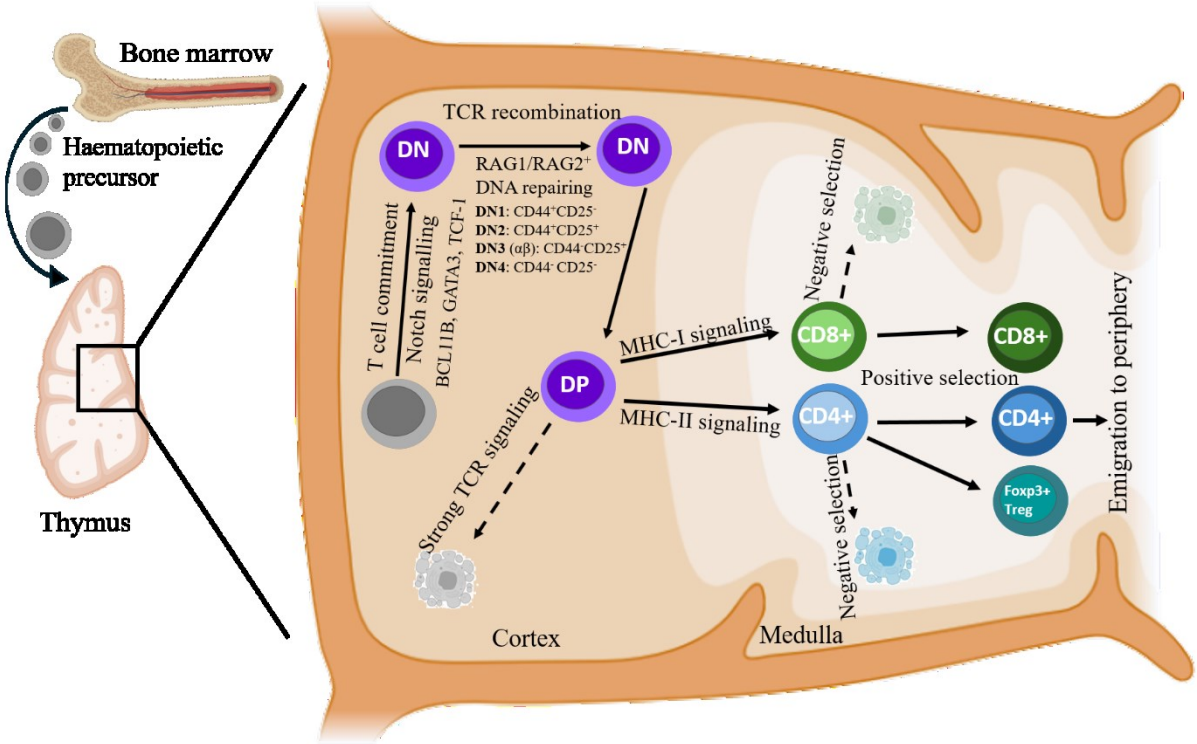


Figure 3. T cell development in thymus. T cell progenitors or precursor cells arrive in the thymus from the bone marrow. They commit to the T cell lineage under the control of Notch signaling and Bcl11b, Gata3, and TCF-1. Thymocytes are in Double Negative stage (DN) ($CD4^-$ $CD8^-$). Thymocytes recombine their TCRs under the control of RAG enzymes and DNA repair machinery to generate a diverse TCR repertoire. Thymocytes are in Double Positive stage (DP) ($CD4^+$ $CD8^+$). TCR recombination is followed by a positive selection of T cells that recognize self-MHC: peptide complexes in the cortex of the thymus. The thymocytes then migrate to the medulla where they are tested for reactivity against self-peptides-by medullary thymic epithelial cells; self-reactive T cells are negatively selected and deleted. Next, developing thymocytes differentiate into $CD4^+$ vs. $CD8^+$ T cells A subset of $CD4^+$ T cells develop into $FOXP3^+$ regulatory T cells (Treg), whereas the others mature into naïve T cells.

1.3 Types of Conventional T cells

Conventional T cells can be broadly classified into various subsets based on their functions and cytokine profiles. These subsets include helper T cells, cytotoxic T cells, and memory T cells.

1. Helper T (Th) cells

Microenvironmental signals, including pathogens and cytokines, trigger specific lineage-determining transcription factors (LDTFs) (Hoyler et al., 2012). LDTFs bind to target DNA

regulatory regions, transitioning target gene loci from inactive to poised. For example, the FOXP3 transcription factor drives Treg polarization. This transition facilitates the differentiation into CD4+ effector T cell subsets, such as T helper 1 (Th1), Th2, Th17, and follicular helper (Tfh) cells. Each subset enhances the immune response to different pathogen families by activating CD8+ T cells, NK cells, B cells, myeloid cells, and non-professional immune cells. Consequently, CD4+ T cells are called "T helper cells". It is now recognized that selective cytokine production is not exclusive to T cells but is also exhibited by innate lymphoid cells (ILCs), which utilize the same LDTFs in a non-antigen-specific manner (Hoyler et al., 2012). Traditionally, T helper subsets were considered fixed states: once a T cell committed to a particular subset, it exclusively produced a specific effector cytokine and could not easily switch phenotypes. Although these concepts help explain certain behaviors of CD4+ T cells, recent immunophenotyping techniques have revealed substantial heterogeneity, complexity, and plasticity within T effector subsets.

A) Th1 cells

Th1 cells produce IFN-γ in response to signals from intracellular microbes (Afkarian et al., 2002). These signals include macrophage-derived IL-12 and macrophage/NK-derived IFN-γ (Afkarian et al., 2002; Fang et al., 2022). IL-12 and IFN-γ activate STAT4 and STAT1, which in turn induce T-bet (encoded by Tbx21) to promote Th1 differentiation. IFN-γ enhances antigen presentation by upregulating MHC-I and MHC-II and promotes B cell class switching to IgG2a, IgG2c and IgG3 (Bossie & Vitetta, 1991; Snapper et al., 1992). Th1 cells express the chemokine receptor CXCR3, which detects CXCL9 and CXCL10, leading to Th1 (Manicone et al., 2008).

B) Th2 cells

Th2-promoting cytokines include IL-4, IL-25, IL-33, and thymic stromal lymphopoietin, while neuropeptides such as substance P, neuromedin U, and CGRP also support Th2 differentiation (Hou et al., 2024; Ouyang et al., 1998; Peine et al., 2016; Y. H. Wang et al., 2007). Other factors like Prostaglandin D2 induce Th2 differentiation through the chemoattractant receptor-homologous molecule (CRTH2). GATA3 is the LDTF that drives Th2 differentiation (Onodera et al., 2010). Other Th2-promoting transcription factors include STAT6, Notch, IRF-4, Growth factor independent-1 (Gfi-1), and Bcl11b (Nakayama et al., 2017; Onodera et al., 2010; J. A. Walker & McKenzie, 2018).

Th2 cells produce type 2 cytokines: IL-4, IL-13, and IL-5 which chemoattracts eosinophils, basophils, and mast cells (J. A. Walker & McKenzie, 2018). IL-4 promotes B cell growth, immunoglobulin class switching to IgG1, IgG4, and IgE, and aids further Th2 differentiation (Akiyama et al., 2018; Bergstedt-Lindqvist et al., 1988; Lebman & Coffman, 1988). IL-13 increases mucus secretion at secretory sites such as airway and gut epithelial cells. Th2 cells attain full effector capacity in tissues by interacting with various cells, including ILCs and neurons, which produce Th2-promoting factors in response to allergens, helminths, and other stimuli (Nakayama et al., 2017; Punnonen et al., 1993; J. A. Walker & McKenzie, 2018).

C) Th17 cells

Th17 cells produce the signature cytokine IL-17A, as well as IL-17F and IL-21 (Kolls & Sandquist, 2018). Extracellular bacteria and fungi promote Th17 differentiation by inducing IL-6, IL-21, IL-23, IL-1β, and TGF-β production (Dong, 2008; Hirahara et al., 2010a; Veldhoen et al., 2006). IL-6, IL-23, IL-1β, and TGF-β enhance the expression of Rorγt, while IL-6, IL-21, and IL-23 activate STAT3(Z. Chen & O'Shea, 2008; Hirahara et al., 2010b; Korn et al., 2009). Other factors that promote Th17 differentiation include RORα, BATF, IRF4, aryl hydrocarbon receptor, IκBζ, HIF-1, and RUNX1. Th17 cells highly express the chemokine receptor CCR6, which binds to CCL20, expressed by epithelial cells, synoviocytes, and Th17 cells within inflamed tissues. After migrating to inflamed tissues, Th17 cells indirectly recruit myeloid cells by inducing G-CSF, CCL2, and chemokines, making IL-17 an essential link between innate and adaptive immunity. Th17 cells are implicated in autoimmune diseases such as psoriasis and spondyloarthritis (Z. Chen & O'Shea, 2008; Zambrano-Zaragoza et al., 2014).

D) T follicular helper (T_{FH}) cells

T_{FH} cells are effector subsets defined by selective cytokine production and are identified by CXCR5 expression (Moser, 2015). CXCR5 allows Tfh cells to localize in B cell-rich follicles and germinal centers, where they support B cell responses (Crotty, 2011; Johnston et al., 2009; Vinuesa et al., 2016). The signals promoting Tfh differentiation differ from those inducing other effector subsets because Tfh cells require dual activation events involving ICOS-ICOSL signaling, mediated first by dendritic cells (DCs) and then by B cells (Rasheed et al., 2006). This interaction is described in detail in the results section of 'MAIT cells role in adaptive response'. Cytokines and transcription factors enhancing Tfh differentiation in mice include IL-6 and IL-27 via STAT3; TGF-β, Activin A, IL-12, and IL-23 promote human Tfh differentiation through STAT3 and STAT4 (Crotty, 2011, 2019). Other Tfh-promoting factors

include PD-1, the ubiquitin ligase ITCH, and transcription factors BATF, IRF4, TCF1/LEF1, and ASCL2. Most of these stimuli act by inducing BCL6, which limits the differentiation of other subsets. The factors that inhibit Tfh differentiation and function include FOXO1, FOXP1, and KLF2 (Bi et al., 2017; J. Y. Lee et al., 2015; Shi et al., 2017).

Tfh cells enhance humoral immunity majorly by producing IL-4 and IL-21 that supports B cell proliferation, survival, and differentiation into plasmablasts or germinal center (GC) cells (Crotty, 2011; Mintz & Cyster, 2020; Vinuesa et al., 2016).

E) Regulatory T (Treg) cells

Tregs promote peripheral and central tolerance (Sakaguchi et al., 2008). However, they do not orchestrate responses to specific pathogens. Most naturally occurring Tregs (nTregs) develop in the thymus when T cells with intermediate-affinity TCRs for self-peptides encounter self-antigens and develop into antigen-specific suppressive cells (Owen et al., 2019; Schmitt & Williams, 2013). Intermediate-affinity TCRs can respond to self-antigens, so nTregs activate and proliferate at lower self-antigen concentrations than their effector counterparts. A smaller group of Tregs derives from T effector cells, differentiating into peripherally derived Tregs (pTregs) outside the thymus (Dikiy & Rudensky, 2023; Owen et al., 2019; Sakaguchi et al., 2008; Shevyrev & Tereshchenko, 2020). In vitro, IL-2 and TGF-β strongly induce Treg development (Horwitz et al., 2008). Both nTregs and pTregs are defined by FOXP3 expression; inactivating FOXP3 mutations cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX), characterized by severe autoimmunity affecting various organs and food allergies (Bennett et al., 2001; Rajendeeran & Tenbrock, 2021; Torgerson et al., 2007).

Exogenous IL-2 and CD25 are crucial for Treg survival and suppression (Chinen et al., 2016; Tang et al., 2015). Checkpoint molecules CTLA4 and PD-1 are key modulators of Treg function (Kumagai et al., 2020; L. S. K. Walker, 2013); CTLA4 inhibition is highly toxic to Tregs, enhancing antitumor responses. PD-1 inhibition, conversely, boosts the suppressive activity of tumor-resident Tregs.

2. Cytotoxic T lymphocytes (CTLs)

Cytotoxic T lymphocytes (CTLs) express CD8 on their surface and thus are also called $CD8^+$ T cells, cytolytic T cells, or Killer cells. The TCR of $CD8^+$ T cells gets activated by recognizing antigens derived from cancer cells or pathogen-infected cells by interacting with MHC Class I. The interaction allows activation along with proliferation (Giles et al., 2023; Koh et al., 2023).

Factors like IL-7, IL-15, and ICOS help maintain the $CD8^+$ T cell population (Le et al., 2018; Rubinstein et al., 2008). In response to activation, $CD8^+$ T cells release cytotoxins like perforins, granzymes, and granulysin which immediately trigger the caspase cascade responsible for the apoptosis of target cells (Kochenderfer et al., 2010; McLane et al., 2019). CTLs have revolutionized the field of oncoimmunology leading the path for advanced cancer treatment like the use of checkpoint inhibitors like antagonists of programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA4) as well as Chimeric Antigen Receptor (CAR) T cells based therapy (Kochenderfer et al., 2010; McLane et al., 2019).

3. Memory T cells

Memory T cells are a type of T lymphocyte that provides long-term immunity by "remembering" past infections or vaccinations. They play a critical role in the adaptive immune response, allowing the body to respond more rapidly and effectively upon re-exposure to a pathogen (Sallusto et al., 2004).

Types of Memory T Cells

A. Central Memory T Cells (T_{CM}): These cells are primarily found in lymphoid organs. They have high proliferative potential and can quickly differentiate into effector T cells upon reactivation. They express markers such as CCR7 and CD62L, which help them home to lymphoid tissues (Sallusto et al., 2004).

B. Effector Memory T Cells (T_{EM}): Found in non-lymphoid tissues and blood, T_{EM} cells lack CCR7 and CD62L but express receptors that allow them to migrate to peripheral sites of infection. They are poised for immediate effector functions like cytokine production and cytotoxic activity.

C. Resident Memory T Cells (T_{RM}): These cells reside permanently in non-lymphoid tissues, such as the skin or mucosal surfaces, providing localized immune surveillance. They express markers like CD103 and CD69, which help them remain in tissue (Mueller & Mackay, 2016).

D. Stem Cell-like Memory T Cells (T_{SCM}): T_{SCM} cells have stem cell-like properties, including the ability to self-renew and differentiate into other memory and effector T cell subsets. They express markers such as CD95, CD62L, and CCR7 (Gattinoni et al., 2011).

Memory T cells can quickly respond to previously encountered antigens, providing a faster and more robust immune response compared to naive T cells. They can persist in the body for years or even decades, contributing to long-term immunity against pathogens and are a key component of the effectiveness of vaccines. Memory T cells are formed following an immune response when a subset of activated T cells does not undergo apoptosis but instead transitions into a memory state. They are maintained through a balance of survival signals, occasional low-level activation, and homeostatic proliferation. Memory T cells are crucial in vaccine design and infectious disease management. They also play roles in autoimmune diseases and are targets in cancer immunotherapy due to their ability to retain antitumor memory.

1.4 Unconventional T cells

Besides these conventional T cells, several T-cell populations have been identified that differ in terms of antigen recognition and function and are called unconventional T cells (UTCs). These UTCs include T cells that interact with non-traditional antigen-presenting molecules. Unlike conventional T cells, which have highly diverse and variable TCRs, some of these unconventional T cells express limited and semi-invariant TCRs (Arstila et al., 1999). This means that their TCRs are less varied and often fixed, recognizing a narrower range of antigens. These T cells often display antigen-independent responses that are driven by cytokine sensing rather than direct antigen recognition. This allows them to respond quickly to infection or other immune challenges without the need for specific antigen recognition.

The MHC system, governing most T lymphocyte activity, shapes our general understanding of T cell responses. Yet, unconventional, non-MHC-restricted T cells do not follow the MHC paradigm mentioned above. These include αβ and γδ T cells recognizing highly conserved antigen-presenting molecules such as CD1a, CD1b, CD1c, CD1d, MR1, HLA-E, HFE, H2-M3, and H2-Q9.

Unconventional T cells can also be referred to as Invariant or Innate-like T cells.

- Invariant T cells

The term "invariant" refers to the restricted diversity of their TCRs. In contrast to conventional T cells, which undergo extensive recombination to create a vast array of TCRs capable of recognizing diverse peptide antigens, invariant T cells have a fixed, or invariant, TCR α or γ chain paired with a limited set of β or δ chains. This limited TCR variability means that all invariant T cells within a given subset express almost identical TCRs, allowing them to recognize the same or similar antigen structures.

- Innate-like T cells

Unconventional T cells are called "innate-like" because they can respond rapidly to pathogens or stress signals in a manner similar to innate immune cells (like macrophages or natural killer cells), rather than relying on the slower, antigen-specific response typical of conventional adaptive T cells. They can quickly produce cytokines and exert effector functions without requiring the long process of clonal expansion and differentiation, which is characteristic of conventional T cells. Unlike conventional T cells, which have highly diverse T-cell receptors

(TCRs) due to random recombination, innate-like T cells typically have semi-invariant or limited TCR diversity. This allows them to recognize a restricted set of antigens or stress signals.

Recent observations have revealed that T cells with conventional restriction can also exhibit innate-like characteristics. This means that even T cells that recognize antigens through conventional MHC I or MHC II molecules can behave in ways like innate immune cells, which respond more generally and immediately to pathogens. An illustrative example of this phenomenon is the T_{MIC} population (MHC II-restricted, innate-like, and commensal-specific T cells) found in the intestines of both humans and mice. These T_{MIC} cells are restricted by MHC II molecules yet exhibit behaviors that are typical of innate immune cells, such as rapid response and recognition of commensal bacteria. This demonstrates that innate behaviors and unconventional restrictions are not always directly correlated and can occur independently.

In this thesis, I focus on two major lineages of Unconventional T cells: **Natural killer T (NKT) cells and mucosal-associated invariant T (MAIT) cells**. Besides NKT and MAIT cells, there are other lineages of UTCs like gamma-delta (γδ) T cells, Chron's associated invariant (CAIT) T cells, and Germline-encoded mycolyl lipid-reactive (GEM) T cells which are not focused in this manuscript.

2. NKT cells

2.1 NKT cell discovery

NKT cells are a unique subset of T lymphocytes that share surface markers and functional properties of both, T and natural killer (NK) cells. On the one hand, NKT cells exhibit MHC-based restriction and thymus-dependent selection, a property confined to T lymphocytes. And on the other hand, NKT cells express surface markers such as CD161 (NK1. 1) and CD122 (IL-2 receptor β). NKT cells were discovered in 1986 based on the presence of a unique (TCR) α-chain gene which is invariant in mice (Imai et al., 1986; Koseki et al., 1989). (TCR) α-chain of NKT cells is encoded by Vα14Jα18 in mice and Vα24Jα18 in humans. TCRα-chain of NKT cells is associated with a limited repertoire of TCRβ-chains, mainly Vβ8.2 in mice and Vβ11 in humans (Bendelac et al., 2007; Kawano et al., 1997b). By analyzing the frequency of Vα14Jα18 expression in different strains of laboratory mice at steady state, it was found that NKT cells were clonally expanded under physiological conditions without any external antigen stimulation (Koseki et al., 1990). In 1997, it was found that NKT cells recognize glycolipid antigens presented by the CD1d (Kawano et al., 1997a). The ligand was composed of a glycosylceramide-containing α-anomeric sugar (hereafter referred to as α-GalCer) with a longer fatty acyl chain (C_{26}) and sphingosine base (C_{18}) (Kawano et al., 1997a). The first crystallized structure of NKT TCR with CD1d/ α-GalCer shed light on the molecular entities involved in efficient bonding (Borg et al., 2007a). The ligand discovery led to the development of CD1d tetramer which revolutionized the field of NKT cells study (Gumperz et al., 2002). Since then, several groups have emerged describing the role of NKT cells in mice and humans during pathogen breach by rapidly producing a broad range of cytokines such as IFN-γ, IL-4, IL-17, IL-2, IL-5, IL-6, IL-10, IL-13, IL-21, TGF-β, and GM-CSF (Brigl et al., 2003a; Matsuda et al., 2008; Stetson et al., 2003; Yang et al., 2020). By secreting Th1 and Th2 cytokines, NKT cells display important roles in innate and adaptive immunity in infection and cancer (Fujii et al., 2007; Hara et al., 2021; Matsuda et al., 2008). Some studies showed that α-GalCer leads to pro-tumoral activity and now there is mounting evidence suggesting the role of NKT cells in anti-tumoral activity and cancer immunosurveillance (Kawano et al., 1998; Morita et al., 1995; B. J. Wolf et al., 2018). The fact that NKT cells are not restricted to polymorphic human leukocyte antigen (HLA) makes them an excellent therapeutic target for adoptive transfer as the transfer does not lead to graft-versus-host disease due to rejection. Many groups have successfully generated Chimeric antigen receptor (CAR) T- NKT and demonstrated their full potential in combating different cancers (Heczey et al., 2014; Y. Liu et al., 2022; Moraes

Ribeiro et al., 2024; Rotolo et al., 2018; Y. Wang & Li, 2024). The diverse effector functions make NKT cells a potential target for clinical translation.

2.2 NKT cell TCR and CD1d

The NKT TCR-α chain (Vα24-Jα18 in humans, Vα14-Jα18 in mice), facilitates CD1d-dependent lipid recognition (Almeida et al., 2019; Borg et al., 2007b; Guo et al., 2016) (Kawano et al., 1997a). Unlike the TCR-α chain, the TCR-β chain (Vβ2, 7, 8 in mice and Vβ11 in humans) is more diverse. However, it does not play as dominant a role in CD1d recognition as the TCR-α chain (Borg et al., 2007b; Guo et al., 2016).

The TCR recognizes the CD1d molecule, which is responsible for presenting lipid antigens, such as glycolipids. The TCR structure allows NKT cells to distinguish between different lipids presented by CD1d, aiding in immune responses to various pathogens. Crystal structures of the NKT TCR-CD1d complex have revealed that the TCR-α chain forms a "straight" docking angle over the CD1d molecule, which differs from the diagonal docking typical of conventional TCR-MHC interactions (Almeida et al., 2019; Borg et al., 2007b). This orientation allows the invariant TCR-α chain to engage both the CD1d molecule and the lipid antigen in a manner that is highly specific for lipid recognition, particularly microbial glycolipids, and self-lipids like α-galactosylceramide. The conserved nature of the NKT TCR enables recognition of a broad range of lipid antigens with minimal structural adjustments. Despite the invariant TCR-α chain, the flexibility of the TCR-β chain allows for fine-tuning in antigen recognition, giving NKT cells a versatile role in immune responses against diverse lipid antigens.

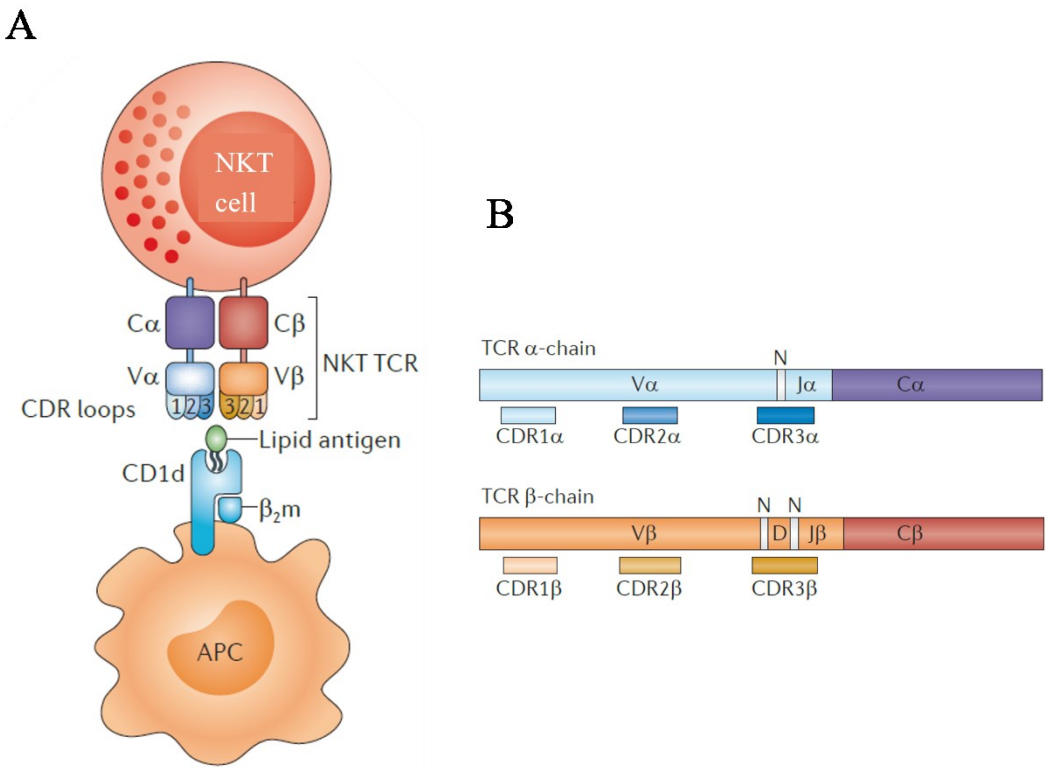


Figure 4. Structure of NKT cell TCR and CD1d. (A) A schematic representation of an NKT cell with variable (V) region gene segments in the α- and β-chains of their T cell receptors (TCRs) that recognize CD1d-restricted antigens. **(B)** The αβ TCR is composed of two chains, with the V domains containing the complementarity-determining region (CDR) loops. The CDR3 loops are encoded by multiple gene segments and also contain non-templated (N) regions, which add further diversity to the TCR repertoire. β2m, β2-microglobulin; APC, antigen-presenting cell; C, constant; D, diversity; J, joining. Scheme from (Rossjohn et al., 2012)

2.3 NKT cell development

NKT cells develop within the thymus soon after birth by positive selection following recognition of self-glycolipid antigens in complex with CD1d expressed by DP cortical thymocytes (Das, Sant'Angelo, and Nichols 2010). The TCRa chain of NKT cells combines with a restricted set of β chain comprised of either Vb7, Vb8, or Vb2. Because of CD1d interaction, Egr1 and Egr2 (early growth response proteins) are expressed which directly bind and activate the promoter of Zbtb16 that encodes PLZF (promyelocytic leukaemia zinc finger

protein) transcription factor (Seiler et al., 2012). Thus, PLZF is a key regulator for NKT cell development in the thymus.

The old model of NKT cell maturation was defined by the expression of CD24, CD44, and NK1.1. The least mature NKT cells are defined as CD24 hi CD44 lo NK1.1 – (Stage 0), followed by $CD24^{lo}$ $CD44^{lo}$ $NK1.1^{-}$ (Stage 1), $CD24^{lo}$ $CD44^{hi}$ $NK1.1^{-}$ (Stage 2) and finally $CD24^{lo}$ $CD44^{hi}$ $NK1.1^{+}$ (Stage 3). Upon reaching Stage 2, NKT cells acquire an activated phenotype and leave the thymus to finally finish the maturation process in the periphery, where they can acquire NK cell-specific markers (Stage 3) or remain NK1.1 negative (Das et al., 2010).

2.4 NKT cells distribution

NKT cells have a distinctive distribution throughout the body. They constitute 0.5% of total T cells in the blood and peripheral lymph nodes (LNs), and 2.5% in the spleen and mesenteric LNs (Bendelac et al., 2007). Their strategic localization includes the inter-follicular areas of LNs and the spleen's red pulp and marginal zone, where they are positioned near pathogen entry points (Barral et al., 2012; Gaya et al., 2018; Kastenmüller et al., 2012). Remarkably, NKT cells can make up to 30% of total T cells in the liver, a preference partly due to their high expression of CXCR6, aligning with CXCL16 expression in liver sinusoids. Here, they typically patrol sinusoids and become active in response to lipid antigens, specific cytokines, or adrenergic signals during events like a stroke (Geissmann et al., 2005; W. Y. Lee et al., 2010a; Wong et al., 2011).

In humans, NKT cells are not predominantly found in the liver but can represent up to 10% of total T cells in the omentum (Lynch et al., 2009). They also inhabit the lungs, skin, gastrointestinal tract, and adipose tissue. In the lungs, NKT cells are initially within the vasculature but migrate to the lung parenchyma upon activation to aid the inflammatory response (Scanlon et al., 2011a). Conversely, in the skin, intestine, and adipose tissue, NKT cells tend to mitigate inflammation by producing IL-10 (Lynch et al. 2014; Olszak et al. 2014; McKee, Mattarollo, and Leggatt 2014).

2.5 Activation of NKT cells

An NKT cell can be activated by a cognate interaction between its TCR and ligand or indirectly by cytokines or by endogenous ligands.

Lipid antigens that are recognized by NKT cells :

NKT cells can bind to a variety of lipid-based antigens complex with CD1d, including a-Gal-Cer, microbial ligands, and self-antigens. a-GalCer, also known as KRN7000, was first identified in a marine sponge for its anti-metastatic properties and is the best-known lipid antigen for activating NKT cells (Kronenberg 2005; Kawano et al. 1997). It has an a-anomeric sugar attached to acyl and sphingosine chains, leading to rapid production of Th1 and Th2 cytokines upon binding. It has been used therapeutically to enhance pathogen clearance by secretion of pro-inflammatory cytokines (Tupin et al., 2007). Whereas, in autoimmunity, a-Gal-Cer ameliorates the disease by secretion of anti-inflammatory cytokines like IL-10 (Van Kaer, 2005).

Microbial glycolipids such as those from Sphingomonas spp. and Mycobacterium bovis, and cholesteryl-a-glucoside from Helicobacter pylori, also activate NKT cells, which respond to both microbial and inflammatory stimuli (Mattner et al., 2005a). Synthetic analogs of a-GalCer have been developed to induce specific cytokine responses, offering therapeutic potential for conditions like cancer, allergy, and autoimmunity. These include derivatives like OCH, which promotes Th2 responses, and other analogs designed to induce Th1 or Th2 cytokine responses (O'Keeffe et al., 2015).

The endogenous self-antigens that are recognized by NKT cells:
One defining characteristic of NKT cells is their autoreactivity, or ability to react to self-antigens presented by CD1d in the absence of exogenous antigens (Exley et al., 1997). Various self-lipid antigens, primarily glycosphingolipids and phospholipids, have been identified as stimulatory for NKT cells. The first endogenous glycolipid activator, iGB3, was shown to be crucial for NKT cell development, although its significance is debated (Zhou et al., 2004) because humans lack the functional enzyme, iGb3 synthase which is required for producing iGb3, and mice deficient for iGb3 synthase have normal levels of NKT cells (Christiansen et al., 2008; Porubsky et al., 2007). Other self-antigens include ganglioside GD3 (D. Y. Wu et al., 2003) and several phospholipids like phosphatidylcholine and lysophosphatidylcholine.

Lysophosphatidylcholine, found in inflammatory conditions, activates NKT cells to secrete G-MCSF (Fox et al., 2009). In another study, it was demonstrated that α-Gal-Cer and α-Glu-Cer are intracellularly produced in small amounts by immune cells making them major endogenous ligands that can be recognized by NKT cells (Kain et al., 2014). The intracellular levels of these two ligands are kept in check by the lysosomal enzyme α-Galactosidase. The presentation of endogenous lipids and/or cytokines produced by APCs is sufficient to induce NKT cell activation (Brigl et al., 2003b).

NKT cells that can become activated solely by cytokines in the case of *Pseudomonas aeruginosa* infection, the production of IL-12 and IL-18 by APCs can indirectly activate NKT cell (Kastenmüller et al. 2012).

These various activation mechanisms and the strategic positioning of NKT cells in peripheral and secondary lymphoid organs enable them to respond to diverse pathogenic threats swiftly.

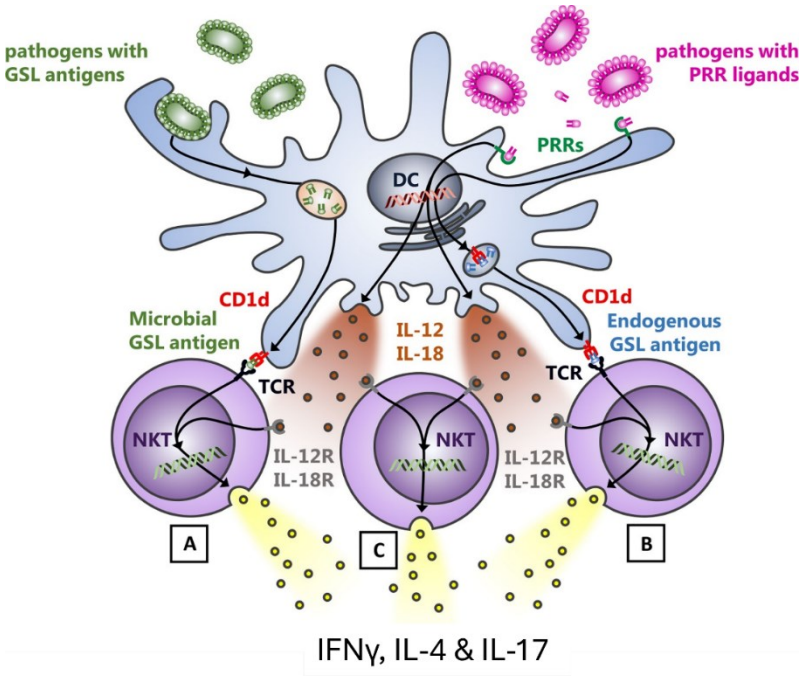


Figure 5. Modes of NKT cell activation upon bacterial infection. Following the uptake and digestion of bacteria by myeloid cells such as DCs, three mechanisms predominantly drive the activation of NKT cells: **(A)**, bacteria-derived glycosphingolipid (GSL) antigens presented by APCs are sufficient to induce the release of IFN-γ, IL-4, and IL-17 by NKT cells via CD1d mediated presentation of the ligand to the NKT cell TCR. **(B)**, endogenous GSL

presentation *via* CD1d to the NKT cell TCR in response to infection and subsequent augmented NKT cell auto-reactivity. **(C)**, the cytokine-driven, TCR-independent activation of NKT cells triggered by IL-12 and IL-18 release by APCs. This interaction also further enhances the release of cytokines by NKT cells, activated by cognate TCR engagement. Scheme from (Vogt & Mattner, 2021)

2.6 NKT cell subsets

NKT cells are divided into two major types: type I and type II, each with distinct roles in immune regulation. There are three subsets of type-I NKT cells that have been broadly described: NKT1, NKT2, and NKT17. NKT1 primarily produces interferon-gamma (IFN-γ) and also shows the ability to produce tumor necrosis factor-alpha (TNF-α), contributing to inflammatory responses (Townsend et al., 2004). NKT1 cells express the transcription factor T-bet, which is critical for their development and drives the production of IFN-γ. These cells typically express NK1.1 (in mice). NKT1 cells are primarily involved in antitumor immunity and in responses against intracellular pathogens. NKT1 cells are found in various tissues, including the liver, spleen, and thymus, and are enriched at sites of inflammation or infection.

The development of NKT2 cells is guided by the transcription factor GATA3, and their function is crucial in maintaining immune balance, especially in contexts requiring type 2 immune responses. NKT2 cells are distinguished from other NKT subsets based on their cytokine profile and transcription factors. They predominantly secrete IL-4 and other type 2 cytokines, like IL-13, playing a role in promoting Th2 immune responses. This positions them as key players in immune regulation, particularly in allergy, asthma, and tissue repair (Brennan et al., 2013; Watarai et al., 2012). However, recent study using scRNA sequencing approach describe NKT2 cells as a transient branching point toward the generation of NKT1 with IL-4 and IFN-γ production capabilities whereas NKT17 cells show IL-17 production tendency (Baranek et al., 2020).

Whereas NKT17 cells are characterized by their production of IL-17 which is a hallmark of this subset. They also show the tendency to produce IL-21. NKT17 cells express the transcription factor RORγt, which is essential for IL-17 production and the development of this subset (Brennan et al., 2013; Watarai et al., 2012). NKT17 cells express markers like CCR6**,**

which is associated with the migration of IL-17-producing cells to mucosal tissues and sites of inflammation. They may have low or absent expression of NK1.1 (in mice) or CD161 (in humans), distinguishing them from the NKT1 subset.

It was shown in 2009 that sulphatides, a major component of the myelin sheet and pancreatic beta-cells could stimulate a subset of type-II NKT cells (Blomqvist et al., 2009) or by certain endogenous hydrophobic lipids (Nishioka et al., 2018). Due to the scarcity of type-II NKT cells and lack of specific markers to identify, their biology has not been studied much.

2.7 NKT cell interaction with other cells

Dendritic cells (DCs) were the first immune cells described to interact with NKT cells in a CD1d-dependent manner (Fujii et al., 2004). They constitutively express CD1d and activate NKT cells in the spleen and lungs following α-GalCer exposure (Barral et al., 2012; Scanlon et al., 2011b). In vitro studies have shown that TLR ligands induce DC-mediated presentation of endogenous lipids and production of IL-12, leading to NKT cell activation (Mattner et al., 2005b). The interaction between DCs and NKT cells involves not only CD1d-TCR binding but also CD40-CD40 ligand interactions, resulting in strong IFN-γ production by NKT cells and further IL-12 production by DCs. These cytokines enhance responses by conventional CD4+ and CD8+ T cells, and promote DC cross-presentation. This bidirectional interaction amplifies both the innate and adaptive immune responses (Fujii et al., 2004; Hermans et al., 2003; Semmling et al., 2010).

Macrophages in various organs also interact with NKT cells through CD1d. In the liver, NKT cells, which comprise a significant portion of total T cells, are activated by macrophage subsets, such as Kupffer cells and stellate cells, following Borrelia sp. Infection (W. Y. Lee et al., 2010b). In the lymph nodes (LNs), subcapsular sinus (SCS) macrophages present exogenous lipids on CD1d to resident NKT cells after particulate α-GalCer administration and activate NKT cells via IL-18 secretion (Barral et al., 2010). These interactions are reciprocal; for instance, NKT-derived IFN-γ induces iNOS (Inducible nitric oxide synthase) production in lymph node SCS macrophages and enhances phagocytosis by pulmonary macrophages (Kastenmüller et al., 2012; Nieuwenhuis et al., 2002). NKT-derived IL-10 can also promote an anti-inflammatory phenotype in adipose tissue macrophages (Lynch et al., 2015).

Neutrophils express CD1d and can interact with NKT cells, particularly during inflammation when the acute-phase protein serum amyloid A1 (SAA1) induces the production of IL-10-producing neutrophils. Under these conditions, neutrophils activate NKT cells by presenting endogenous ligands on CD1d. NKT cells can reverse the suppressive phenotype of neutrophils and promote IL-12 production, which helps balance immunosuppressive neutrophil frequencies (De Santo et al., 2010). Additionally, NKT cells can recruit neutrophils to infected tissues via the production of CXCL2, independent of CD1d interactions (Nieuwenhuis et al., 2002).

NKT cells also interact with B cells through CD1d. Splenic marginal-zone B cells express the highest levels of CD1d among antigen-presenting cells, but their role in NKT activation during infection remains uncertain (Faunce & Stein-Streilein, 2002). A specific NKT cell subset, known as NKT_{FH} cells, provides cognate help to B cells by presenting exogenous glycolipids on CD1d (Chang et al., 2011; King et al., 2011). This interaction leads to rapid immunoglobulin production and some affinity maturation, though it does not generate long-term B cell memory. Thus, NKT cell cognate help may enhance early antibody responses during infection (Barral et al., 2012; Harwood et al., 2012; Vomhof-DeKrey et al., 2014). Additionally, NKT cells provide non-cognate help to B cells via the secretion of IL-4, IL-5, IL-6, IL-13, IL-21, and CD40 ligand (Coquet et al., 2008).

More recently, Innate lymphoid cells (ILCs) were shown to acquire lipids in vitro and in vivo and load lipids on CD1d to mediate presentation to NKT cells driving IL-22 secretion (Saez de Guinoa et al., 2017).

NKT cells interact with various non-immune cells across different tissues, influencing processes such as tissue homeostasis, repair, metabolism, and inflammation. Some examples of such cells that have been investigated to interact with NKT cells are adipocytes, hepatocytes, fibroblasts, keratinocytes, etc (Beaudoin et al., 2002; Kamata & Tada, 2023; Marrero et al., 2018; Satoh & Iwabuchi, 2024).

3. MAIT cells

3.1 MAIT discovery:

The discovery of MAIT cells was around the same time as NKT cells in 1993. The investigation of NKT cells by Bendelac and Lantz team from human samples was performed when S.Porcelli reported an abundant TRAV1-TRAJ33 TCRα chain with invariant CDR3 length and amino acid sequence with a limited spectrum of antigens in human $CD4^{-}CD8^{-}$ (DN) T cells (Porcelli et al., 1993). Lantz's team found that the usage of TRAV1-TRAJ33 TCRα was > 200-fold in DN T-cell fraction in human blood. (Lantz & Bendelac, 1994; Tilloy et al., 1999). These cells were also observed in cattle and were present in very low numbers in the commonly used mouse models implying the conservation of these T cells in mammalian species. They were also reported to be restricted to a β_2m-containing molecule, not involving MHC-I, MHC-II, CD1, or TAP, suggesting the recognition of nonpeptidic ligands. In 2003, Treiner, Duban, and colleagues named these cells MAIT cells because they are enriched within mucosal sites (Treiner et al., 2003). The same study identified the restricting element of MAIT cells as the MHC-Ib molecule MR1. MR1 molecule is highly conserved between species and found in a folded conformation at the cell surface. Studies that followed reported that MAIT cells respond to diverse bacteria and yeast (Gold et al., 2010; Le Bourhis et al., 2010). It was in 2012 when Kjer-Nielson et al. demonstrated that MR1 presented intermediates in the vitamin B and folic acid synthesis pathway to MAIT cells (Kjer-Nielsen et al., 2012). Soon after MR1-tetramers were developed that defined MAIT cells expressing *TRAV1- 2* with other *TRAJ* genes along with *TRAJ12* and *TRAJ20* (Reantragoon et al., 2013). The ternary complex (a structure composed of three entities) of a MAIT cell TCR/MR1 antigen was elucidated in the same year allowing a better understanding of the docking footprint (protein-protein interaction-based structural study) leading to the specific reactivity of MAIT cell and their similarities/dissimilarities to the other invariant T cells (López-Sagaseta et al., 2013a). This paved the way to identifying potent stimulatory ligands of MAIT cells 5-A-RU (5-amino-6-D-ribitylaminouracil) derived in the riboflavin synthesis pathway. They show that 5-A-RU indirectly activates MAIT cells by binding with small molecules like glyoxal or methylglyoxal in a non-enzymatic reaction forming 5-(2-oxoethylideneamino)-6-d-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)- 6-d-ribitylaminouracil (5-OP-RU), respectively (Corbett et al., 2014). With the help of MR1-antigen-loaded tetramers, heterogeneity and phenotypical properties of MAIT cells were identified across tissues in C57BL/6 and BALB/c mice

(Rahimpour et al., 2015). It was also the first time when different subsets of MAIT cells RORγt^{+} IL-17A–producing MAIT cells and T-bet^{+} IFN-γ–producing MAIT cells were reported. As a result of these findings, it was established that MAIT cells display a mixed type 1 and type 17 phenotype and function (Dusseaux et al., 2011a). Studies detailing the phenotype of MAIT cells led to the discovery of the presence of the transcription factor PLZF as the innate-like functionality governing factor. In subsequent work from the Pellicci group, a highly detailed MAIT cell developmental pathway in the thymus of humans and mice was identified (Koay et al., 2016). Early studies suggested that viruses do not induce MAIT cell activation as it was determined at that point that MAIT cells respond to riboflavin metabolites, as viruses do not synthesize riboflavin. However, it was later found that MAIT cells can respond to, or be modulated by many different viruses (Cosgrove et al., 2013; Saeidi et al., 2016; Van Wilgenburg et al., 2016) indirectly through cytokines such as IL-18, IL-12, IL-15, type I interferons, IL-23, IL-7, etc. (Hinks & Zhang, 2020).

Along with infection, many studies reported alterations in the number or function of MAIT cells during non-microbial diseases like multiple sclerosis, celiac disease, diabetes, metabolic disorder and inflammatory bowel disease (Abrahamsson et al., 2013; Hiejima et al., 2015; Tominaga et al., 2017; Toubal & Lehuen, 2021). However, it remains unclear if MAIT cells would have a pathogenic or protective role in these diseases. MAIT cells have also been linked to allergies and asthma. (Lezmi et al., 2018; Ye et al., 2020). MAIT cells have also been broadly investigated in the context of tumor immunity and several ongoing studies on the subject reflect their potential in the development of therapeutics (Gentles et al., 2015; Ling et al., 2016; Nitta et al., 1990; Peterfalvi et al., 2008).

Although they were discovered 30 years ago, it has only been a decade since MAIT cells have started to capture the attention of immunologists and it is likely that with the advancements in technology, we will have a solid basis to understand the biology of MAIT cells in health and diseases across species.

3.2 The (semi) invariant T cell receptor of MAIT cell and MR1

The specificity of the MAIT cell TCR for MR1-loaded antigens is attributed to its distinct CDR3 characteristics **(Figure 6)**. The TCRα chain predominantly recognizes antigens within the MR1 binding groove, regardless of Vα/Jα pairing, and this recognition requires a tyrosine at residue 95. This tyrosine is essential for effective TCRα chain-mediated antigen interaction (Corbett et al., 2014; Eckle et al., 2015). Some MAIT cells use TRAV1-2-TRAJ33 rearrangements without the typical Tyr95α residue but still react to MR1-5-OP-RU **(Figure 6)**, suggesting other factors contribute to antigen recognition (Held et al., 2015). The TCRα chain commonly pairs with Vβ2 or Vβ13 (*TRBV20* and *TRBV6*, respectively), while the CDR3β primarily interacts directly with the MR1 molecule. The TCRβ chain provides the greatest source of variability in the MAIT TCR repertoire. Up to 25% of MAIT TCRs use TCRβ chains outside of TRBV6 or TRBV20 (Eckle et al., 2014; Gold et al., 2014; Held et al., 2015).

The CDR3β region among MAIT cell TCRs variability ranges from 9 to 19 amino acids with no discernible motifs, influencing MR1 recognition and antigen binding affinities. (Eckle et al., 2014; Lepore et al., 2014). In mice, the TCR structure is similar: Vα19 (*Trav1*) pairs with Jα33 (*Traj33*) to form the ligand-binding TCRα chain, which then pairs preferentially with Vβ6 or Vβ8 (*Trbv19* or *Trbv13*, respectively) (Rahimpour et al., 2015; Tilloy et al., 1999). This architectural consistency of the MAIT cell TCR and its position within the TCRα locus is broadly conserved across mammalian species (Boudinot et al., 2016a).

The basal expression of unloaded/empty MR1 is lower compared to the other presenting molecules (S. Huang et al., 2008) and its transport is independent of tapasin (proteins required for antigenic peptide transport across endoplasmic reticulum). Thus, the biological processes involved in the presentation of metabolites by MR1 were unknown for a long time. McWilliam et al. described the mechanism of how MR1 monitors extracellular microbial metabolites from within the cell (McWilliam et al., 2020). Other presenting molecules such as MHC II and CD1a-d migrate to the endosomal compartment associated with their endosomal ligands that are replaced by endocytosed antigens in an APC. However, MR1 accumulates in the endoplasmic reticulum in a large proportion until the carbonyl group of the ligand binds the lysine residue (K43) present on the MR1 binding cleft forming a Schiff base (a compound with double bond linking carbon and nitrogen). The interaction is highly selective as evidenced by the presence of very few ligands presented by MR1. As a result of this covalent binding, MR1

undergoes conformational change due to the neutralization of K43 lysine and is trafficked to the cell surface. This switches ‘on’ the MR1 presentation pathway on the surface. The ligands randomly binding the other residues to the MR1 cleft induce little or no MR1 surface expression. This complex of MR1-riboflavin pathway-derived metabolite on the cell surface has a half-life of 2-4 hours after which they are internalized and delivered to the endocytic route for degradation. A small number of molecules can recycle and return to the surface, potentially after acquiring new ligands in endosomes. The ligands are not produced by the APCs. Instead, they are released from phagosomes with internalized pathogens or from an infected cell. However, the processes involved in the transport of these ligands to the lumen of the endoplasmic reticulum are still unknown (McWilliam & Villadangos, 2017). Upon clearance of pathogens, the availability of the MR1-ligand complex wanes, and the MR1 pathway returns to the ‘off’ state.

Ligand-bound MR1 places itself between the 2′−OH group and the Tyr95α of the MAIT TCR. Along with this interaction, Tyr95α of the MAIT TCR also forms hydrogen bonding with Tyr152 from MR1 (Awad et al., 2020). This hydrogen-bonding network between Tyr95α, Tyr152, and 5-OP-RU is conserved among all the known ternary complexes of MAIT-TCR with MR1 presenting 5-OP-RU. Asad et al coined the ‘interaction triad’ by Awad et al (Awad et al., 2020)

Even though the TRAV1-TRAJ33 iTCRα and MR1 are highly conserved, they were deleted from the genome of several animals such as Carnivora (dogs, cats, bears, ferrets) and lagomorphs (rabbit, pika) and in the armadillo (Boudinot et al., 2016). A remnant pseudogene of *Mr1* is present in Carnivora lacking TRAV1, suggesting that TRAV1 was omitted before the inactivation of MR1. One possibility for this deletion would be due to limited adaptation to bacterial fermentation caused by their specific diet that induces intestinal hyperoxia. Germain et al proposed a hypothesis that the higher oxygen availability that increases vitamin metabolites might have a deleterious contra-selection of MAIT cells (Germain et al., 2025a).

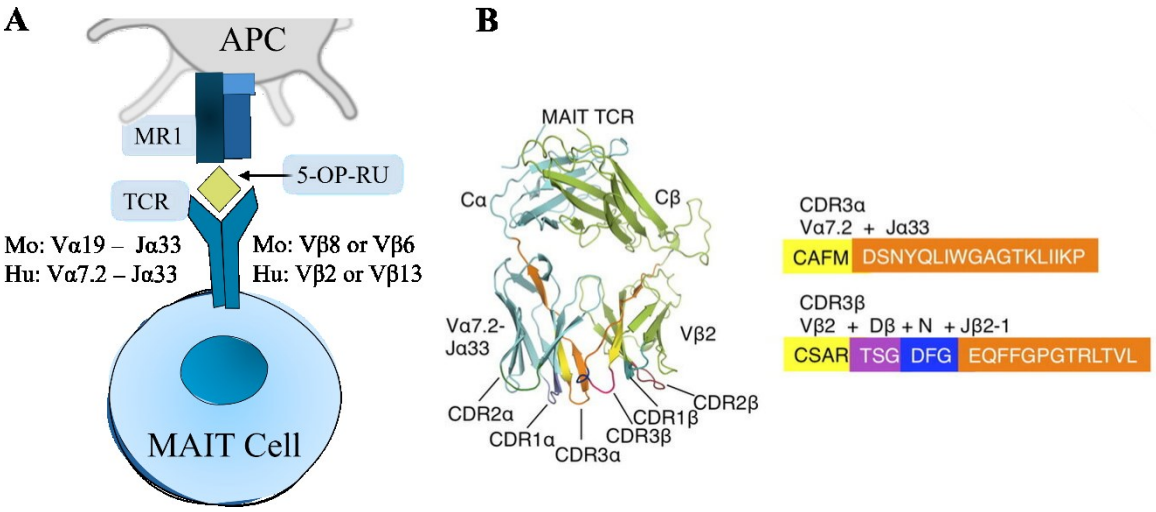


Figure 6. The MAIT TCR structure and docking orientation on MR1. (A) The TCR of MAIT cell (composed of TCRα and TCRβ) recognizes vitamin metabolite (5-OP-RU) presented by APC via MR1. **(B)** Ribbon diagrams of the complex structures of the MAIT TCR, Structure of MAIT TCR and amino acid composition of CDR3 loops. TCRα chain, cyan; TCRβ chain, light green; CDR1α, purple; CDR2α, dark green; CDR1β, teal, CDR2β, ruby; CDR3 loops, color coded according to their genetic origin (right) Figure 6B adapted from (Reantragoon et al., 2012).

3.3 Thymic development of MAIT cells

Like most T cells including NKT cells, MAIT cells develop in the thymus (Martin et al., 2009; Tilloy et al., 1999). Like type-I NKT cells, the development of MAIT cells requires interacting with MR1 expressed on CD4+CD8+ cortical thymocytes and SLAM-SAP pathway activation.

A three-stage sequence in both mice and humans maps the development journey of MAIT cells (Koay et al., 2016) **(Figure 7)**. In mice, the earliest cells (stage 1) are defined as $CD3^+$, MR1 tetramer positive (MR1-tet^+), $CD24^+$, and $CD44^-$ ($CD3^+$MR1-$tet^+CD24^+CD44^-$). These cells are found only in the thymus and dominate in very young (2-week-old) mice. Stage 2 MAIT cells are defined as $CD3^+$MR1-$tet^+CD24^-CD44^-$. The progression to stage 2 involves the presentation of ligands by MR1 and the microRNA miR181a/b-1 (Winter et al., 2019). Ligands presented by MR1 originate from microbiota proven by a significantly reduced number of MAIT cells in the germ-free mice (Constantinides & Belkaid, 2021; Kjer-Nielsen et al., 2012; Legoux et al., 2020a). This stage also determines the survival, and/or expansion of MAIT cells beyond stage 1. The next maturation step sees the emergence of stage-3 MAIT cells, defined as $CD3^+$MR1-$tet^+CD24^-CD44^+$, which more closely resemble mature MAIT cells in the periphery. At this point, MAIT cells begin to express classical markers such as CD218 (IL-18R), CD44, and PLZF (Koay et al., 2016; Winter et al., 2019). The transition from stage 2 to stage 3 is regulated by multiple factors, including PLZF, MR1, IL-18, miR181a/b-1, and undefined microbial factors. PLZF expression is associated with tissue homing and residency, as well as rapid responses to antigen and TCR-independent stimuli via cytokines, which are characteristics of MAIT cells likely controlled by this transcription factor (Beaulieu & Sant'Angelo, 2011).

Stage 3 is a crucial stage where the pathway to development of MAIT cells bifurcates based on the expression of transcription factor Tbet or RORyt. The sublineages at this stage are the IFN-γ-producing T-bet^+ (MAIT-1) sub-lineage or the IL-17A-producing RORγt^+ (MAIT-17) (Koay et al., 2016; Rahimpour et al., 2015). A study from 2023 claims that TAK1 which is a member of the mitogen-activated protein (MAP) kinase kinase kinase determines MAIT1 and MAIT17 sublineage polarization (Krevh et al., 2023). Another study hints that MicroRNA-155 is the key regulator of MAIT1 and MAIT17 cell differentiation (T. Liu et al., 2021). The molecular pathway involved in fate decision-making requires future studies.

In humans, three similar stages of MAIT cell development can be identified using different cell-surface markers. Stage 1 is defined as $CD3^{+}MR1\text{-}tet^{+}CD27^{-}CD161^{-}$, stage 2 as $CD3^{+}MR1\text{-}tet^{+}CD27^{+}CD161^{-}$, and stage 3 as $CD3^{+}MR1\text{-}tet^{+}CD27^{+}CD161^{+}$. There are a few common features that MAIT cell development passage has in humans and mice: stage 1 cells are present only in the thymus, stage 2 cells are present at low frequency in the periphery, and the transition from stage 2 to stage 3 involves upregulation of PLZF and IL-18R.

MAIT cells undergo further maturation and expansion after leaving the thymus. MAIT cells expand gradually after leaving the thymus, peaking around early adulthood and then gradually decreasing so that by age 70, they usually represent less than 1% of T cells (Koay et al., 2016). Peripheral maturation of MAIT cells involves transitioning from the expression of CD8αβ to CD8αα and a marked increase in cytokine-producing potential. The drivers of peripheral maturation, expansion, and homeostasis of MAIT cells remain unknown (Koay et al., 2016; Leeansyah et al., 2014).

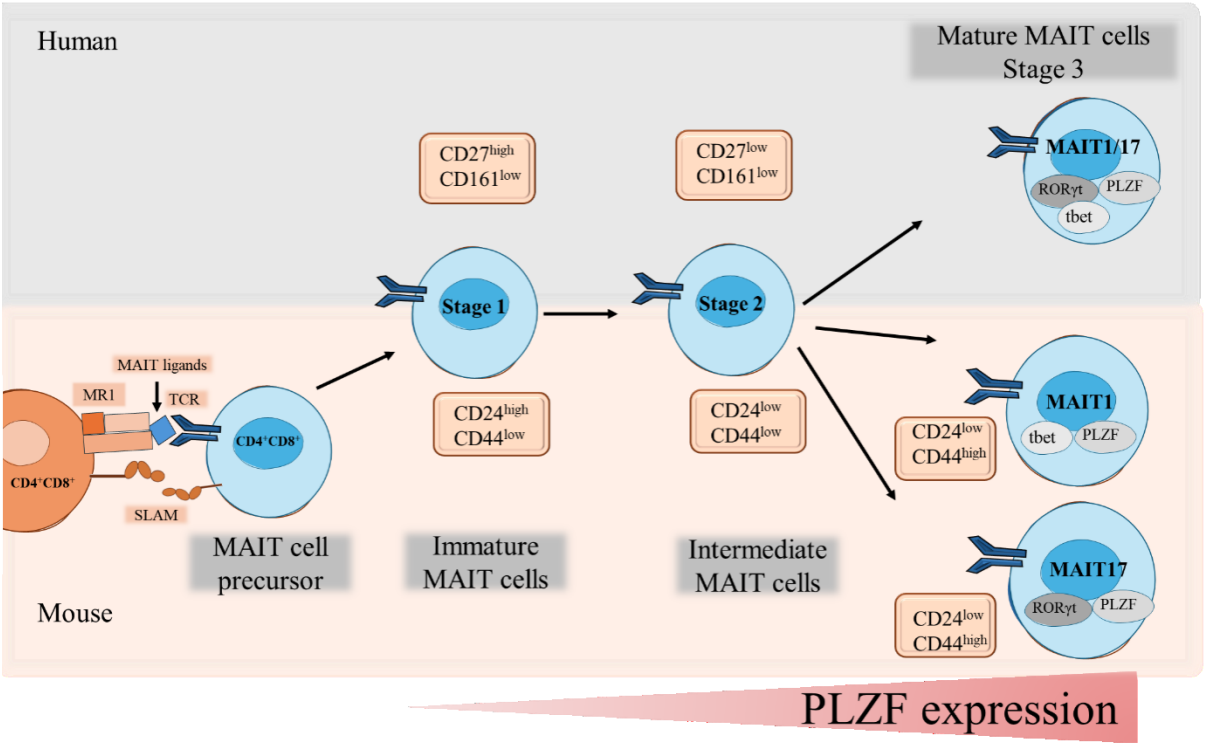


Figure 7. MAIT cell development in mouse (bottom) and human (top) thymus. Three stage development steps that MAIT cells undergo in the thymus are common in humans and mice. MAIT cell precursor is activated by MAIT ligand presented by $CD4^{+}CD8^{+}$ thymocytes via their TCR along with SLAM-SLAM interactions. There is a progressive expression of the PLZF transcription factor throughout MAIT cell development. Scheme adapted from (Pellicci, Koay, and Berzins 2020).

3.4 MAIT cell ligands

Unlike classical T cells, the TCR of MAIT cells recognizes non-peptidic antigens in the context of MR1.

a) Classical MAIT cell ligands

MAIT cells in mice and humans can respond to diverse bacterial and yeast species in an MR1-dependent manner (Le Bourhis et al., 2010) indicating that MAIT cells are capable of sensing antigens common to a diverse range. In 2012, the first MR1 ligands of non-peptidic nature were identified (Kjer-Nielsen et al., 2012) by studying microbial biosynthesis of vitamins. Riboflavin is an essential biosynthesis pathway for the metabolism of living organisms. Riboflavin is produced as a result of sequential steps of enzymatic reactions wherein GTP is processed into the intermediate 5-A-RU (5-amino-6-D-ribitylaminouracil) with the help of ribA and ribD enzymes **(Figure 8)**. The ligands identified by Kjer-Nielsen and team included 6-formyl pterin (6-FP) (a metabolite of vitamin B9 also known as folic acid) and several ribityllumazines and pyrimidine-based intermediates (Corbett et al., 2014). While 6-FP and its analog acetyl-6-FP bind to MR1 and increase its surface expression, they do not trigger MAIT-TCR signaling. However, genetic experiments have shown that deleting the key enzymes in the riboflavin pathway like ribD stops MAIT cell responses to *Lactococcus lactis*, *Salmonella enterica* serovar Typhimurium, *Escherichia coli* (Kurioka et al., 2018; Tastan et al., 2018). These studies identified 5-A-RU as a crucial intermediate in riboflavin biosynthesis of microbial and fungal species, necessary for producing lumazine- and pyrimidine-based antigens. Pyrimidine antigens are formed through the non-enzymatic condensation of 5-A-RU with methylglyoxal or glyoxal, resulting in 5-(2-oxopropylideneamino)- 6-d-ribitylaminouracil (5-OP-RU) and 5-OE-RU 5-(2-oxoethylideneamino)-6-d-ribitylaminouracil (5-OE-RU), respectively (Corbett et al., 2014; Kjer-Nielsen et al., 2012). Unless bound to MR1, these pyrimidine molecules are highly unstable with the ability to spontaneously cyclize into lumazines. These ligands were the first known agonists of MAIT cells.

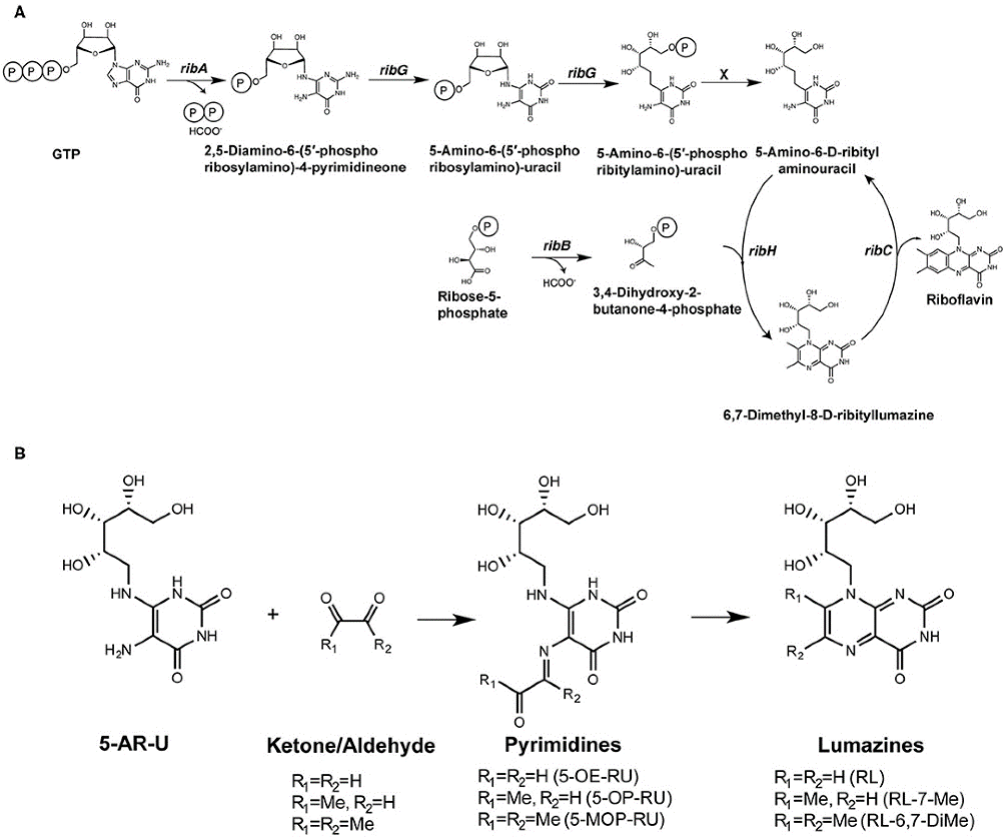


Figure 8 . Riboflavin and riboflavin derived MAIT ligands synthesis pathway. A. Riboflavin synthesis pathway. **B.** MAIT ligands formation from spontaneous reaction of the intermediary metabolite 5-A-RU and a small dicarbonyl molecule. Scheme from A. Corbett et al. 2020.

b) Non-classical ligands:

The crystallized ternary structure of MAIT TCR-MR1-Ag allowed a thorough investigation of the MR1 binding pockets (López-Sagaseta et al., 2013b) by several groups. 5-OP-RU and 6-FP are compounds containing pyrimidine heterocycles as scaffolds. Keller et al. investigated other chemical scaffolds from preexisting pharmacological compounds that can bind MR1 *in silico* using virtual docking and identified about 80 modulators with stimulatory or inhibitory effects (Keller et al., 2017). The modulators were further analyzed *in vitro* for their binding capacity to MR1 and MAIT TCR activation/inhibition. The potent modulators identified were diclofenac (a widely used non-steroidal anti-inflammatory drug), an aspirin analog 3-FSA (3-formylsalicylic acid), and 2,4-DA-6FP (2,4-diamino-6-formylpteridine) which is a derivative of the immunomodulatory drug methotrexate. Compared to 5-OP-RU, diclofenac's activation

potency was weaker since 10^7 times more concentrated diclofenac than 5-OP-RU led to MAIT cell activation. Conversely, compounds such as 3-FSA and 2,4-DA-6FP displayed antagonistic behavior by inhibiting the activation of MAIT cells. The reactivity of MAIT cells toward these drugs flagged the possibility of their role in certain drug-related hypersensitivity responses (Keller et al., 2017; Veerapen et al., 2021).

Harriff et al. used mass spectrometry and molecular networking approach to identify MR1 ligands of microbial origin by expressing chimeric soluble human platform MR1 (hpMR1) in insect cells infected with *E.coli* or *M.smegmatis* or without any bacteria (Harriff et al., 2018). They analyzed the molecules eluted from chimeric soluble MR1 during the intracellular infection and found that 6-(2-carboxyethyl)-7-hydroxy-8-ribityllumazine (photolumazine I) and 6-(1H-indol-3-yl)-7-hydroxy-8- ribityllumazine (photolumazine III) can also bind MR1 and activate MAIT cells. The identification of these non-uracil ligands opens the possibility of several unidentified antigens with the potential to modulate MAIT cells.

We know that intrathymic selection of MAIT cells is MR1-dependent (Koay et al., 2016) which also happens in germ-free mice (Treiner et al., 2003) where they display a reduced number of MAIT cells but are not completely absent (Legoux et al., 2019) . This raises the possibility of a non-microbial origin or a self-ligand aiding the selection process of MAIT cells. Recently, Ito et al. 2024 reported that sulfate bile acids can be presented by MR1 to MAIT cells (Ito et al., 2024). They demonstrated that cholic acid 7-sulfate (CA7S) which is biosynthesized by sulfate conjugation of cholic acid led to agonistic effects on MAIT cells. Structurally, CA7S is completely different from the reported MR1 ligands, and functionally, the interaction is as weaker as diclofenac where a 10^5–fold higher concentration than 5-OP-RU resulted in the activation of MAIT cells. Their work also hinted that the transcriptomic profile of MAIT cells stimulated with CA7S or 5-OP-RU is different, where CA7S treatment led to an upregulation of homeostatic genes compared to the pro-inflammatory signatures in the cells with 5-OP-RU treatment. There is also evidence that MR1 binds to some tumor-associated antigens as well as to non-riboflavin-based antigens which are presented to an atypical subset of MR1-restricted T cells that are not MAIT cells (Crowther et al., 2020; Lepore et al., 2017; Meermeier et al., 2016). The MR1 binding groove pocket displays plasticity to accommodate a range of ligands that are still not documented. Thus, it seems likely that the diverse list of MR1 ligands will be appreciated for understanding the immunological importance of MAIT cells in the future.

3.5 Distribution of MAIT cells

MAIT cells are abundant in humans but they are present in lower frequencies in common laboratory murine models (Koay, Su, et al., 2019; Rahimpour et al., 2015). In this section, I will summarize the distribution of MAIT cells in the tissues of both organisms in parallel.

MAIT cells develop primarily in the thymus accounting for about 0.16% in humans and even fewer (0.01%) in mice. These frequencies in the thymus are primarily a consequence of microbiota exposure as there is a significantly reduced MAIT cell number in the germ-free mice (Constantinides & Belkaid, 2021). The intrinsic properties of MAIT cells seem to be a key factor for determining their tissue tropism. Indeed, thymic MAIT cells express the *Runx3*-related transcription factor which imprints the tissue-resident memory signatures of conventional CD8+ T cells (Milner et al., 2017). Furthermore, tissue localization correlates with established effector functions in the thymus under the regulation of PLZF expression (Thomas et al., 2011). PLZF expression in conventional T cells induces a tissue-resident profile, indicating PLZF's regulatory role over MAIT residency.

MAIT cells preferentially reside in non-lymphoid organs post-thymus exit (Salou et al., 2019a) and undergo further modifications in the peripheral tissues. For instance, human MAIT cells initially lack the memory marker CD45RO but acquire it in peripheral tissues (Youssef et al., 2018). In adults, MAIT cells may constitute up to 10% of circulating T cells and 45% in certain peripheral tissues, such as the liver. They are also abundant in mucosal barriers such as in the lung which is discussed in detail in the ‘MAIT cells are the mucosal barrier’ section. In humans, MAIT cells are prevalent in organs like the liver (20-50%), lungs (2-4%), skin (2-7%), and intestine (10%) but less so in the lymphoid organs like the spleen (1%) and lymph nodes (<0.2%) (Rahimpour et al., 2015; Salou et al., 2019b).

Additionally, a dominance of RORγt expressing subsets of MAIT cells is observed in murine models in different organs. They may represent upto 60% in the lungs and nearly all of them express RORγt in the colon lamina propria **(Figure 9)**.

Within the identical tissue, subsets of MAIT cells demonstrate varying localization; for instance, within the pulmonary system, MAIT1 cells are found within the vascular compartment, whereas MAIT17 cells are located outside the vascular system. This distinction suggests that the T-bet and RORγt transcriptional programs significantly affect the tissue tropism of MAIT cells (Legoux et al., 2020b; Salou et al., 2019b).

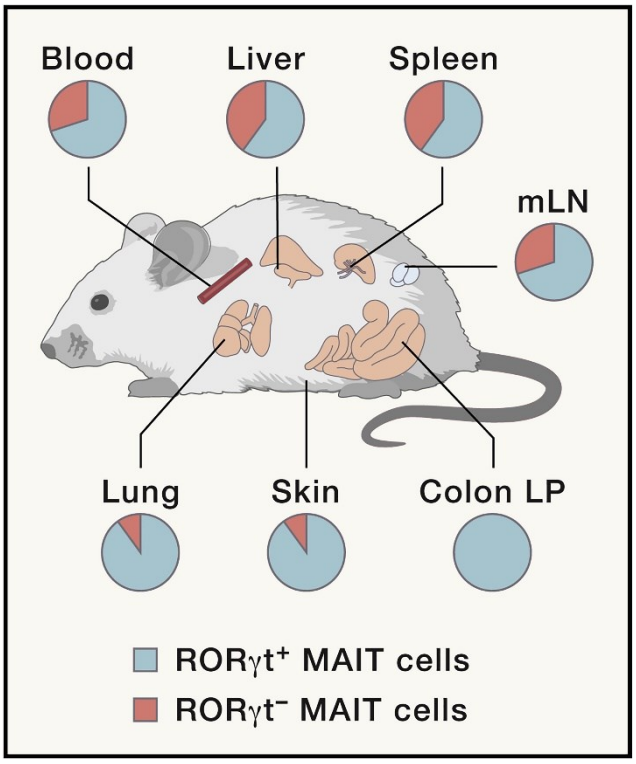


Figure 9. Percentages of MAIT1 (in red) and MAIT17 (in blue) cells among total MAIT cells in different organs. Scheme from (Legoux et al., 2020).

3.6 Tools to study

The identification of MAIT cells relied on the combination of antibodies targeting CD3, TRAV1-2, and CD161 (in the case of humans) until recently when 5-OP-RU-loaded MR1 tetramers were developed (Reantragoon et al., 2013). The comprehensive study on the 'normal' laboratory mice using MR1 tetramers concluded that the distribution of MAIT cells in mice is 10-100 fold less abundant than in humans (Cui et al., 2015a; Rahimpour et al., 2015; Tilloy et al., 1999). To overcome this hurdle, $TRAV1^{+}TRAJ33^{+}$ and MR1-knockout mice were introduced (Croxford et al., 2006; Kawachi et al., 2006). However, the MAIT cells in this $TRAV1^{+}TRAJ33^{+}$ TCR transgenic model did not mimic the features of MAIT cells in non-transgenic or human MAIT cells. The MAIT cells in this transgenic model have a naïve phenotype, did not express PLZF transcription factor, and are not MR1-dependent in contrast to naturally occurring MAIT cells (Rahimpour et al., 2015; Sakala et al., 2015). Lantz and his team screened several strains of mice and found that CAST/EiJ mice generated 10 times more

MAIT cells than does the C57BL/6J mice (Cui et al., 2015a). The progenies after crossing CAST/EiJ mice with C57BL/6J mice crossed for over 10 generations, and the B6-MAIT^{CAST} congenic strain was generated that revolutionalized the approach to study MAIT cells. The MAIT cells in these congenic mice are PLZF+ and MR1-dependent. For our studies on MAIT cells, we only use these B6-MAIT^{CAST} congenic mice unless mentioned. Koay et al. generated TRAJ33-knockout mice in which MAIT cells are absent yet retain MR1 expression (Koay, Gherardin, et al., 2019). To study the mobilization of MAIT cells, many teams utilize in vivo stimulation of MAIT cells with 5-OP-RU (Z. Chen et al., 2017a) or with bacteria such as *Salmonella* Typhimurium (Z. Chen et al., 2017a), *Legionella longbeachae* (H. Wang et al., 2018) and *Francisella tularensis* (Meierovics et al., 2013a) and with *Escherichia coli* (Soudais et al., 2015). In addition, intranasal stimulation with of 5-OP-RU in combination with lipopolysaccharide (LPS) can also lead to pulmonary expansion of MAIT cells (López-Rodríguez et al., 2023). Lantz's team recently generated a conditional MR1 Tg mouse (Lox-STOP-Lox-MR1) for Cre-mediated overexpression of MR1 (Morr et al., 2024). This model will be useful for understanding the strength of TCR signaling in the future.

3.7 Activation of MAIT cells

MAIT cells are innately active as soon as they leave the thymus (Koay et al., 2016). MAIT cells undergo further maturation and expansion as a result of the cues they receive from their surroundings. These cues could be either the cognate ligands that interact directly with the MAIT TCR (TCR-dependent activation) or inflammatory stimuli in the absence of TCR-mediated antigen recognition.

a) TCR-dependent activation

As described above, MAIT cells do not recognize traditional peptides like conventional T cells. The activation of MAIT cells through TCR requires the presentation of vitamin metabolites by MR1. The vitamin metabolites are derived as a result of the riboflavin pathway that nearly 80% of known bacteria and fungi undergo (Boudinot et al., 2016b). Given the scrutinizing process of the antigen presentation (as described in the 'MAIT cell ligand' section of this manuscript), there are very limited ligands that form a successful 'interaction triad' (Awad et al., 2020). 5-OP-RU and 5-OE-RU are two of the few ligands broadly studied for their agonistic effect on MAIT cells. The enzymes of the riboflavin synthesis pathway are grouped in a highly regulated

operon in Gram-positive bacteria. However, these enzymes are dispersed in the genome of Gram-negative bacteria meaning that there may be subtle differences in the nature or the abundance of the intermediary compounds that could be generated. Several studies have demonstrated that MR1-dependent activation occurs in gram-positive and gram-negative bacteria (Meermeier et al., 2018). Moreover, i*n vitro* and *in vivo* experiments of various *Streptococcus pneumonia* clinical isolates indicated that the genetic differences in riboflavin metabolism among these isolates determine the response generated by MAIT cells (Hartmann et al., 2018). Bacteria that have been shown to induce MR1-dependent MAIT cell activation due to the presence of the riboflavin pathway are *Mycobacteria* (Le Bourhis et al., 2010), *Clostridiodes* (Marquardt et al., 2021), *Staphylococcus* (Cooper et al., 2022), *Escherichia* (Le Bourhis et al., 2010), *Salmonella* (Howson et al., 2018) species, *Proteus mirabilis* and *Klebsiella oxytoca* (Constantinides & Belkaid, 2021). On the other hand, some bacteria that do not lead to MAIT cell activation like *Listeria monocytogenes* (Gold et al., 2010), *Enterococcus faecalis,* and *Streptococcus* Group A (Le Bourhis et al., 2010).

There is limited literature on the fungal species that act as stimuli for MAIT cells. The species that have been described to activate MAIT cells are Candida albicans, Candida smoothie, Mucorales, and Aspergillus (Böttcher et al., 2021; Jahreis et al., 2018a, 2020).

b) TCR-independent activation of MAIT cells

Along with TCR, MAIT cells also express several cytokine receptors. And like NKT cells, they can be triggered upon exposure to different cytokines. Ussher et al. demonstrated that MAIT cells can be activated with the help of IL-18 and IL-12 stimulation (Ussher et al., 2014). Other cytokines that have been described to trigger MAIT cells are IL-7, IL-15, type-I Interferons, and IL-23 (Hinks & Zhang, 2020; López-Rodríguez et al., 2023; Rahimpour et al., 2015; Slichter et al., 2016). Due to the presence of one or in combination of these cytokine receptors, MAIT cells' anti-microbial functions extensively range from non-riboflavin pathway-dependent bacteria and fungi to viruses (Van Wilgenburg et al., 2016; Wilgenburg et al., 2018). The synergistic effect of different combinations of these cytokines can have a preferential effect. For example, in the case of IL-15 in synergy with either IL-12 or IL-18 triggers IFN-y production by MAIT cells (Van Wilgenburg et al., 2016) or IL-1 combined with IL-7 can polarize MAIT cells to produce IL-17A (Turtle et al., 2011). Along with activation cues, IL-23 has been shown to promote the expansion of MAIT cells (H. Wang et al., 2019a). This implies that cytokine signaling is more effective than TCR-dependent signaling in delivering a potent

MAIT cell capable of performing effector functions post-activation as well as for expansion **(Figure 10)**.

TCR-dependent and -independent pathways act together to achieve complete MAIT cell activation. Upon stimulation, MAIT cells significantly upregulate the expression of activation markers CD69 and CD25 (Le Bourhis et al., 2010). Additionally, the secretion of cytolytic molecules, including granzyme B and perforin, is markedly enhanced, which likely contributes to the MAIT cells' ability to eliminate infected cells (Dusseaux et al., 2011a; Kurioka et al., 2015; Leeansyah et al., 2015).

Following stimulation, MAIT cells also produce a range of pro-inflammatory cytokines, such as IL-17, IFN-γ, TNF, and granulocyte-monocyte colony-stimulating factor (GM-CSF) (Dias et al., 2016; Hackstein & Klenerman, 2023; López-Rodríguez et al., 2023). These cytokines are thought to bolster the anti-microbial response by acting on myeloid cells (Dusseaux et al., 2011a; Hinks & Zhang, 2020; Le Bourhis et al., 2010). Furthermore, there is an increased secretion of chemokines like CCL3, CCL4, XCL1, and CXCL16, indicating that MAIT cells may play a pivotal role in orchestrating the broader immune response (Legoux et al., 2020b).

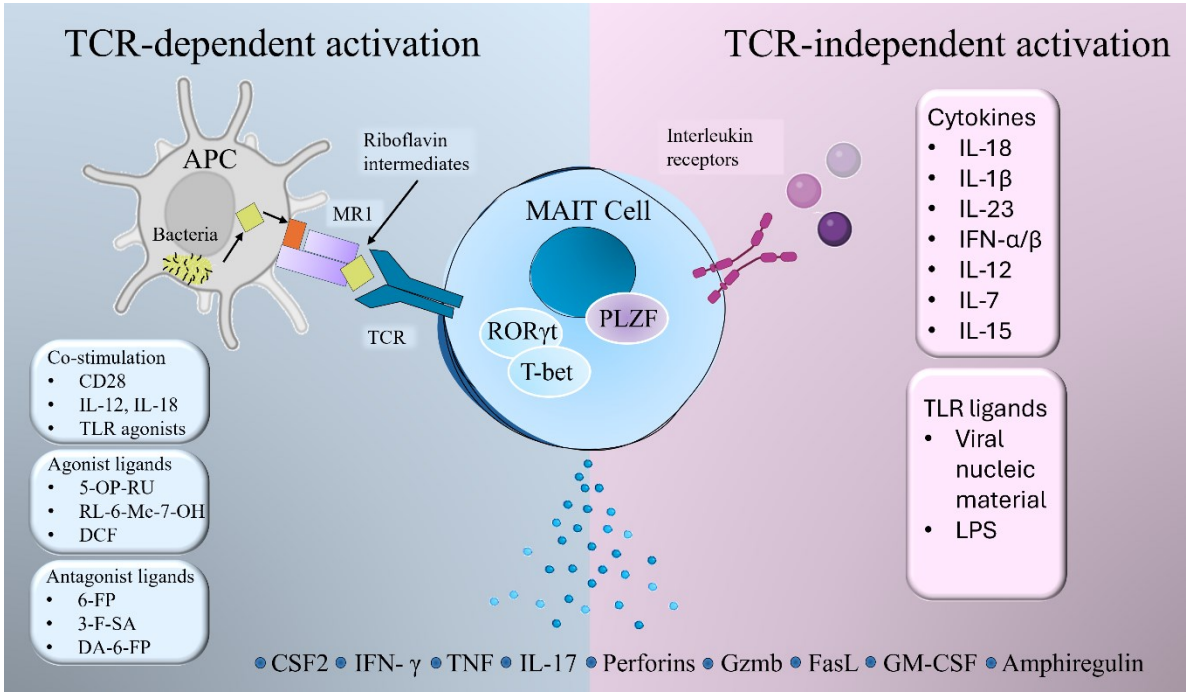


Figure 10. MAIT cell activation. Left, TCR-dependent MAIT cell activation involving MR1-mediated presentation of riboflavin derivatives in conjunction with co-stimulation. Co-stimulation may include CD28 and/or cytokines such as IL-12, and IL-18, and can be induced by danger signals including TLR agonists detected by APCs that enhance MR1 presentation (top). Examples of MR1-ligands are agonists (middle) or non-agonists (bottom) for MAIT cells. Right, TCR-independent MAIT cell activation involves inflammatory cytokines and TLR ligands such as viral DNA and LPS. In response to the stimuli and transcription factors: RORγt, T-bet, or PLZF, MAIT cells release cytokines like IL-17, TNF, and factors like GM-CSF, and Amphiregulin. 3-F-SA, 3-formylsalicylic acid; APC, antigen-presenting cell; DCF, diclofenac; RL-6-Me-7-OH, 7-hydoxy-6-methyl-8-D-ribityllumazine; LPS, lipopolysaccharide; IFN, Interferon; Gzmb, Granzyme; TLR, Toll like receptor; IL, Interleukin; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor.

3.8 MAIT cell subsets

The MAIT cell subsets are defined by their distinct functional profiles, particularly based on the transcription factors they express and the types of cytokines they produce. The three main subsets of MAIT cells—MAIT0, MAIT1, and MAIT17—are distinguished by their roles in immune responses and their developmental pathways.

a) MAIT0 Cells

MAIT0 cells are considered a "naive" or uncommitted subset of MAIT cells, often regarded as an intermediate population that can differentiate into MAIT1 or MAIT17 subsets depending on environmental cues and signaling pathways (Jabeen & Hinks, 2023a). These cells generally have not yet fully committed to expressing either the T-bet (TBX21) transcription factor associated with the MAIT1 subset or the RORγt (RORC) transcription factor associated with MAIT17 cells. They often express PLZF (promyelocytic leukemia zinc finger), a transcription factor important for MAIT cell development and tissue homing (Pellicci et al., 2020). MAIT0 cells are less functionally specialized than MAIT1 or MAIT17 cells and have limited cytokine-producing capabilities. MAIT0 cells are typically at the earlier developmental stages and can further differentiate based on external signals, such as exposure to specific microbial antigens or cytokines.

b) MAIT1 Cells (T-bet+ MAIT Cells)

MAIT1 cells are the Th1-like subset of MAIT cells that are involved in antiviral and antibacterial responses by producing pro-inflammatory cytokines, particularly IFN-γ . MAIT1 cells are defined by high expression of T-bet (TBX21), a transcription factor crucial for Th1-type immune responses like the production of Th1 cytokines and support cell-mediated immune functions. IFNγ is the hallmark cytokine produced by MAIT1 cells, which plays a crucial role in activating macrophages and promoting the clearance of intracellular pathogens like viruses and bacteria (Lamichhane et al., 2019; López-Rodríguez et al., 2023; Souter et al., 2019). Another important cytokine produced by MAIT1 cells is TNF-α which contributes to inflammation and immune responses against infections. They may also produce granzyme B to induce apoptosis in infected cells.

They are critical for host defense against intracellular pathogens, including viruses (such as Influenza or SARS-CoV-2) and bacteria (like Mycobacterium tuberculosis). These cells can

get activated in the presence of IL-12 and IL-18, which are often secreted in response to infections.

c) MAIT17 Cells (RORγt+ MAIT Cells)

MAIT17 cells are the Th17-like subset of MAIT cells, primarily involved in responses against extracellular bacteria and fungi, and associated with the production of IL-17A (Pisarska et al., 2020). MAIT17 cells express high levels of RORγt (RORC), a transcription factor essential for Th17-type immune responses. RORγt promotes the production of IL-17A and other Th17-associated cytokines. IL-17A is the signature cytokine of MAIT17 cells, driving inflammation and recruiting neutrophils to sites of infection, which is especially important for controlling extracellular pathogens (Sobkowiak et al., 2019; Souter et al., 2019). MAIT17 cells can also produce IL-22 which plays a role in maintaining mucosal barriers and protecting against bacterial infections. MAIT17 cells have also been reported to produce GM-CSF that contributes to immune cell recruitment and activation .

MAIT17 cells are primarily involved in mucosal immunity and are important for defending against extracellular bacteria and fungi. They are important in maintaining barrier immunity, particularly in the gut and other mucosal surfaces (Germain et al., 2025b; Nel et al., 2021; Sobkowiak et al., 2019). Differentiation into MAIT17 cells is driven by RORγt expression and exposure to cytokines like IL-23 and IL-6. Microbial exposure, especially to bacterial-derived antigens presented by MR1, influences the shift toward the MAIT17 lineage. **MAIT0 to MAIT1** transition is driven by factors such as T-bet expression and exposure to IL-12 and IL-18 resulting in enhanced Th1-like response.

MAIT0 to MAIT17 transition is regulated by RORγt and involves exposure to IL-23 and IL-6 and boosts mucosal immunity (Kurioka et al., 2017).

Besides transcription factors, surface expression of CD8 and CD4 has also been widely studied to track pathogen-driven immune response. For example, in mice, MAIT cells populations include $CD4^{+}CD8^{-}$, $CD4^{-}CD8^{-}$, $CD8\alpha^{+}CD8\beta^{-}$ and $CD8\alpha^{+}CD8\beta^{+}$, and in common laboratory C57BL/6 mice, $CD4^{-}CD8^{-}$ MAIT cells are the main population in peripheral lymphoid organs, while in BALB/c mice, there is a higher frequency of $CD4^{-}CD8^{+}$ MAIT cells (Rahimpour et al., 2015).

Recent studies demonstrate that mucosal-associated invariant T (MAIT) cells can co-express both T-bet (TBX21) and RORγt (RORC), especially in humans and non-rodent species (Bugaut

et al., 2024; Loh et al., 2024). This dual expression suggests that MAIT cells possess functional plasticity, allowing them to mediate both Th1-type (IFN-γ production) and Th17-type (IL-17A production) immune responses. While in humans, sheep, and cattle, MAIT cells frequently co-express these transcription factors, in rodents, T-bet and RORγt are generally restricted to distinct subpopulations (Bugaut et al., 2024). This functional diversity contributes to their broad immune response capabilities (Loh et al., 2024).

3.9 MAIT cells: infection and diseases

As mentioned above, MAIT cells can respond to various pathogens with or without MR1 presentation.

a) Bacterial infection

Many bacterial species with or without intact riboflavin have been shown to activate MAIT cells. The accumulation of MAIT cells at the site of infection at the early stages of infection suggests that they are rapidly recruited (Z. Chen et al., 2017b; Constantinides & Belkaid, 2021; Cui et al., 2015b; Meierovics et al., 2013b; H. Wang et al., 2018). There are indications that MAIT cell recruitment occurs from the peripheral blood to the lung since a reduced number of MAIT cells in the peripheral blood was observed in the case of pulmonary infection with *Salmonella* Typhimurium and *Francisella tularensis* (Z. Chen et al., 2017b; Meierovics et al., 2013a). Some clinical studies with human samples support this theory of recruitment from the periphery as well (Grimaldi et al., 2014; Loh et al., 2016). However, in the case of acute pulmonary *L. longbeachae* infection, MAIT cells were observed to proliferate locally—both in the tissue and the dLN (H. Wang et al., 2018). It was reported by Constantinides *et al.* that upon infection with *Staphylococcus epidermidis*, there was an expansion of skin MAIT cells even in the absence of lymph nodes (Constantinides & Belkaid, 2021). These two different dynamics of MAIT cells can be associated with factors such as the dose and nature of the pathogen, the cellular composition of the infection site itself, the response of APCs in MR1 presentation or not, and the cytokine cues they receive.

Subsequent studies in mice have revealed that MAIT cells display a tendency to provide protection against bacterial pathogens as seen in the case of *Klebsiella pneumoniae*, *F. tularensis*, *E. coli*, *L. longbeachae,* and *Mycobacterium bovis* (Chua et al., 2012; Georgel et al.,

2011; López-Rodríguez et al., 2023; H. Wang et al., 2018). Interestingly, MAIT cells do not seem to be essential for the control of infection with *E. coli, Shigella dysenteriea,* and *Yersinia enterocolitica* (Georgel et al., 2011). A human subject with no detectable MAIT cells due to a homozygous mutation of MR1 was reported to have a history of severe bacterial pneumonia and refractory *Campylobacter* gastroenteritis (Howson et al., 2020). In line with this, recurrent skin and lung infections caused by *Staphylococcus aureus* and by the yeast *Candida albicans* were found in Signal transducer and activator of transcription (STAT)3-deficient individuals that reportedly have partial loss of MAIT cells resulting in lower IL-17 (Béziat et al., 2018; R. P. Wilson et al., 2015).

The anti-bacterial functions are primarily due to the effector functions of MAIT cells upon encounter by the production of IL17A, IFN-γ, and TNF which could be MR1 dependent or independent response or the combination of the two stimuli. Other immune components are modulated as a result of MAIT cell activation. For example, during *F. tularensis* infection, MAIT cells promoted the differentiation of monocyte-derived DCs that supported the priming of recruited more protective CD4 T cells (Meierovics et al., 2013b).

Conversely, in the case of *Helicobacter pylori* infection in mice, MAIT cell recruitment and IL-17A production augmented the gastric pathology compared to the MAIT-deficient (D'Souza et al., 2018) animals. In another case, a short nucleotide polymorphism (SNP) of MR1 conferring increased susceptibility to *Mycobacterium tuberculosis* was identified in a case-population genetic study of active tuberculosis in Vietnamese adults (Seshadri et al., 2017).

b) Fungal infection

Along with bacteria, several fungal species depend on riboflavin biosynthesis that results in production of MAIT cell precursors. MAIT cell activation in vitro and in vivo has been demonstrated by *Candida albicans, C. glabrata*, *Saccharomyces cerevisiae*, and *Aspergillus fumigatus* (Gold et al., 2010; Hernández-Santos et al., 2018; Jahreis et al., 2018b; Le Bourhis et al., 2010). IL-17A production by MAIT cells. Hernández-Santos *et al.* reported a mild increase of IL-17A^{+} MAIT cells in murine pulmonary infection with *Blastomyces dermatitidis* (Hernández-Santos et al., 2018). However, to date, no studies demonstrate the role of MAIT cells in the control of fungal infection. Many pulmonary diseases such as COVID-19 could lead to a secondary fungal infection (Raffaelli et al., 2022). At the same time, MAIT cells tend

to persist at the infection sites after the resolution of the pathogen (H. Yu et al., 2020a). Together, these studies hint that MAIT cells can be targeted to lead protective immunity against fungal pathogens by IL-17A production.

c) Viral infection

Early studies reported that MAIT cells were unresponsive to viral infection (Gold et al., 2010; Le Bourhis et al., 2010) which made sense when it was revealed that MAIT cells respond to riboflavin metabolites (Corbett et al., 2014; Kjer-Nielsen et al., 2012). It was found in the subsequent years that both, murine and human MAIT cells can be triggered by cytokines such as IL-18, IL-12, IL-15, IL-1, IL-7, type I interferons, IL-23 that are released majorly by myeloid cells in response to viral infection as they bear the corresponding receptors (Ussher et al., 2018). Many viral infections lead to MAIT cell activation (*IL-18 Dependent MAIT Cell Activation in COVID19*, n.d.; Loh et al., 2016; Lu et al., 2020a; Ussher et al., 2018; Van Wilgenburg et al., 2016; Wilgenburg et al., 2018). However, there are a limited number of studies describing their anti-viral role. A study showed that MAIT-deficient mice exhibited higher mortality and a significant reduction in body weight following a lethal respiratory Influenza virus infection. Furthermore, these defects were rescued upon adoptive transfer of MAIT cells in MAIT-deficient mice (Wilgenburg et al., 2018). They demonstrated that MAIT cells confer protection against Influenza by producing IFN-γ. Another study with an HIV model in macaques showed that MAIT cell frequency correlated with lower viral loads (Murugesan et al., 2020). MAIT cell frequency and ability to produce cytokines were also found to co-relate with SARS-COV-2 disease severity (Flament et al., 2021) .

In contrast, IL-17-producing MAIT cells in the broncho-alveolar lavage fluids were attributed to disease severity in children with community-acquired pneumonia (Lu et al., 2020b).

d) Response to microbiota

A reduced number of MAIT cells in germ-free mice directly implies that there is a crosstalk between MAIT cells and microbiota (Martin et al., 2009; Treiner et al., 2003). In other words, MAIT cells are maintained by recognizing the commensal microbiota thus, contributing to immune protection at mucosal sites. The presence of MAIT cells has been linked to the integrity of the gut, reduced dissemination of gut microbes, and improved progress of diabetes in the nonobese diabetic (NOD) mouse model (Rouxel et al., 2017a). Recently, it was shown that microbial diversity might affect MAIT cell activation via riboflavin utilization by increasing microbial acid stress impairing riboflavin availability by increasing the riboflavin demand

(Krause et al., 2020). Another study demonstrated that MAIT cells shape microbiota composition as the altered microbiota in MAIT-deficient mice confers resistance to *Clostridium difficile* infection (A. D. Smith et al., 2019). Though there are no conclusive studies, recent work from El Morr et al. highlighted a key role played by MAIT cells in colitis by inducing tissue-repair functions (Morr et al., 2024). These studies open the door for exploring the role of MAIT cells in diseases with dysbiosis of the gastrointestinal microbiome like inflammatory bowel disease (IBD).

e) Sterile inflammation

MAIT Cells in Diabetes

Type 1 Diabetes (T1D): MAIT cells may contribute to the pathogenesis of T1D due to their pro-inflammatory functions, particularly via IFN-γ and TNF-α production (Petersone & Walker, 2017). Studies have shown an increase in MAIT cell frequency and activation in patients with T1D, leading to an exacerbation of pancreatic β-cell destruction. MAIT cells' interaction with innate immune signals, cytokines, and microbiota-derived metabolites could accelerate immune-mediated damage in T1D. In non-obese diabetic mice, a significant reduction in MAIT cells was associated with accelerated diabetes onset, suggesting their potential regulatory role (Rouxel et al., 2017b).

Type 2 Diabetes (T2D): T2D is characterized by chronic low-grade inflammation. MAIT cells are found in elevated numbers in the adipose tissue of obese subjects and those with T2D (Toubal et al., 2020). In T2D, MAIT cells exhibit an altered cytokine profile, producing high levels of IFN-γ and TNF-α, which contribute to insulin resistance and adipose inflammation. A study by Magalhaes et al. (2015) demonstrated that individuals with T2D had an increased frequency of circulating MAIT cells, which correlated with poorer metabolic outcomes (Magalhaes et al., 2015). The loss of MAIT cell function in T2D patients is also linked to disrupted microbial homeostasis (Jabeen & Hinks, 2023b; Toubal et al., 2020).

Non-alcoholic Fatty Liver Disease (NAFLD): In NAFLD and NASH, MAIT cells respond to metabolic stress and liver inflammation. Hegde et al. (2018) found that MAIT cell depletion attenuated liver fibrosis in murine models, suggesting that their activation may exacerbate sterile liver inflammation (Hegde et al., 2018).

MAIT Cells in Cancer

MAIT cells exhibit both pro- and anti-tumor roles depending on the tumor type and microenvironment (Yigit et al., 2024). In many cancers, such as colorectal and liver cancers, MAIT cells infiltrate the tumor microenvironment, where their presence has been linked to

either suppression or progression of cancer. The secretion of IFN-γ and TNF-α by MAIT cells supports anti-tumor immunity by enhancing cytotoxic responses from other immune cells like NK and $CD8^+$ T cells. However, in some cancers, MAIT cells may contribute to an immunosuppressive environment. In hepatocellular carcinoma (HCC), for example, MAIT cells have been shown to produce IL-17, promoting a pro-inflammatory milieu that supports tumor growth (Ruf et al., 2023).

Colorectal Cancer: MAIT cells are found in higher numbers within colorectal tumors, and their functional capacity is altered, often switching to a more immunosuppressive role. Dusseaux et al. (2011) reported that MAIT cells in colorectal cancer patients exhibited impaired IFN-γ production, while expressing exhaustion markers like PD-1, thus weakening their ability to mount an effective anti-tumor response (Dusseaux et al., 2011b). The tumor microenvironment may further suppress MAIT cell functions through cytokines like TGF-β and IL-10 (Yigit et al., 2024).

MAIT Cells in Autoimmunity

MAIT cells are also implicated in various autoimmune diseases due to their ability to drive inflammation through cytokine production, particularly IL-17 and IFN-γ. Their dysregulated activation can contribute to chronic inflammation and tissue damage seen in autoimmune conditions such as multiple sclerosis (MS), rheumatoid arthritis (RA), and inflammatory bowel disease (IBD).

Multiple Sclerosis (MS): In MS, MAIT cells accumulate in the central nervous system (CNS), where they may promote neuroinflammation by producing IL-17 and IFN-γ. Studies have shown that MS patients have increased frequencies of circulating MAIT cells, and these cells are recruited to sites of inflammation in the CNS, exacerbating disease progression (De Federicis et al., 2024). Elbourhi et al. (2020) observed that MAIT cells in MS patients were highly activated and produced large amounts of pro-inflammatory cytokines, contributing to neuronal damage (De Federicis et al., 2024; Gazali et al., 2020).

Rheumatoid Arthritis (RA): In RA, MAIT cells are enriched in the synovial fluid of affected joints, where they secrete pro-inflammatory cytokines like IL-17, worsening joint inflammation and contributing to bone and cartilage destruction (Li et al., 2022a). A study by Rao et al. (2016) found that MAIT cells in RA patients were more activated and produced higher levels of IFN-γ, correlating with disease severity (Li et al., 2022b). Moreover, MAIT cells can interact with macrophages and dendritic cells within the synovial tissue, amplifying the inflammatory cascade.

Inflammatory Bowel Disease (IBD): MAIT cells are abundant in the gut and play a crucial role in maintaining intestinal homeostasis. However, in IBD, their function is skewed toward an inflammatory profile. Both Crohn's disease and ulcerative colitis patients show elevated levels of MAIT cells in intestinal tissues, where they produce IL-17 and IFN-γ, contributing to chronic intestinal inflammation (Konecny et al., 2023). A study by Hinks et al. (2015) demonstrated that MAIT cell numbers are reduced in the peripheral blood of IBD patients but increased in inflamed gut tissues, indicating a tissue-specific recruitment and activation (Hinks et al., 2019).

3.10 Factors that affect frequency and distribution of MAIT cells

While pathogenic breach and dysbiosis affect the frequency of MAIT cells in the organs and in the circulation, there are several other factors can affect MAIT cells' number and distribution:

1) Sex

There are a handful of studies focused on understanding the distribution of MAIT cells organ-wise in female and male human subjects. Although there is no definitive proof yet that gender-based bias occurs in the case of MAIT cells' distribution, a contraction of $CD8^+$ MAIT cells along with IFN-I producing plasmacytoid DCs was observed in subjects undergoing gender-affirming hormone therapy with testosterone (Lakshmikanth et al., 2024). Therefore, sex hormones as a determinant for the frequencies of MAIT cells is a possibility that cannot be dismissed. There are several instances where sex hormones were involved in controlling the immune response. For example, studies have shown that male mice have fewer Group 2 innate lymphoid cells (ILC2s) in their lungs compared to females, and this reduction is attributed to the presence of androgens (Laffont et al., 2017). Therefore, it cannot be assumed that the responses that are observed and documented in one sex will be mimicked by the other.

2) Age

The distribution of MAIT cells changes with age in both mice and humans, reflecting differences in their development, maturation, and function. In human neonates, MAIT cells are present at low frequencies in the blood (L. J. Walker et al., 2014). Their numbers are relatively low at birth but increase gradually during early childhood. This suggests that MAIT cells develop postnatally, possibly in response to microbial colonization of mucosal surfaces. MAIT cell numbers continue to increase during childhood and peak in adolescence. This expansion

corresponds with increased microbial exposure and maturation of the immune system. In adults, MAIT cells typically comprise 1-10% of $CD3^+$ T cells in peripheral blood (Nikolich-Žugich, 2008; L. J. Walker et al., 2014). However, the frequency and absolute number of MAIT cells start to decline with age, especially after the age of 40. This decline is believed to be associated with immunosenescence and possibly changes in gut microbiota composition. In individuals over 60, there is a marked reduction in both the frequency and functional activity of MAIT cells. This decline may contribute to increased susceptibility to infections and decreased vaccine responsiveness observed in the elderly population.

Similar to humans, neonatal mice have a very low number of MAIT cells, primarily due to their limited exposure to environmental microbes. MAIT cell numbers increase as the mice grow and are exposed to more environmental antigens. This increase continues into young adulthood, with a significant presence in tissues such as the liver and lungs. Older mice (over 1 year) show a decline in MAIT cell frequency, especially in the liver and lungs. The functional capacity of MAIT cells, such as cytokine production and proliferative response, also decreases with age (L. J. Walker et al., 2014).

The mice used in the experiments involving infection and immunization protocols in this manuscript are between 8 weeks and 13 weeks of age. However, as mentioned above, neonatal mice and older subjects have a lower number of MAIT cells. Other immune cells in the body also fluctuate with age. The overall impact of age-related changes including environmental factors such as lifestyle can be a huge factor in the course of MAIT cells' functioning throughout life. Moreover, immunosenescence which are age-related change in the immune system, such as reduced proliferative capacity and altered cytokine profiles, affects MAIT cell function and distribution as mentioned in detail previously.

3) Thymic Output

Thymic output decreases with age, which may limit the replenishment of MAIT cells and contribute to their decline in the elderly. While carrying out experiments related to thymic expansion of MAIT cells for adoptive transfer, I repeatedly observed that thymus from younger mice (4-6 weeks) resulted in more MAIT cells at the end of the culture compared to older mice.

3.11 MAIT cells at the mucosal barrier and the lymphoid connection

As mentioned before, MAIT cells gained their name due to primarily localization at the mucosal sites. Mucosa is the soft tissue that lines the body's canals and organs in the digestive, reproductive, and respiratory systems. The mucosal sites where MAIT cells have been broadly studied are:

1) Digestive system

In the gut, MAIT cells are primarily localized in the intestinal lamina propria and intraepithelial compartments (P. M. Smith & Garrett, 2011). MAIT cells play a crucial role in the gut immune response by recognizing microbial antigens, producing cytokines, mediating cytotoxicity against infected cells, enhancing mucosal defense as well as maintaining tissue homeostasis (Dias et al., 2016; Leng et al., 2019). As mentioned above, the dysbiosis of MAIT cells is associated with several gut related diseases like Inflammatory bowel disease (IBD). A more recent study reported MAIT ligands that are synthesized by aerotolerant bacteria found in the gut lumen (Morr et al., 2024). The interaction of MAIT cells with these ligands leads to the production of anti-inflammatory and tissue-repair mediators, implying their role in reducing intestinal inflammation.

In this study, they also reported that MAIT cells are in an active state within the mesenteric lymph node (draining lymph node of the gut), however, they did not delve into further investigating their functional importance in the lymph node.

2) Reproductive system

MAIT cells are found in the mucosa throughout the female genital tract with a bias towards IL-17 and IL-22 secretion thus providing antimicrobial defense (Gibbs et al., 2017). The dynamics of endometric MAIT cells were also briefly studied in the context of HIV infection, menstrual cycle, and post-menopause (Bister et al., 2021; Gibbs et al., 2022). Another study detailed how MAIT cells play a dual role during pregnancy by inducing tolerance as well as providing anti-microbial defense (Raffetseder et al., 2021). The fact that MAIT cells are present in abundance throughout the female genital tract, unlike other tissues where they display distribution heterogeneity, is intriguing and should attract more groups to investigate their role in this system.

The lymph nodes associated with the female genital tract include inguinal, pelvic, paraaortic, and parametrial nodes (Dabi et al., 2021) that drain different parts of the female reproductive system. Yet, there is a huge literature gap in the studies related to these lymph nodes associated with the female genital tract and MAIT cells.

3) Respiratory system

Within the lung tissue, immune cells are found in all the spaces including the alveoli, the vasculature, and the parenchyma. However, the distribution and localization of MAIT cells within these spaces remain poorly explored. The studies so far suggest that in homeostasis MAIT cells are located primarily in the lung parenchyma in both mice and humans (López-Rodríguez et al., 2023; H. Wang et al., 2018). In fact, MAIT cells are more widely studied in pulmonary infection settings compared to the other systems.

MAIT cells are activated during respiratory infection with bacteria in MR1-dependent and -independent ways. The infection with *Francisella tularensis* and *Legionella longbeachae* leads to MR1-dependent activation of MAIT cells (Meierovics et al., 2013b; H. Wang et al., 2018) that drives IFN-γ production as their major effector cytokine along with GM-CSF that is responsible for the differentiation of monocytes. Ultimately, MAIT cells drive antibacterial functions in these two infection models. In the case of respiratory infection with *Salmonella typhimurium*, the accumulation of MAIT cells and expansion has been reported in both humans and mice, however, these cells are not required for bacterial clearance (Z. Chen et al., 2017b; Raffetseder et al., 2021; H. Wang et al., 2019b). Another group found that respiratory infection with *Klebsiella pneumoniae* leads to the accumulation of MAIT cells with a Th1-transcriptional program in an MR1-independent manner that facilitates the clearance of bacteria (López-Rodríguez et al., 2023). There is a long list of respiratory viruses that lead to the activation of MAIT cells in the absence of MR1 via cytokines such as HIV, Influenza, and COVID-19 (Parrot et al., 2020; Van Wilgenburg et al., 2016; Wilgenburg et al., 2018). MAIT cells provide a protective role in the case of influenza virus strain A/Puerto Rico/8/34/1934 (PR8, H1N1), a conclusion that was based on the observation that infected MAIT deficient mice had impaired survival which was improved by adoptive transfer of pulmonary MAIT cells before infection (Wilgenburg et al., 2018).

While all these studies detailed the role of MAIT cells in the lungs, there is not enough evidence of their role in the mediastinal lymph nodes. In the case of *Mycobacterium tuberculosis*, a prophylactic vaccination of MAIT cells by 5-OP-RU delays bacterial dissemination from

the lungs to the lymph node (Sakai et al., 2021). These findings are crucial since in this particular infection model, the initiation of adaptive immune response relies on antigen production in the lymph node and not in the lung which is significantly boosted by the priming and expansion of MAIT cells (Sakai et al., 2021; A. J. Wolf et al., 2008). A high number of MAIT cells are also found in the lungs and lymph nodes of cattle following endobronchial challenge with *M. bovis* (Edmans et al., 2021).

The lymphatic migration of cells from tissue to the lymph node is an important phenomenon that drives major immune functions in the host upon infection. All the examples mentioned above show that there is a huge lack of knowledge regarding the dynamics of MAIT cells in the lymph nodes. The work of Kastenmueller Wolfgang group highlighted that lymphatic migration of unconventional T cells which is their migration from tissues into their draining lymph nodes plays a big role in fine-tuning the immune cells in such a way that it generates a site-specific immune response (Ataide et al., 2022).

Lymph nodes are key hubs for immune responses enriched with immune cells like T cells, B cells, dendritic cells, and macrophages, making them crucial for pathogen detection, antigen presentation, and immune cell activation. Pathogens that target lymph nodes do so to manipulate or evade the immune response, or to replicate in the rich cellular environment they provide. Tropism of a pathogen in the lymph node can greatly influence the disease progression by dampening the immune responses via different evasion strategies and lead to systemic spread or chronic infection.

AIM OF THE THESIS

In the past fifteen years, numerous investigations have revealed a multitude of roles for unconventional T cells (UTCs), including Natural Killer T (NKT) cells and Mucosal Associated Invariant T (MAIT) cells, in enhancing immune responses during pathological invasions. These roles encompass direct interactions with infected cells through the secretion of antimicrobial peptides, the recruitment of other immune cells to amplify the immune reaction, and the release of factors that sustain homeostasis. Most of these functions have been documented through observations of these UTCs within the primary infection sites, such as the lungs, intestine or skin. UTCs, in conjunction with other immune cells, migrate into the lymphatics into the respective lymph nodes, thereby connecting tissue immunity to their lymph node functionalities. This lymphatic migration represents a critical phenomenon, as it mitigates systemic pathogen spread via the lymphatic pathway by executing their innate capabilities. Consequently, a thorough understanding is only achievable by concurrently examining the dynamics of UTCs within the tissue and their corresponding draining lymph nodes. The overall objective of this thesis is to elucidate the roles that UTCs play within the lymph nodes upon activation and how these roles shape the immune response.

This thesis encompasses three principal objectives:

1) Elucidate the function of NKT cells in orchestrating an immune response across various infection contexts.

The research I present here is an extension of prior investigations conducted by the laboratory, which characterized NKT cells at the intersection of innate and adaptive immunity. My findings in this section contributed to a larger study (now published) that underscored the divergent functions of UTCs (MAIT cells, NKT cells, and Gamma-Delta T cells) across different organs (lungs, intestine, and skin) and their associated draining lymph nodes.

2) Investigate the dynamics of MAIT cells within both the lung and the draining lymph node simultaneously in the context of respiratory infection.

I concurrently examined the activation modalities, functions, and degree of involvement of MAIT cells in the lungs and mediastinal lymph nodes in response to TCR-dependent and TCR-independent stimuli. By addressing the commonalities and distinctions among MAIT cells'

functions in these two organs, I reveal novel roles for MAIT cells in the lymph node that are activated by type-I interferon and triggered by IL-23 and IL-1 signaling to produce IL-17 as a major effector cytokine to mitigate pathogen during respiratory infections. My work in this direction implicates MAIT cells as the primary source of IL-17 in the lymph node for exerting antibacterial functions, a phenomenon describing their function beyond previously described roles in the mucosa.

3) Explore the implications of MAIT cells in adaptive immunity.

Upon recognizing the role of NKT cells in adaptive immunity, I questioned whether MAIT cells, as analogous counterparts to NKT cells (sister cells), would similarly contribute to bridging innate and adaptive immunity. My investigations in this area illuminated the defects in B cell responses resulting from the lack of MAIT cells. Employing various strategies, I set out to unravel the underlying mechanisms by which MAIT cells regulate B cell responses linking innate immunity with adaptive immunity, a phenomenon that has not been previously documented.

RESULTS

CHAPTER 1: Role of NKT cells in host defense

Introduction and background:

NKT cells provide B cell help through cognate and non-cognate interactions, influencing both innate and adaptive humoral immunity, with implications for various diseases.

During infection of mice with *Sphingomonas* NKT cells can provide direct cognate help to B cells (Mattner et al., 2008). Another study from the Batista team demonstrated that NKT cells enhance the adaptive immune response by specific BCR internalization of particulate iNKT antigens (Barral et al., 2008). As mentioned in the introduction of NKT cells, there is a subset of **NKT_{FH} cells** that provides cognate help to B cells by presenting exogenous glycolipids on CD1d (Chang et al., 2011; King et al., 2011). The team of Carola Vinuesa has extensively described the dynamics of Bcl6-dependent NKT_{FH} in providing B-cell help by producing IL-21 (Chang et al., 2011; Vinuesa & Chang, 2013). It was shown in vitro as well as in vivo that sphingolipids results in NKT_{FH} cell expansion, induction of germinal center B cells, and consequently the production of anti-sphingolipid antibodies (Nair et al., 2015). In another study, exposure of NKT cells to *Mycobacterium tuberculosis* antigens resulted in their expression of CXCR5 and release of IL-21 that supported the provision of cognate B cell help for the production of IgG and IgA (C. Wu et al., 2015). With T_{FH}, NKT_{FH} can provide B cell help and induce the production of antibodies to *Clostridium difficile* toxin B in mice (Rampuria et al., 2017).

NKT cells also provide B cell help by enhancing their activation and promoting antibody production without direct antigen recognition as a result of non-cognate interaction. The co-expression of CD1d and MHC class II molecules by DC and CD40 ligand expression by iNKT cells can enhance the production of antibodies specific for the antigen during different infections when α-Gal-Cer is used for priming the immune system (Galli et al., 2007; Ko et al., 2005). NKT cells recruit DCs through CD40/CD40L and cytokines (IFNγ and TNFα) and also rapidly up-regulate the costimulatory molecules CD80/86 and CCR7 on DCs. DCs activate $CD4^+$ T cells through antigen presentation, costimulatory surface molecules, and secreted cytokines and chemokines, such as IL-6, IL-12, and CCL17. T cells activated in this manner

are induced to proliferate and differentiate into Tfh cells to help B cells (Galli et al., 2007). These studies highlight the non-cognate help provided to B cells by NKT cells indirctly.

The previous work done by my supervisor detailed how NKT cells deliver a direct non-cognate help to B cells by producing an early wave of IL-4 to form Germinal centers and further play a role in inducing class-switching of IgG1 Antibody-secreting cells (ASCs) during respiratory infection with Influenza virus (Gaya et al., 2018). Mechanistically, IL-18 derived from $CD169^{+}$ macrophages in the draining lymph node of the lung (mediastinal lymph node) (mLN) triggers IL-4 production by NKT cells in reponse to Influenza virus infection. This early wave of IL-4 is restricted to the periphery of B cell follicles and thus, initiates B cell immunity to infection (Gaya et al., 2018). This section of results is an extension of above mentioned findings of Gaya et al performed by me under his supervision.

Results

1.1 *Activation of NKT cells during viral infection with Influenza and bacterial infection with K.pneumoniae is TCR-independent*

As previously mentioned NKT cells have been reported to respond to different pathogens. We questioned if the TCR usage of NKT would vary in the case of viral versus bacterial triggers. I used Nur77GFP transgenic mouse model to study the role of the TCR of NKT cell in different respiratory immunization settings. The team of Kristin A. Hogquist developed this model to study antigen receptor signaling in vivo called $Nur77^{GFP}$ transgenic mice by inserting a GFP-Cre fusion protein in the start codon of the Nr4a1 (Nur77) gene of a bacterial artificial chromosome (BAC) construct (Moran et al., 2011). Nur77 is an orphan nuclear receptor which is immediately upregulated upon TCR stimulation in thymocytes and T cells (Z. G. Liu et al., 1994). $Nur77^{GFP}$ mice were reported to up-regulate GFP upon antigen receptor stimulation which was proportionate to the strength of TCR stimulation.

I chose 2 x 10^4 cfu of *Klebsiella pneumoniae* 52.145 strain (Kp) for intranasal bacterial infection since it has been shown to produce lipids (H. Huang et al., 2013; Wassef, 1976) but there is no literature on how NKT cells TCR respond to *K.pneumoniae* infection. As a respiratory viral infection model, I used 5 pfu of Influenza virus PR8 (A/Puerto Rico/8/34) strain. As a positive control, I performed an in vivo stimulation with 2µg of α-Gal-Cer.

Based on the previous work mentioned in Gaya et al paper, 3 days post-immunization (dpi) was chosen to assess the GFP in vivo as at this time-point, the activation level of NKT cells in the lymph node peaks. With the help of CD1d loaded tetramer, I was able to identify NKT cells in the lung draining lymph node **(Fig 1A)**. I observed an upregulation of CD69 expression on NKT cells upon immunization with α-Gal-Cer, *K.pneumoniae*, and Influenza compared to the non-immunized (Ni) subjects **(Fig 1B)**. There was a basal level of GFP expression in non-immunized controls **(Fig 1C)**. As expected, more than 60% of NKT cells upregulated GFP in response to a-Gal-Cer immunization indicating an efficient TCR-dependent activation. Surprisingly, we did not observe any upregulation in GFP levels of *K.pneumoniae* infected mice, indicating that the NKT cell activation is independent of CD1d signaling in this infection model. These findings were surprising since *K.pneumoniae*-derived lipids are synthesized during infection (H. Huang et al., 2013; Wassef, 1976) and theoretically should have led to an upregulation of GFP expression. GFP levels in Influenza-infected mice were expected since no Influenza-borne lipids were produced during the infection. These results indicate that during

respiratory infection with *K.pneumoniae* and Influenza, NKT cells are activated in a CD1d-independent manner. The cytokine signaling involved during Influenza infection was investigated in Gaya et al paper. However, we currently do not know the cytokine signaling involved during *K.pneumoniae* infection.

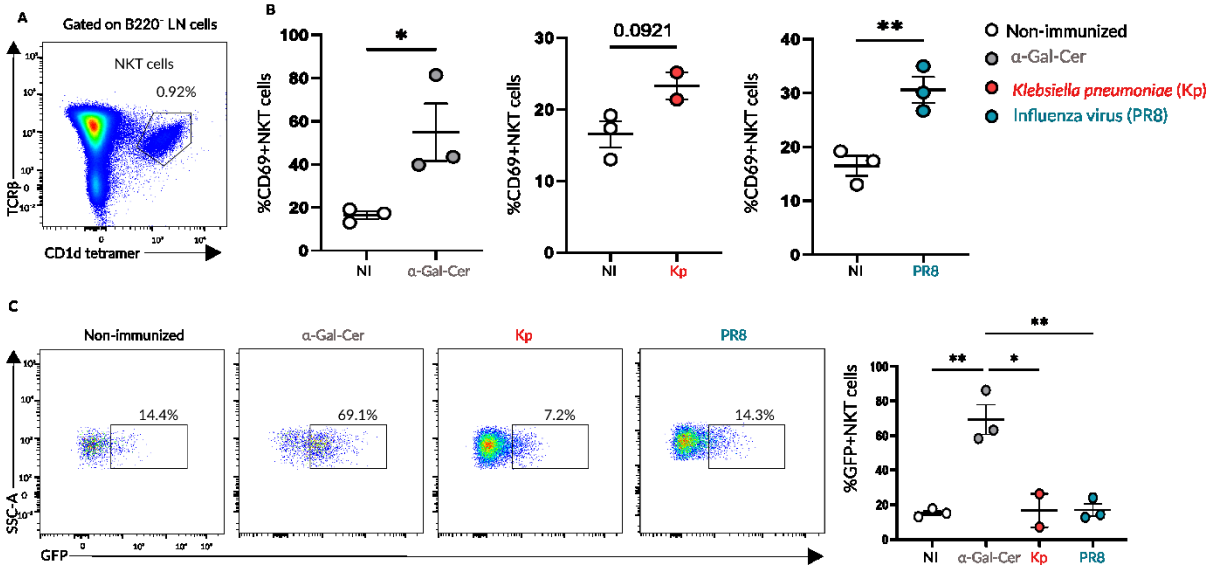


Figure 1. TCR-independent activation of NKT cells upon bacterial and viral infection

(A) Flow cytometry plot showing identification of NKT cells (TCRβ^{+} B220^{-} CD1d-tetramer^{+}) in mediastinal lymph node (mLN) mLN. **(B)** Quantification of the percentage of CD69^{+} NKT cells based on the flow cytometry analysis of mLN of non-immunized (NI), α-Gal-Cer treated, *Klebsiella pneumoniae* or Influenza infected mice after 3 days. **(C)** Flow cytometry analysis of GFP expression 3dpi in mLN of Nur77^{GFP} mice that were non-immunized (Ni), α-Gal-Cer treated, or infected with *Klebsiella pneumoniae* (Kp) or Influenza (PR8). Lines represent mean ± SEM, each dot is a mouse. In figures, asterisks stand for: *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001, and no symbol if the comparison was non-significant.

1.2 Site-specific response generated by NKT cells in skin- and lung-draining lymph nodes

Previous work of the lab showed that NKT cells produce an early wave of IL-4 cytokine upon viral infection (Gaya et al., 2018). To investigate if NKT cells are same capable of producing IL-4 in the draining lymph nodes of different organs, I challenged one group of C57BL/6 mice intranasally and another group of mice subcutaneously with Vaccinia Virus Western reserve strain (VV) with the same dose of infection (2000 pfu) **(Figure 2A)**. I observed significantly higher amount of IL-4 in the mediastinal lymph node (lung draining lymph node) (medLN) compared to the skin-lymph node (sdLN) at day 3 post-infection. In both cases, NKT cells were the major producers of IL-4.

As previously mentioned, IL-4-producing NKT cells are involved in the class-switching of IgG1-producing germinal center (GC) B cells in the mediastinal lymph node following the Influenza virus challenge (Gaya et al., 2018). Consistently, I observed a significant reduction of IgG1-producing germinal center B cells in medLNs after pulmonary VV infection in NKT-deficient (*Cd1d*$^{-/-}$) mice as compared with wild-type (WT) mice at day-9 post-infection **(Figure 2B)**. By contrast, NKT cells were not critical for the generation of IgG1-producing germinal center B cells in the sdLN after VV infection of the skin **(Figure 2B)**. Importantly, activation of CD4 T_{FH} cells was similar in sdLNs of WT and *Cd1d*$^{-/-}$ mice **(Figure 2C)**. Therefore, the early wave of IL-4 produced by NKT cells at day 3 has a significant role in providing B cell help at day 9 by regulating the IgG1 class-switching in the model of respiratory infection. But this phenomenon does not bear any importance for the establishment of the same response during subcutaneous infection.

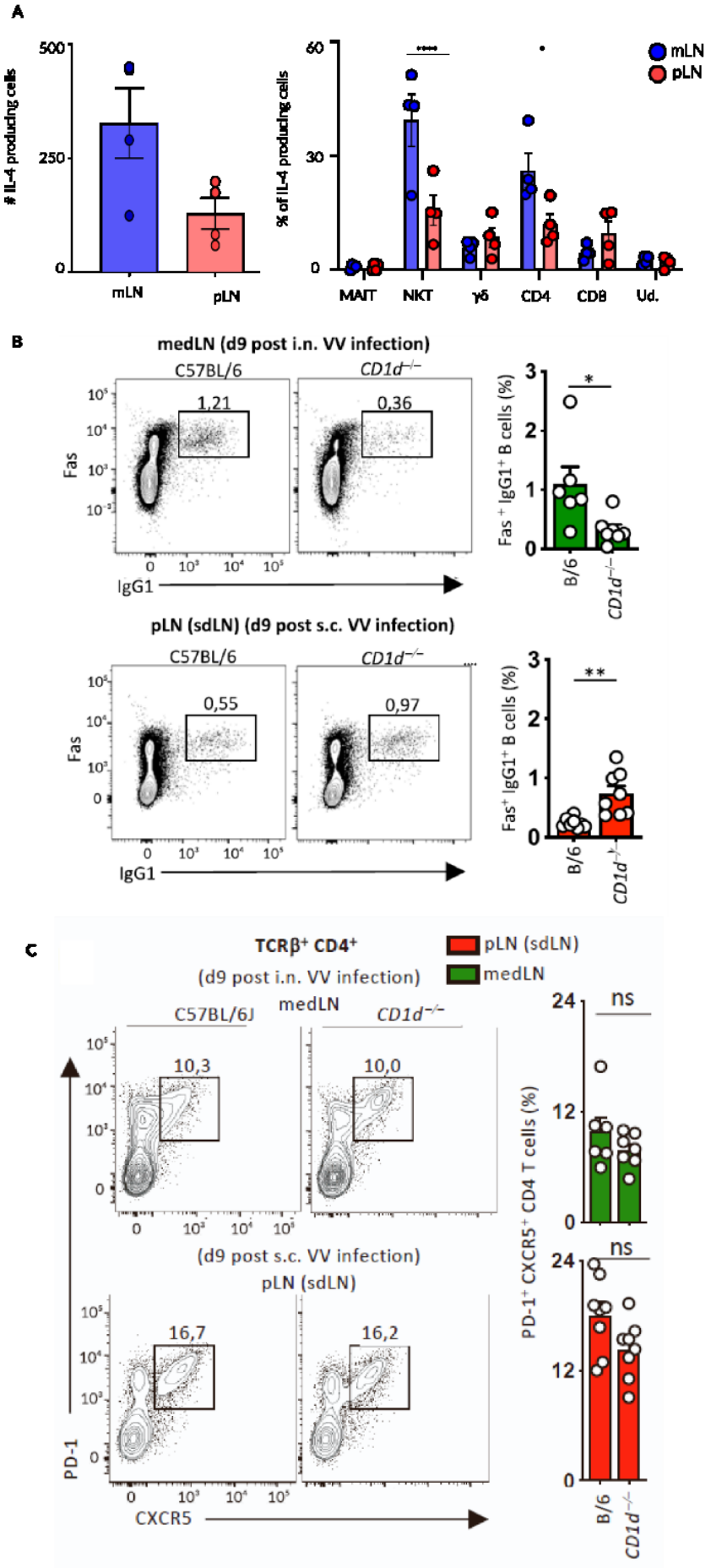

A
IL-4 producing cells
500
250
0
mLN
pLN
% of IL-4 producing cells
60
30
0
****
mLN
pLN
MAIT
NKT
γδ
CD4
CD8
Ud.
B
medLN (d9 post i.n. VV infection)
C57BL/6
CD1d−/−
1,21
0,36
Fas
IgG1
Fas+ IgG1+ B cells (%)
*
B/6
CD1d−/−
pLN (sdLN) (d9 post s.c. VV infection)
C57BL/6
CD1d−/−
0,55
0,97
Fas
IgG1
Fas+ IgG1+ B cells (%)
**
B/6
CD1d−/−
C
TCRβ+ CD4+
(d9 post i.n. VV infection)
medLN
pLN (sdLN)
medLN
C57BL/6J
CD1d−/−
10,3
10,0
(d9 post s.c. VV infection)
pLN (sdLN)
16,7
16,2
PD-1
CXCR5
PD-1+ CXCR5+ CD4 T cells (%)
ns
ns
B/6
CD1d−/−

Figure 2. Site-specific response generated by NKT cells in skin- and lung-draining lymph nodes. (A) Total number of IL-4-producing cells (left) and percentage of IL-4-producing different T cells in the mediastinal lymph node (mLN) and popliteal lymph node (pLN) (right) 3 days post-infection with 2000 pfu of Vaccinia virus. **(B)** Flow cytometry plot and percentage of $IgG1^+$ GC B cells in the mediastinal lymph node (mLN) and popliteal lymph node (pLN) (right) 9 days post-infection with 2000 pfu of Vaccinia virus in WT and $CD1d^{-/-}$ mice. **(C)** Flow cytometry plot and percentage of CD4+ T_{FH} cells in the mediastinal lymph node (mLN) and popliteal lymph node (pLN) (right) 9 days post-infection with 2000 pfu of Vaccinia virus in WT and $CD1d^{-/-}$ mice. Lines represent mean ± SEM, each dot is a mouse. In figures, asterisks stand for: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, $^{****}p<0.0001$, and no symbol if the comparison was non-significant.

GRAPHICAL ABSTRACT

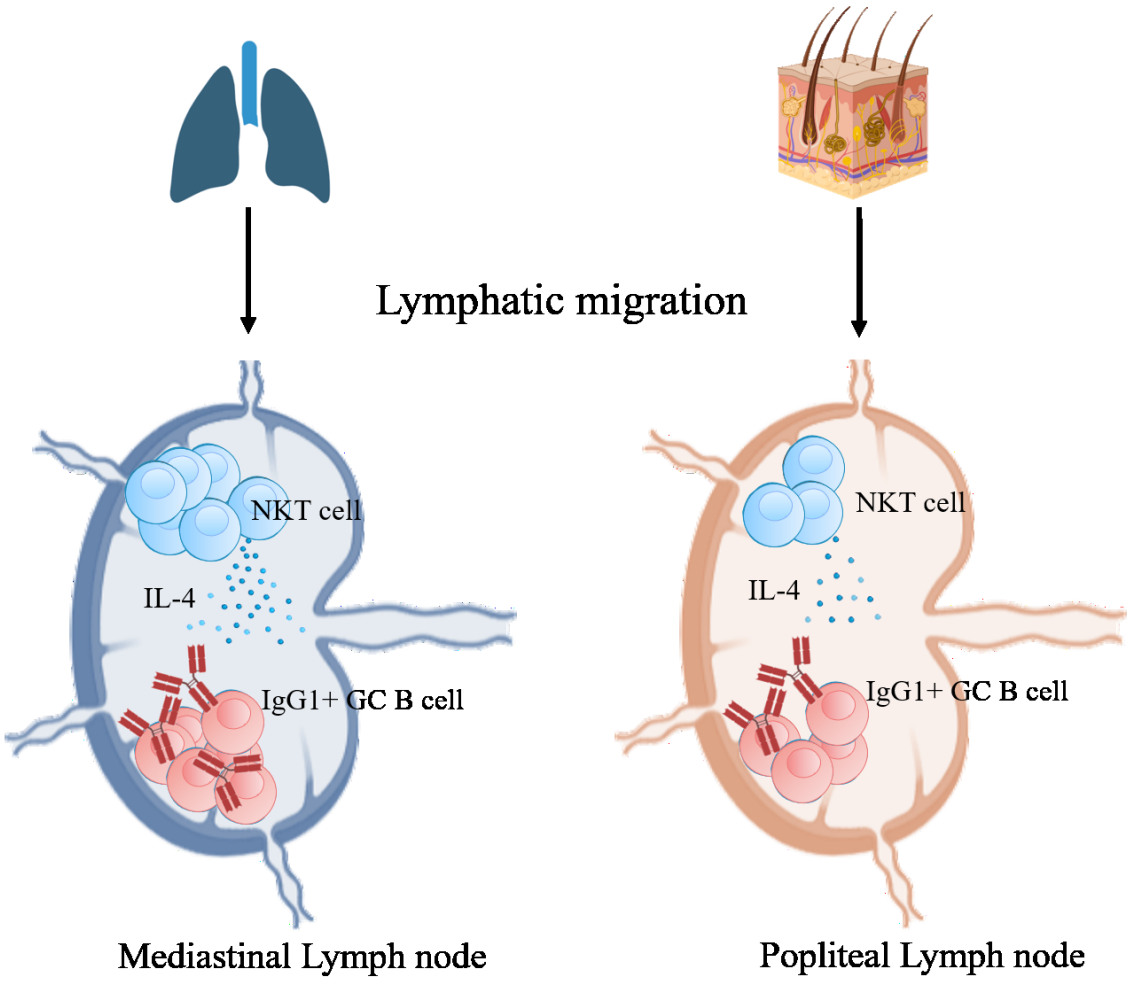


Figure 3. Graphical abstract of site-specific response generated by NKT cells in lung-draining lymph nodes (mediastinal lymph node) (left) and skin-draining lymph node (popliteal lymph node) (right).

Discussion

During viral infection with Influenza, NKT cells get activated in a CD1d-independent manner as a result of IL-18 produced by macrophages in the lymph node (Gaya et al., 2018). However, we do not know which signal is responsible for activating NKT cells in the case *of K. pneumoniae* infection. *K.pneumoniae* infection induces the production of a wide range of cytokines in antigen-presenting cells like TNF-α, IL-1β, IL-6, and IL-10 (W. Yu et al., 2024). It is crucial to understand the phenotype based on the transcriptor factor of NKT cells that are involved during *K.*pneumoniae infection to know the effector functions and hypothesize the signal responsible for their activation. For example, IL-1 and IL-23 have been implicated in inducing activation of NKT cells where they induce Th17-functions (Doisne et al., 2011). IL-12 and IL-18 cytokines are responsible for inducing IFN-γ during *Mycobacterium tuberculosis* infection and participate in pathogen clearance (Sada-Ovalle et al., 2008). By using a combination of techniques like generating knock-out models or performing in vivo blocking of cytokine signaling that have been described to be induced during *K.pneumoniae* infection, the potential cytokine signaling can be deduced.

I observe that there is an optional requisite of NKT cells in terms of promoting the adaptive response in two separate organs. Lung-draining lymph node require IL-4-producing NKT cells to support the class-switching of IgG1 GC B cells. However, there may be another source that facilitates class-switching IgG1-GC B cells in the skin-draining lymph node. Our work done on NKT cells led to collaboration with the team of Kastenmueller and was published as part of a large-scale study on the lymphatic migration of Unconventional T cells in different immunization models (Ataide et al., 2022). One may ask why there is this optional requisite of NKT cells in the two different lymph nodes. One reason could be that IL-4 production is more important for Th2-based immune response in the respiratory system but the same level of response in the case of subcutaneous infection might lead to negative effects. For example, an override by Th2-response that produces IL-4 can drive disease progression and contribute to evasion by malignant cells in the case of cutaneous T-cell lymphoma (Mazzetto et al., 2024) or lead to barrier dysfunction in the case of atopic dermatitis (Silverberg & Kantor, 2017). Moreover, other T cells that display the tendency to produce IL-4 like γδT cells are associated with escalating the symptoms of atopic dermatitis (Fagundes et al., 2023). Therefore the positioning and frequency of IL-4-producing NKT cells in different organs are indeed beneficial for the host.

The goal of our study with Kastenmueller was to determine the potential role that UTCs might play in the intricate linkage between tissue immunity and their associated lymph node functions. The tissues and lymph nodes that were involved in this study are lung and mediastinal lymph node, skin and skin-draining lymph node, and gut with mediastinal lymph nodes. The findings of this study revealed a fascinating divergence in both the local composition and the cytokine production capabilities of UTCs across all these lymph nodes and this divergence proved to have a profound and substantial impact on the immune responses that were ultimately generated within those tissues. These notable local differences emerged as a direct consequence of the continuous migration of UTCs through the lymphatic system to the LNs that drain specific tissues, which in turn led to the establishment of a tissue-specific composition of UTCs within those draining LNs. Remarkably, this migratory behavior was not only evident but also was found to be conserved across different T cell receptor (TCR)-based lineages, including NKT cells, MAIT cells, and γδ T cells, as well as across varying effector states, thereby suggesting that these cells share interrelated and interconnected functions that are essential for their roles in immunity. In summary, these findings compellingly illustrate that UTCs are in a constant state of migration via the lymphatic route to the lymph nodes that drain specific tissues, where they successfully form functional units that are capable of generating characteristic immune responses that are uniquely tailored to their site of origin, thereby establishing a crucial link between tissue immunity and the overall function of lymph nodes. This fundamental connection not only enhances our understanding of immune system dynamics but also underscores the importance of UTCs in orchestrating localized immune responses that are vital for effective immune surveillance and response across different tissue environments. Ultimately, this work sheds light on the sophisticated interplay between tissue-specific (site-specific) immunity and lymph node function, revealing the critical role that UTCs play in maintaining the delicate balance required for a robust and adaptive immune response in the body.

CHAPTER 2: Role MAIT cells in innate immunity

INTRODUCTION

The immune system comprises a diverse array of cellular components, each playing a unique role in defending the body against pathogens. Unconventional T cells have emerged as a diverse group of lymphocytes that provide rapid immune responses to microbial threats. This group comprises three main lineages: γδ T cells, natural killer T (NKT) cells, and mucosal-associated invariant T (MAIT) cells, all of which possess characteristics that distinguish them from conventional $CD4^+$ and $CD8^+$ T cells (Godfrey et al., 2015). These lineages exhibit limited T cell receptor (TCR) diversity, leading to 'public' antigen specificities. Their TCRs can detect exogenous and endogenous lipids, small-molecule metabolites, and specially modified peptides, rather than classical peptide antigens. These ligands are recognized in the context of non-polymorphic molecules such as CD1, MR1, and MHC class Ib. Interestingly, unconventional T cells can further exert antigen-independent responses driven by cytokine sensing, allowing them to respond quickly to infections or other immune challenges without the need for specific antigen recognition (M. Lee et al., 2020).

Unconventional T cells have the unique capacity to differentiate into polarized effector cells in the thymus and subsequently accumulate in peripheral tissues, especially in those exposed to external stimuli and microbiota like the lungs, liver, skin, and intestine (M. Lee et al., 2020). In these locations, they can often outnumber $CD4^+$ and $CD8^+$ T cells with a defined antigen-specificity. In recent years, MAIT cells have risen as crucial players in maintaining barrier tissue homeostasis due to their functional plasticity. In the skin, MAIT cells promote wound healing through amphiregulin production, while in the gut, they monitor microbial balance and promote gut integrity through IL-22 secretion ((du Halgouet et al., 2023; Gao & Zhao, 2024; Zheng et al., 2023). In the lungs, MAIT cells combat both bacterial and viral pathogens by directly killing infected cells, though the release of cytotoxic effectors, or by engaging other immune cells, through the production of pro-inflammatory cytokines such as TNF-α and IFN-γ (Le Bourhis et al., 2010; Van Wilgenburg et al., 2016). All these studies underscore the significance of MAIT cells at the frontline of immune defense.

Upon infection, unconventional T cells can migrate from barrier tissues to local draining lymph nodes via the lymphatic route, where they shape both innate and adaptive immune responses (Ataide et al., 2022). Our previous research demonstrated that NKT cells within the lymph

node interfollicular areas deliver an early wave of IL-4 to activated B cells, initiating class-switching to IgG1 and germinal center reactions (Gaya et al., 2018). However, the role of MAIT cells in modulating local immune responses upon their arrival in lymph nodes remains unclear.

In this study, we simultaneously tracked MAIT cell dynamics in the lungs and mediastinal lymph nodes following intranasal exposure to vitamin B-derived metabolites, influenza virus, or Klebsiella pneumoniae. All stimuli induced robust MAIT cell activation, although the requirement for TCR signaling and the resulting transcriptional programs differed significantly. Yet, in all cases, lymph node MAIT cells switch their chemokine receptor expression by downregulating CXCR6 and upregulating CCR7 transcripts, which favors their retention in lymph nodes and prevents their migration back to barrier tissues. Remarkably, MAIT cell effector programs diverged between the lungs and local lymph nodes: while in the lungs, type I interferon drives an IFN-γ/cytotoxic program, in lymph nodes, IL-1 and IL-23 synergistically induce an IL-17-biased effector program. Notably, MAIT cells constitute the principal and non-redundant source of IL-17 in local lymph nodes, which is essential for controlling bacterial but not viral burden. Therefore, while MAIT cells are crucial at barrier tissues, our findings show that their migration into local lymph nodes is essential for controlling pathogen spread and shaping local immune responses.

RESULTS

2.1 Pulmonary and local lymph node MAIT cells predominantly produce IL-17 during respiratory infection

To investigate the dynamics of MAIT cells in barrier tissues and local lymphoid organs, we challenged B6.CAST mice intranasally and studied immune responses in both the lungs and draining mediastinal lymph nodes (mLN) (Figure 1 A). B6.CAST mice possess MAIT cell numbers similar to those found in humans, allowing us to accurately identify MAIT cells and study their physiological relevance in our experimental setup. We administered intranasally with 0,14 nmoles of 5-OP-RU, a vitamin B-derived ligand recognized by the MAIT cell TCR, 5 plaque-forming units (PFU) of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain, or 2 x 10^4 colony-forming units (CFU) of *Klebsiella pneumoniae* KP52.145 strain. After 3 days, we harvested the lungs and mediastinal lymph nodes and identified MAIT cells by gating on $CD44^+$ cells and using a loaded MR1 tetramer in two different fluorophores. This approach allows us to unequivocally identify MAIT cells, as double-positive cells are absent in B6.CAST $MR1^{KO}$ mice **(Figure 1A)**.

We observed that all stimuli induced the activation of lung and lymph node MAIT cells, as evidenced by the upregulation of surface activation markers such as CD25 and CD69 **(Figures 1 B, C, and D)**. To assess whether this activation was dependent on TCR signaling, we crossed B6.CAST mice with Nur77-GFP mice, as Nur77 expression can be used as a surrogate of TCR signaling. We found that 5-OP-RU administration into B6.CAST Nur77-GFP mice strongly induced GFP expression in MAIT cells compared to non-immunized animals, confirming that vitamin B metabolites activate MAIT cells through their T cell receptor **(Figure 1E)**. In contrast, both influenza virus and *Klebsiella pneumoniae* infection did not induce upregulation of Nur77-GFP, indicating that these pathogens activate MAIT cells independently of their TCR and potentially through a cytokine pathway **(Figure 1E)**.

Next, we analyzed the cytokines produced by MAIT cells in response to different stimuli in the lungs and lymph node. We found that both TCR-dependent and -independent stimuli induced the production of IFN-γ and IL-17 with a dominance for IL-17 production in MAIT cells in response to infection with Influenza and *Klebsiella pneumoniae* in the lungs and lymph node **(Figure 1F)**.

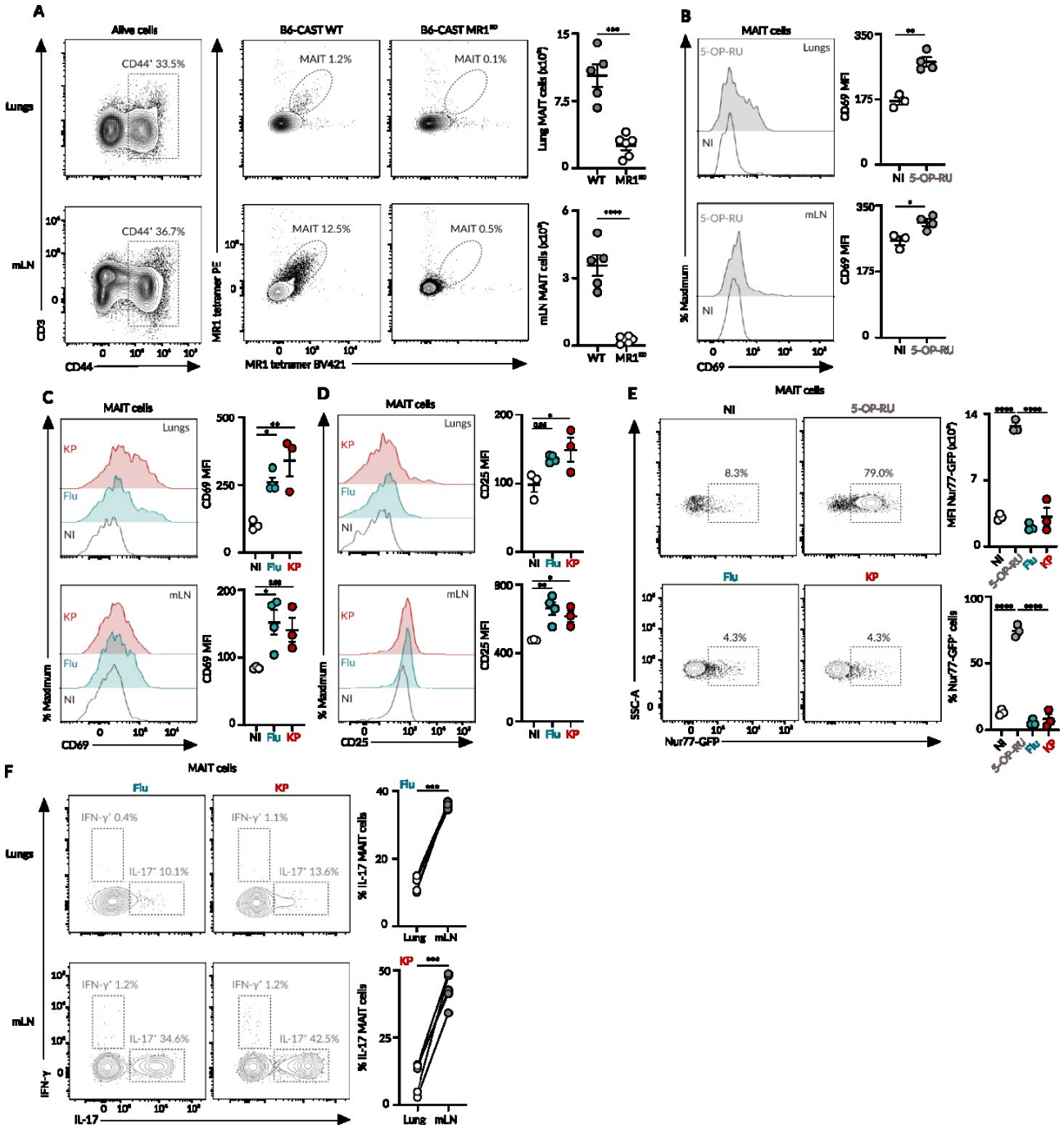


Figure 1. Pulmonary and mediastinal lymph nodes MAIT cells display Th17-type effector functions during respiratory infection. (A) Flow cytometry plots and quantification of MAIT cells in the lung (top) and mediastinal lymph node (bottom) of B6-MAIT^{CAST} mice (hereafter, referred to as WT) and MR1^{KO} mice identified as CD3^{+} CD44^{+} further double positive for staining with MR1-tetramer^{+} coupled with PE and BV421. **(B and C)** Mean fluorescence intensity of MAIT cells' CD69 in the lungs (top) and mLN (bottom) of B6-MAIT^{CAST} mice 3 days post-immunization with 5-OP-RU or non-immunized (NI) (B), Non-immunized or Influenza (Flu) or *Klebsiella pneumoniae* (KP 52.145 strain) (C). **(D)** Mean fluorescence

intensity and quantification MAIT cells' CD25 in the lungs (top) and mLN (bottom) of B6-MAIT^{CAST} mice non-immunized (NI) mice, or mice 3 days post-immunization with Influenza (Flu) or *K. pneumoniae* (KP). **(E)** Flow cytometry plots, mean fluorescence intensity (top), and quantification (bottom) of GFP in MAIT cells in the mediastinal lymph nodes of B6.CAST Nur77-GFP mice non-immunized (NI), 5-OP-RU immunized, Influenza infected (Flu) and *K. pneumoniae* (KP). **(F)** Flow cytometry plots of IFN-γ and IL-17 in MAIT cells in the lung and mediastinal lymph node of B6-MAIT^{CAST} mice upon Influenza (Flu) or *Klebsiella pneumoniae* (KP) infection (left), percentage of IL-17 producing MAIT cells in the lung and mediastinal lymph node (mLN) upon 3 days post-infection with Influenza (Flu) (top) or *Klebsiella pneumoniae* (KP) (bottom). Lines represent mean ± SEM, each dot is a mouse. In figures, asterisks stand for: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, $^{****}p<0.0001$, and no symbol if the comparison was non-significant.

2.2 MAIT cells are the principal and non-redundant source of IL-17 in lymph nodes

To explore the relevance of IL-17 production by MAIT cells relative to other cytokine-producing T cells in lungs and lymph nodes, we crossed B6.CAST mice with IL-17-GFP animals and treated them with either 5-OP-RU, influenza virus, or *K. pneumoniae* to observe the kinetics of IL-17 production at day 3, day 6, and day 9 post-treatment in the two organs. We observed a gradual increase in the IL-17 production in the lungs from day 3 to day 9 **(Figure 2A)**. Interestingly, we observed the opposite in the lymph nodes with a robust IL-17 production at day 3 which declined by day 6 **(Figure 2B)**. Next, we harvested lungs and mediastinal lymph nodes on day 3 post-treatment and performed Uniform Manifold Approximation and Projection (UMAP) analysis to visualize the cell composition of the IL-17 GFP^+ population in each organ. In the lungs, the primary IL-17 producers were CD4 T cells and γδ T cells, with CD8 T cells, NKT cells **(Figures 2 C and D)**, and MAIT cells contributing to a lesser extent. In stark contrast, we found that in lymph nodes, MAIT cells were the predominant source of IL-17 at early stages of infection, representing over 50% of IL-17-producing cells **(Figure 2 C and D)**.

Previous studies have shown that in the absence of one unconventional T cell population, other innate-like T cells can compensate for their function. To investigate whether IL-17 production by MAIT cells was essential or could be counterbalanced by other cells, we measured IL-17-producing cells in the lungs and lymph nodes after influenza or *Klebsiella* infection in both B6.CAST wild-type (WT) and B6.CAST $MR1^{KO}$ mice, the latter of which lack MAIT cells. While $IL\text{-}17^+$ cell numbers in the lungs were similar in both mouse strains, there was a significant reduction in $IL\text{-}17^+$ cells in the lymph nodes of $MR1^{KO}$ mice **(Figures 2 E and F)**. This finding demonstrates that MAIT cells play a unique and irreplaceable role as a source of IL-17 in lymph nodes.

Collectively, these experiments establish that MAIT cells are the primary and indispensable source of IL-17 in lymph nodes at early stages of infection.

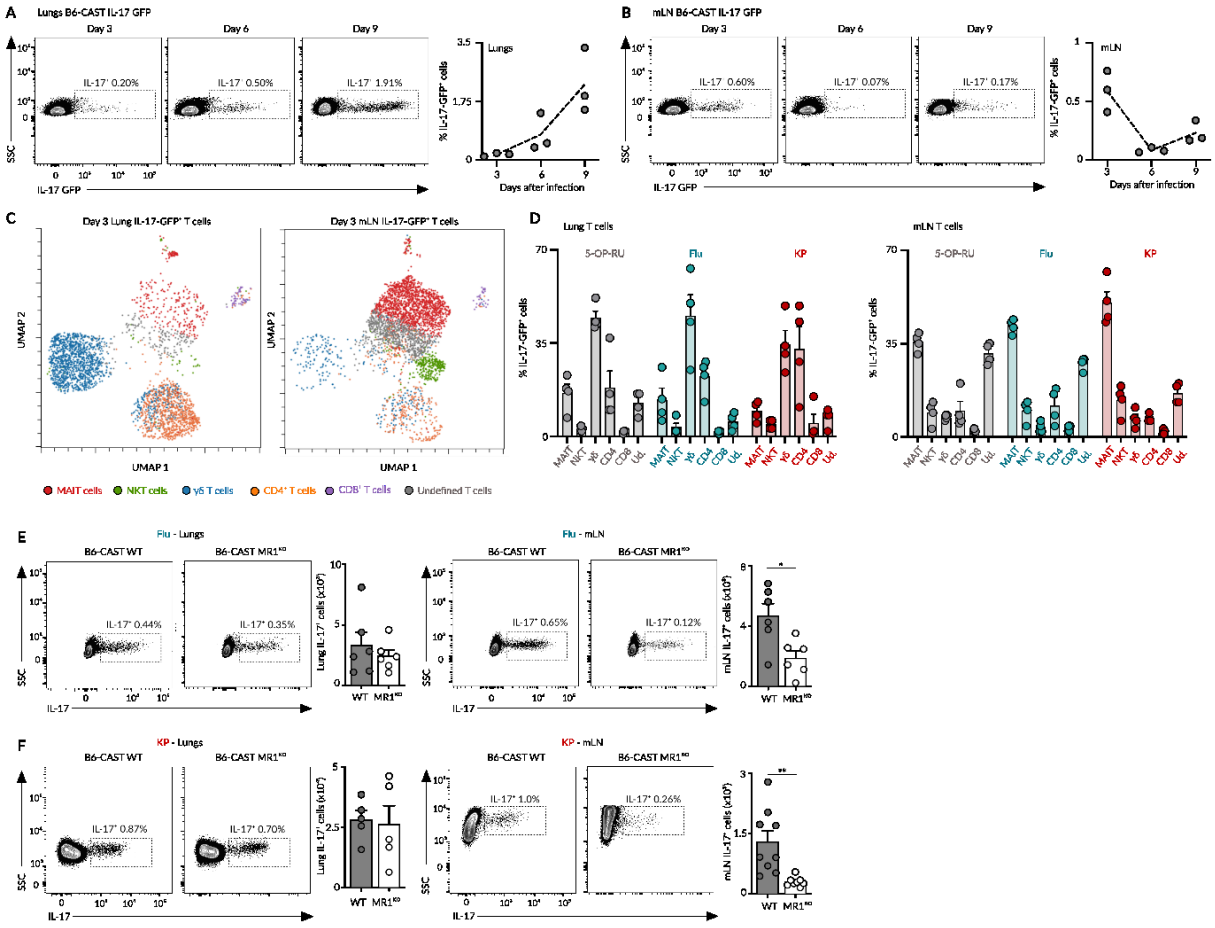
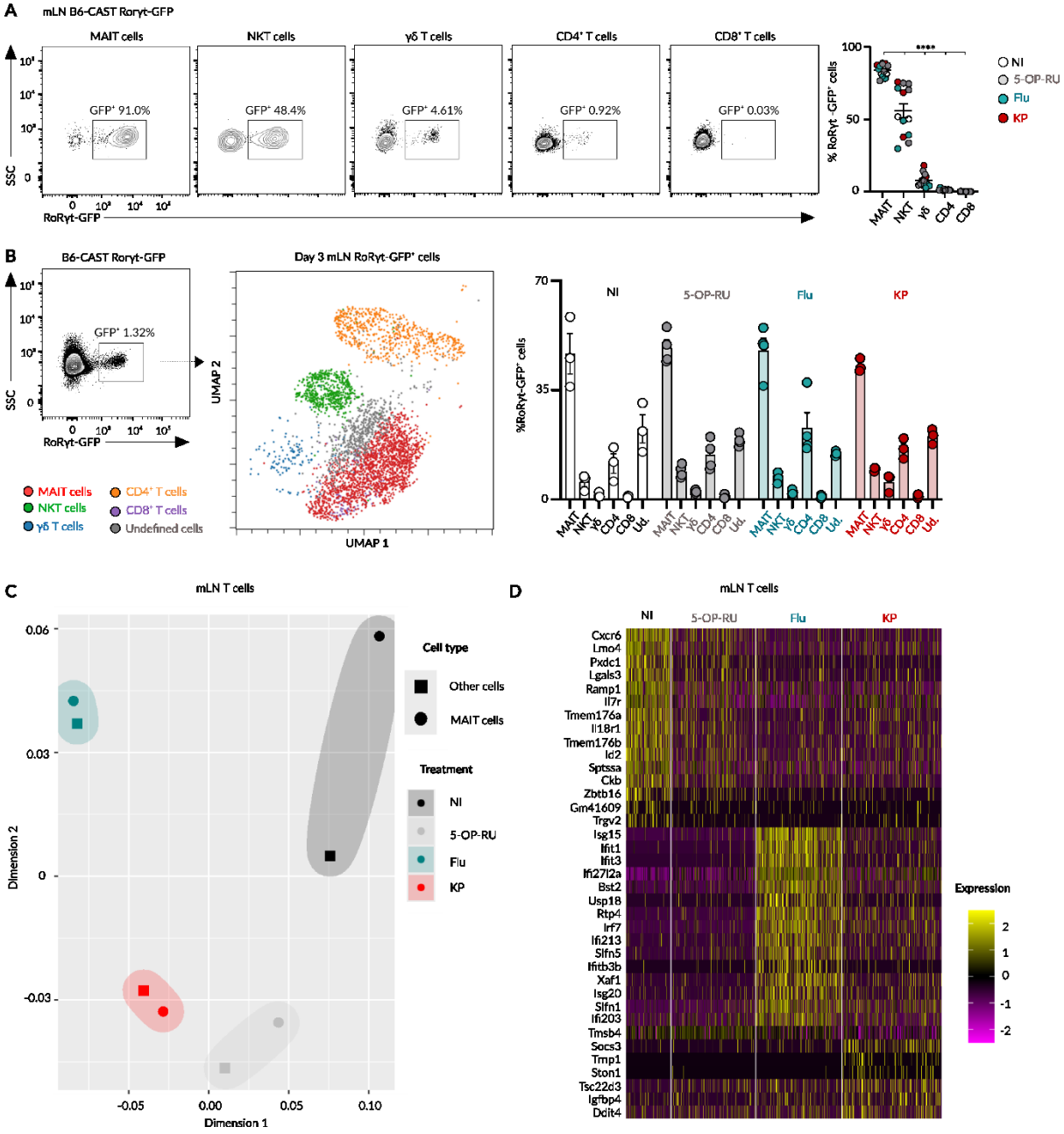


Figure 2. MAIT cells are the principal and non-redundant source of IL-17 in lymph nodes. **(A and B)** Flow cytometry plots and quantification of IL-17 GFP+ cells in the lung (A) and mediastinal lymph node (B) of B6.CAST IL-17-GFP mice at 3, 6, and 9 days post-infection with Influenza. **(C)** UMAP visualization of IL-17 GFP producing subsets colored by cell types in the lung (left) and mLN (right) concatenated from non-immunized, 5-OP-RU treated, Influenza and *K.pneumoniae* infected B6.CAST IL-17-GFP mice at 3 days post-treatment (n=4). **(D)** Percentage of different subsets of IL-17 GFP positive cells in the lung (left) and mLN (right) upon immunization with 5-OP-RU, Influenza (Flu), and *K.pneumoniae* (KP). **(E and F)** IL-17 GFP positive cells in the lung (left) and mLN (right) of B6-CAST-WT and B6-CAST-MR1^{KO} mice 3 days post-infection with Influenza (Flu) (top) and *K.pneumoniae* (KP) (bottom). Lines represent mean ± SEM, each dot is a mouse. In figures, asterisks stand for: *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001, and no symbol if the comparison was non-significant.

2.3 MAIT cells acquire distinct transcriptional signatures according to the nature of the stimulus

The production of IL-17 is a signature property of the RORγt expressing MAIT17 subset. The dominance of this subset was reflected in the B6-CAST *Rorc*-GFP reporter mouse model, where about 90% of the total MAIT cells in the lymph nodes expressed GFP in response to stimulation with 5-OP-RU, influenza virus, or *K. pneumoniae* treated mice as well as in the non-immunized animals **(Figure 3A)**. At the same time, we observed the dominance of the prevalence of RORγt expressing MAIT cells over other T cells like NKT and γδT cells **(Figure 3B)**. To understand if these RORγt expressing MAIT cells display differences in their transcriptomic profile, we performed single-cell RNA sequencing on the MAIT cells from the lymph nodes of B6.CAST *Rorc*-GFP mice treated with 5-OP-RU, influenza virus, or *K. pneumoniae* and non-immunized subjects based on the expression of MAIT cell specific TRAV1 gene. As the cytometry data shows that MAIT cells in the lymph node predominantly express RORγt, we sorted all the GFP-positive cells without MR1-tetramer staining to avoid 5-OP-RU-based TCR downstream signaling. MAIT cells were identified by the presence of MAIT cell-specific TCR TRAV1 gene sequence in the GFP-positive cells sorted 3 days post-treatment. To broadly asses the profile of MAIT cells and other cells included in the sorting based on the treatment, we performed a multiple dimensions scaling (MDS) analysis. MDS analysis revealed that there is no overlap between the cells (both MAIT and other cells) based on the treatment implying a diverse transcriptomic profile **(Figure 3C)**. In other words, except for the MAIT cells in non-immunized mice, there is a close similarity between MAIT and other cells in the immunized mice. Analyzing the differentially expressed genes (DEGs) in all the conditions, allowed detailed descriptions of the varying MAIT cells' transcriptomic profiles **(Figure 3C)**. MAIT cells clustered differently during all 4 conditions indicating that based on the nature of the stimuli, a very diverse and strategic set of downstream gene profiles are imprinted on a MAIT cell. For example, Influenza virus infection induced a strong anti-viral response in the MAIT cells with an upregulation of several interferon-related genes such as *ISG15, IFIT1, IFIT3, and IRF7* **(Figure 3C)**. Whereas, MAIT cells in the *K.pneumoniae* infected mice displayed an upregulation of genes related to tissue homeostasis such as *SOCS3* **(Figure 3D)**. In order to understand direction of the pathways related to the genes involved in response to viral and bacterial infection, we performed a G0 pathway enrichment analysis. MAIT cells in Influenza infected mice displayed a strong anti-viral response by upregulating

genes stimulated by interferon **(Figure 3E)**. On the other hand, MAIT cells during bacterial infection reflected genes upregulation in response to glucocorticosteroid stimuli. These diverse programing in MAIT cells from two different pulmonary pathogens imply a strong pathogen specific response mediation. In summary, these results indicate that the functions of MAIT cells go beyond tissue repair in response to immune triggers.



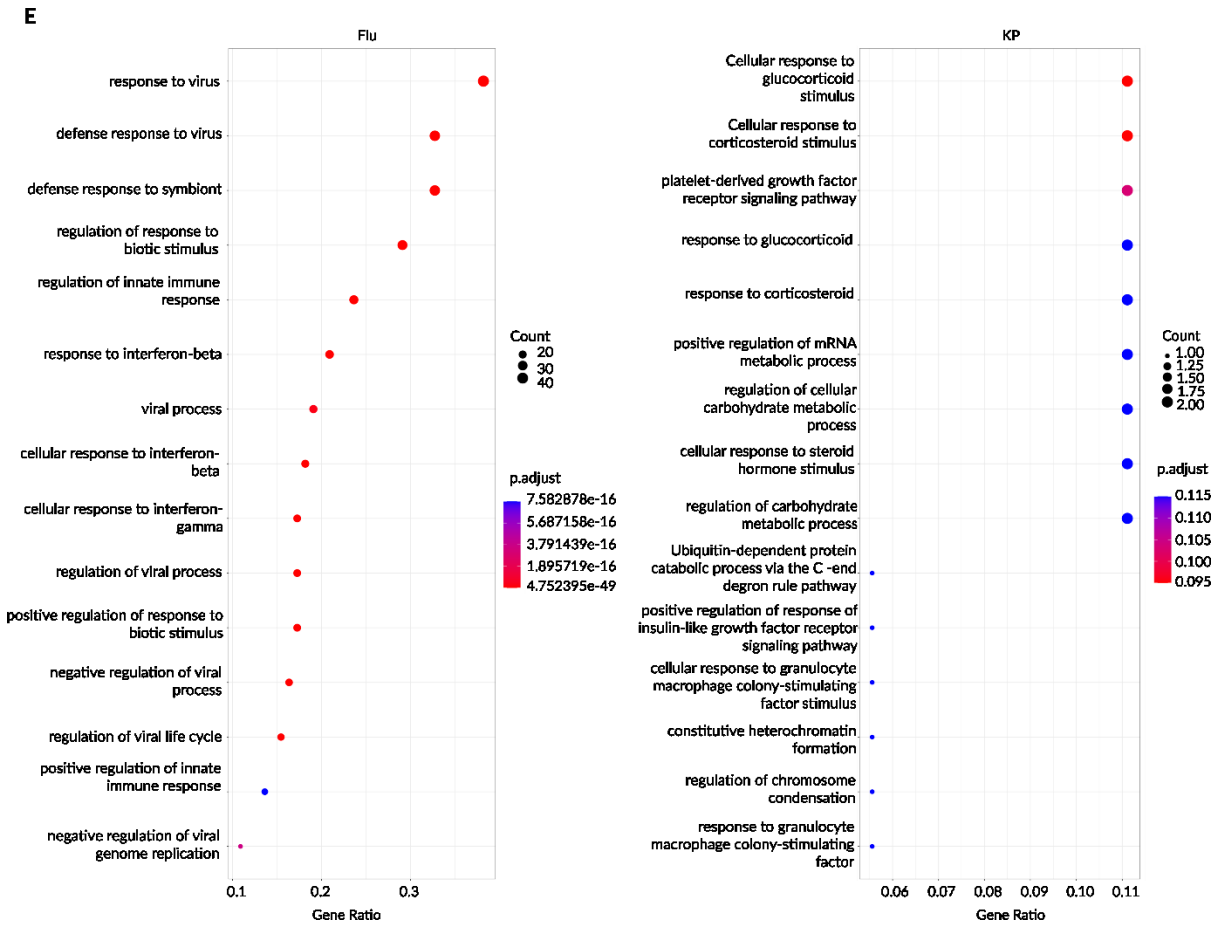


Figure 3. Stimulation-based transcriptional signatures acquired by MAIT cells. (A) Flow cytometry plots and quantification of RORγt-GFP positive cells in the mediastinal lymph node (mLN) of B6-CAST RORγt-GFP mice in non-immunized (NI), and 5-OP-RU stimulated, Influenza (Flu) and *K.pneumoniae* (KP) infected mice after 3 days. **(B)** Flow cytometry plot of RORγt-GFP positive cells in the mediastinal lymph node (mLN) of B6-CAST RORγt-GFP mice used for sorting (left), UMAP visualization (middle), and quantification (right) of IL-17 GFP producing subsets colored by cell types in the mLN from non-immunized, 5-OP-RU treated, Influenza and *K.pneumoniae* infected B6.CAST RORγt-GFP mice at 3 days post-stimulation. **(C, D)** Multiple dimension scaling (MDS) plot of MAIT cells and other non-MAIT cells (C) and Differentially expressed genes (DEG) of MAIT-TCR-expressing cells from RORγt-GFP sorted population in the mediastinal lymph node (mLN) of B6-CAST RORγt-GFP mice in non-immunized (NI), and 5-OP-RU stimulated, Influenza (Flu) and *K.pneumoniae* (KP) infected mice after 3 days using single-cell RNA sequencing (n=4). **(E)** Gene pathway analysis of MAIT cells RORγt-GFP sorted population in the mediastinal lymph node (mLN)

of B6-CAST RORγt-GFP mice in Influenza (Flu) (left) and *K.pneumoniae* (KP) (right) infected mice after 3 days using single-cell RNA sequencing (n=4). Lines represent mean ± SEM, each dot is a mouse. In figures, asterisks stand for: *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001, and no symbol if the comparison was non-significant.

2.4 The MAIT cell/IL-17 axis is essential to control lymph node bacterial load

In response to infection, pulmonary infiltration of MAIT cells corresponds to enhanced protection from pathogens (Meierovics et al., 2013b; H. Wang et al., 2018). Lung-draining mediastinal lymph node is crucial for immune responses in the respiratory tract. Pathogens may escape the lumen to enter the lymphatics, making the draining lymph node the last resort for pathogen filtering. To investigate the spatial location of MAIT cells in the lymph nodes, we visualized the MR1-tetramer stained cells in B6-CAST *Rorc*-GFP reporter mice treated with 5-OP-RU, Influenza, and *K.pneumoniae* **(Figures 4A, B and C)**. Tetramer based staining has been previously decribed by the team for the identification of NKT cells (Courey-Ghaouzi & Gaya, 2022). I employed the same strategy to identify MAIT cells by incubating the lymph nodes harvested from mice with MR-1 tetramer coupled with PE fluorochrome before fixing the tissue. Based on the MR1-tetramer and GFP co-staining, MAIT cells localize in the interfollicular/T-cell zone in the lymph node.

To determine if the positioning of MAIT cells in the lymph node benefits the organism during infection, we measured the viral and bacterial load in the lymph nodes and lungs of B6.CAST wild-type (WT) and B6-CAST $MR1^{KO}$ mice post infection with Influenza and *K.pneumoniae*. Since MAIT cells produce IL-17 as their major cytokine, we included B6.CAST $IL\text{-}17^{KO}$ mice in our analysis. The lack of MAIT cells did not affect the viral load in the lungs or lymph nodes upon Influenza virus infection **(Figure 4D)**. Interestingly, B6-CAST $MR1^{KO}$ mice failed to control the expansion of bacteria in the lymph node resulting in the leakage of bacteria into the bloodstream **(Figure 4E)**. Even though there was no significant decrease in the bacterial load in the lungs, the presence of bacteria in the blood coincided with the deteriorated health of mice.

Moreover, B6-CAST $IL\text{-}17^{KO}$ mice displayed ineffective bacteria control in the lungs, lymph nodes and in blood, suggesting further possible mechanisms for anti-bacterial MAIT cell functions **(Figure 4F)**. To decipher if IL-17 produced by MAIT cells confer this protection, I performed an the adoptive transfer of thymus derived and in vitro expanded MAIT cells from

B6.CAST wild-type mice and B6-CAST IL-17^{KO} mice in C57BL/6 mice (they harbor 10x less MAIT cells compared to CAST mice) and challenged them with *K.pneumoniae* after the transfer. I observe more bacterial load in the lymph node of mice that received MAIT cells from B6-CAST IL-17^{KO} mice as opposed to C57BL/6 mice that received adoptive transfer of MAIT cells from B6-CAST IL-17^{KO} mice **(Figure 4G)**. This enhanced protection in the mice with MAIT cells from B6-CAST IL-17^{KO} mice seems partly due to a slightly (but not statistically significant) increased IL-17 production in the lymph node upon MAIT cells' transfer **(Figure 4G)**. Thus, these data indicate that MAIT cells drive their protective role during *K. pneumoniae* infection in an IL-17-dependent manner. However, IL-17-producing MAIT cells are dispensable in case of viral infection with Influenza virus.

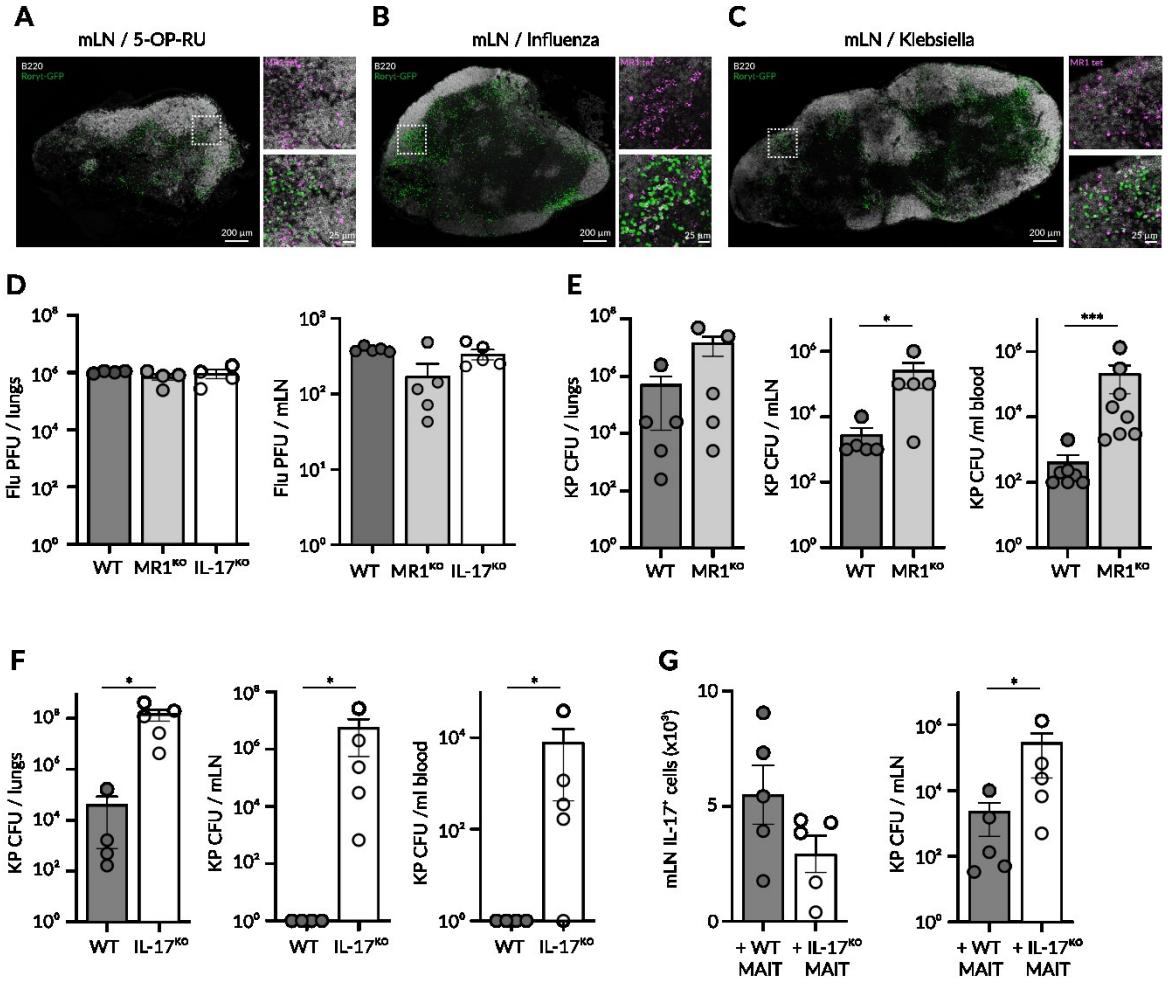


Figure 4. MAIT cells provide protection against bacterial infection by IL-17 secretion

(A, B, and C) Confocal images of MR-1 tetramer (magenta), B220 (white), and RORγt-GFP (green) stained mediastinal lymph nodes of B6-CAST RORγt-GFP mice 3 days post-

stimulation with 5-OP-RU (A), Influenza (B) and *K.pneumoniae* (C). **(D)** Viral burden measured as Plaque forming unit (PFU) on MDCK cells in the lung based on the number of plque counted (left) and mediastinal Lymph node based on the ImageJ analysis on crystal violet zone non-occupied region per lymph node relative to the non-immunized control (right) of B6-CAST-WT, B6-CAST-MR1^{KO}, and B6-CAST-IL-17^{KO} mice 3 days post-infection with Influenza virus (Flu). **(E and F)** Bacterial burden measured as Colony forming unit (cfu) in the lungs, mLN, and blood B6-CAST-WT and B6-CAST-MR1^{KO} (E) and B6-CAST-WT and B6-CAST-IL-17^{KO} after 3 days of infection with *K.pneumoniae*. **(G)** 8 x 10^5 Thymic-MAIT cells from B6-CAST-WT or B6-CAST-IL-17^{KO} were adoptively transferred (i.v.) in B57BL/6 mice and infected with *K.pneumoniae* after 12 hours of transfer. Total IL-17-producing cells (left) and bacterial burden measured as cfu in the mediastinal lymph node 3 days post-infection. Lines represent mean ± SEM, each dot is a mouse. In figures, asterisks stand for: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, $^{****}p<0.0001$, and no symbol if the comparison was non-significant.

2.5 MAIT cells require IL-1R and IL-23R signaling to produce IL-17 in lymph nodes

So far, we have demonstrated that MAIT cells colonize the lymph node as a consequence of draining from the lung post-infection and contribute significantly to the antibacterial response via the production of IL-17. Our experiments with B6.CAST Nur77-GFP mice revealed that *K.pneumoniae* infection activates MAIT cells in a TCR-independent manner and potentially through a cytokine pathway **(Figure 1E)**. MAIT cells display several cytokine receptors on their surface that have been shown to act independently or in synergy to activate MAIT cells when their TCR is not involved (Hinks & Zhang, 2020; *IL18 Dependent MAIT Cell Activation in COVID19*, n.d.; Jesteadt et al., 2018; López-Rodríguez et al., 2023; H. Wang et al., 2019a). To dissect the signal that leads to the activation of MAIT cells as well as drives the production of IL-17 upon *K.pneumoniae* infection responsible for the protective functions we took advantage of knock-out and in vivo blocking models. Firstly, we generated B6-CAST-IL-18^{KO} mice and measured the activation of MAIT cells and IL-17 production by MAIT cells in response to *K.pneumoniae* infection in the lymph node. We found no significant differences in the percentage of activated MAIT cells (CD69^{+}) and IL-17 production between B6-CAST-WT

and B6-CAST-IL-18^{KO} mice in the lymph node **(Figures 5A and B)**. Pulmonary MAIT cells depend on type-I interferon signaling for activation post-infection with *K.*pneumoniae (López-Rodríguez et al., 2023). To check if lymph node residing MAIT cells depend on type-I interferon signaling for their activation, we generated B6-CAST-IFNAR^{KO} mice. We observed a significant reduction in the expression of CD69 indicating a reduced MAIT cell activation in B6-CAST-IFNAR^{KO} mice in response to *K.peumoniae* infection in the lymph node with no changes in the IL-17 production **(Figures 5C and D)**. This shows that MAIT cells require type-I interferon signaling for activation but this signaling pathway is not responsible for IL-17 production by MAIT cells. Next, we performed an in vivo blocking of IL-1 and IL-23 signaling separately in B6-CAST-WT mice and compared the IL-17 production by injecting monoclonal anti-mouse IL-1R and anti-mouse IL-23 antibodies throughout the infection. The individual blocking of these two signals did not affect the IL-17 production by MAIT cells **(Figure 5E and F)**. Interestingly, a combination of IL-1R and IL-23 blocking antibody cocktail led to a significant decrease in IL-17 production by MAIT cells compared to mice treated with Isotype control **(Figure 5G)**. Moreover, blocking IL-1R and IL-23 signaling also increased the bacterial load in the animals, which remained unchanged in the absence of IL-18 signaling **(Figure 5H)**. Interestingly, B6-CAST-IFNAR^{KO} mice that displayed unaltered IL-17 levels compared to B6-CAST-WT also had a significantly increased bacterial load in the lymph nodes **(Figure 5H)**. These results indicate that IL-1 and IL-23 act in synergy to induce IL-17 production in MAIT cells which in return aids bacteria control. While type-I interferon signaling is responsible for the activation of MAIT cells in the lymph node, this signaling is not required for IL-17 production by MAIT cells.

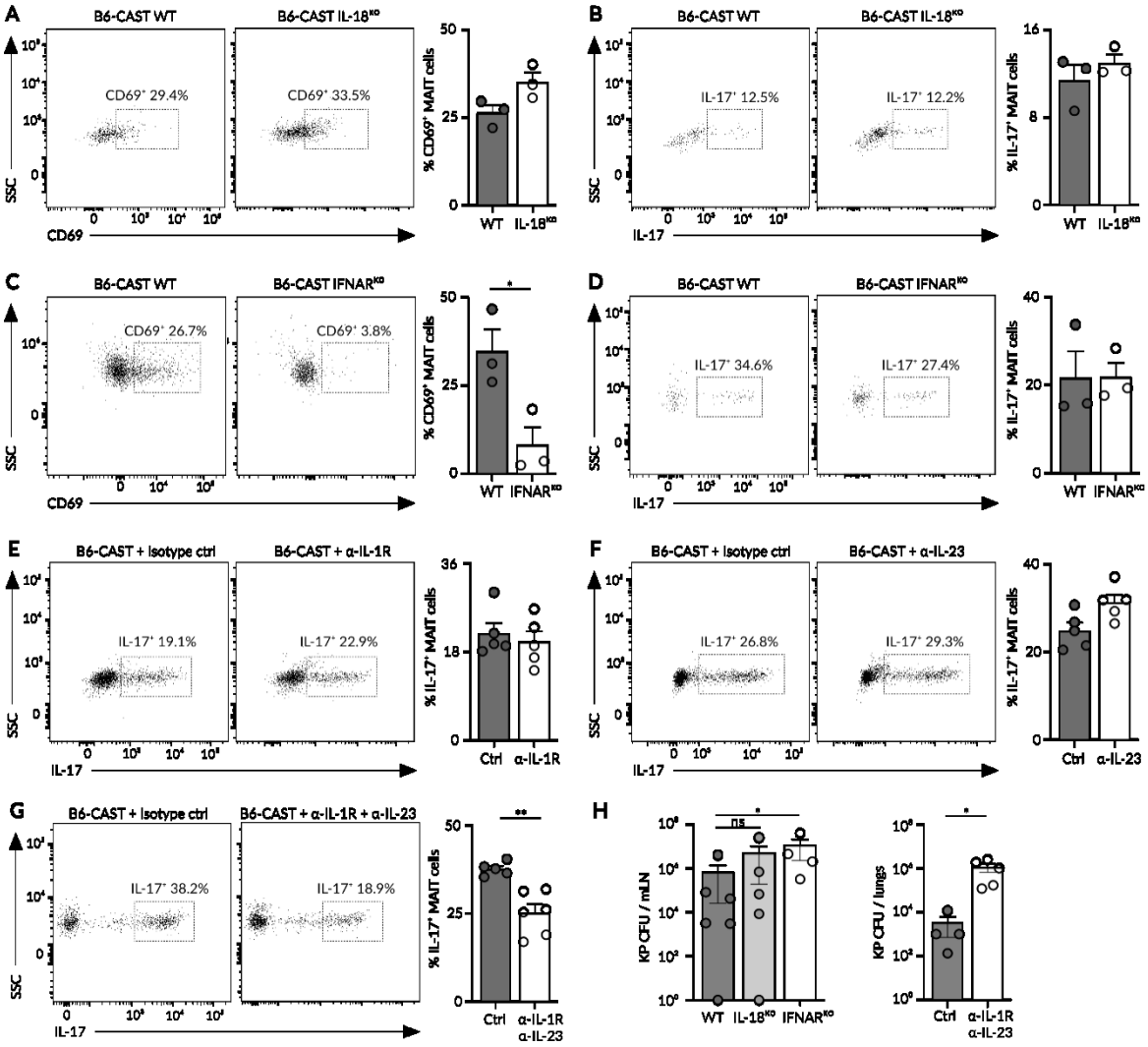


Figure 5. IL-1 and IL-23 signals act in synergy to induce IL-17 production in the MAIT cells of lymph node. (A and B) Flow cytometry plots and percentage of CD69 (A) IL-17-producing MAIT cells in the mediastinal lymph node of B6-CAST-WT and B6-CAST IL-18^{KO} mice 3 days post-infection with *K.pneumoniae*. **(C and D)** Flow cytometry plots and percentage of CD69 (A) IL-17-producing MAIT cells in the mediastinal lymph node of B6-CAST-WT and B6-CAST IL-18^{KO} mice 3 days post-infection with *K.pneumoniae*. **(E)** Flow cytometry plots and percentage of IL-17-producing MAIT cells in the mediastinal lymph node of B6-CAST-WT treated i.v. with isotype control and α-IL-1R after 3 days of infection with *K.pneumoniae*. **(F)** Flow cytometry plots and percentage of IL-17-producing MAIT cells in the mediastinal lymph node of B6-CAST-WT treated i.v. with isotype control and α-IL-23 after 3 days of infection with *K.pneumoniae*. **(G)** Flow cytometry plots and percentage of IL-17-producing MAIT cells in the mediastinal lymph node of B6-CAST-WT treated i.v. with isotype control and combination treatment α-IL-1R and α-IL-23 after 3 days of infection with

K.pneumoniae. **(H)** Bacterial burden measured as Colony forming unit (cfu) in the mLN of B6-CAST-WT, B6-CAST IL-18^{KO} and B6-CAST-IFNAR^{KO} (left) and B6-CAST-WT treated i.v. with isotype control and combination treatment α-IL-1R and α-IL-23 (right) after 3 days of infection with *K.pneumoniae*. Lines represent mean ± SEM, each dot is a mouse. In figures, asterisks stand for: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, $^{****}p<0.0001$, and no symbol if the comparison was non-significant.

2.6 MAIT cells undergo chemokine receptor switch to stay in lymph node

To identify the chemokine receptors responsible for the positioning of MAIT cells as a result of an immune response, we used our scRNA-seq data on MAIT cells from the lymph nodes of B6.CAST *Rorc*-GFP mice treated with 5-OP-RU, influenza virus, or *K. pneumoniae* and non-immunized mice. Amongst all the chemokine receptors, the expression levels of CXCR6 and CCR7 were found to be significantly affected in response to the three stimuli above. We observed a downregulation of CXCR6 expression in MAIT cells of the lymph nodes in response to stimuli that was accompanied by an upregulation of CCR7 in the same conditions **(Figure 6A)**. This implies that CXCR6 signaling which is attributed to the influx of MAIT cells in the lung (H. Yu et al., 2020b) is intrinsically downregulated to enable MAIT cells' entry into the lymphatics followed by an increase of CCR7 that retains their localization in the lymph node. To test this hypothesis, we used homozygous *CXCR6* EGFP Knock-in mice on C57BL/6 background (CXCR6^{GFP}) and compared the percentage of MAIT cells in the lymph node compared to C57BL/6 (WT) mice at 3 days post-infection with *K.pneumoniae*. CXCR6^{GFP} mice had a significant reduction in the total number of MAIT cells in the lymph node along with a slightly reduced but not statistically significant decrease in the lungs upon infection **(Figure 6B)**. We also quantified MAIT cells in the lymph node and lungs of CCR7^{KO} on B57BL/6 background compared to B57BL/6 and observed a reduced tendency of MAIT cell number in the lymph node of CCR7^{KO} mice in the same infection setting with no differences in the MAIT cell number in the lungs **(Figure 6C)**. Therefore, we deduce that the CXCR6 receptor allows MAIT cells to enter the lymph node and the lung via circulation. At the same time, the CCR7 receptor may also be involved in the retention of MAIT cells in the lymph node as we observe a declining trend (not statistically significant) of MAIT cells in the lymph node of CCR7^{KO}.

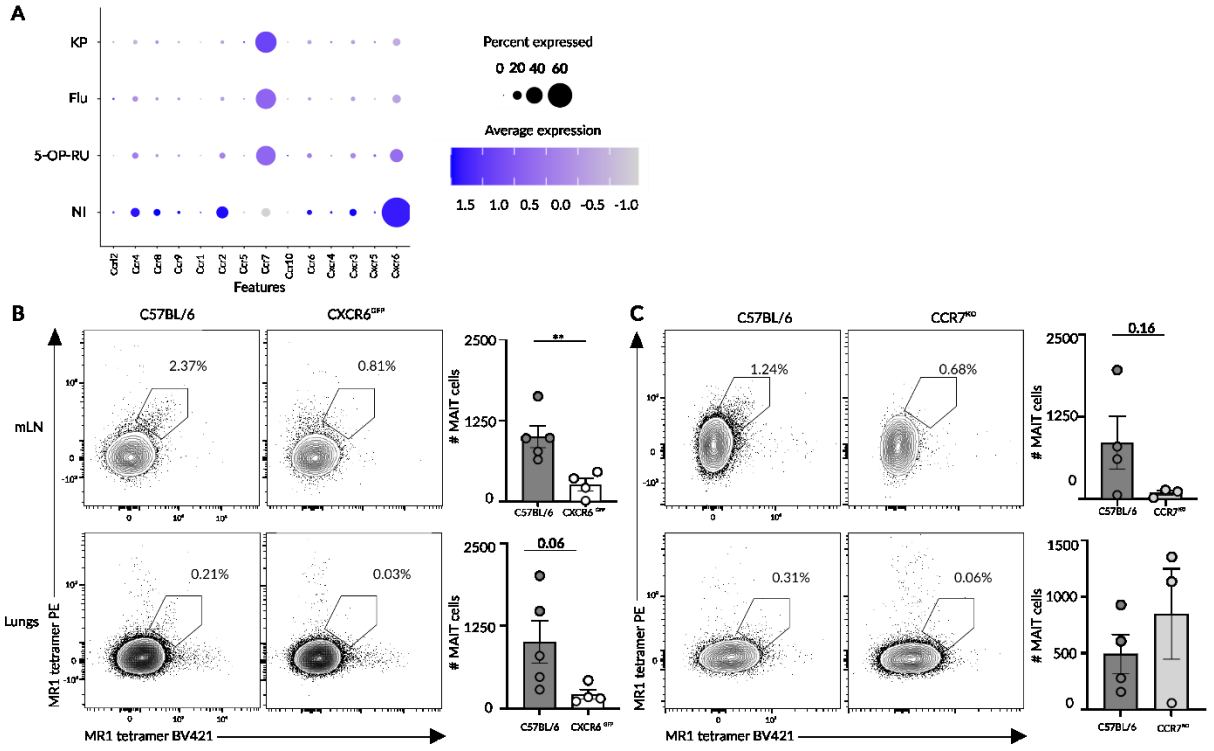


Figure 6. CXCR6 and CCR7 dependent localization of MAIT cells in the lymph node. **(A)** Transcriptomic expression of different chemokine receptors in the MAIT-TCR-expressing cells from RORγt-GFP sorted population in the mediastinal lymph node (mLN) of B6-CAST RORγt-GFP mice in non-immunized (NI), and 5-OP-RU stimulated, Influenza (Flu) and *K.pneumoniae* (KP) infected mice after 3 days using single-cell RNA sequencing (n=4). **(B)** Flow cytometry plots and quantification of MAIT cells in the lymph node (upper panel) and lungs (bottom panel) of C57BL/6 and CXCR6^{GFP} after 3 days of infection with *K.pneumoniae*. **(C)** Flow cytometry plots and quantification of MAIT cells in the lymph node (upper panel) and lungs (bottom panel) of C57BL/6 and CCR7^{KO} after 3 days of infection with *K.pneumoniae*. Lines represent mean ± SEM, each dot is a mouse. In figures, asterisks stand for: *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001, and no symbol if the comparison was non-significant, unpaired two-tailed *t* test.

2.7 Lack of MAIT cells does not interrupt infiltrating immune cells and their functions

MAIT cells with cytotoxic programming induce antimicrobial functions. But in the case of MAIT cells in the lymph node, we observe a Th17-type response where MAIT cells protect *K.pneumoniae* by IL-17 secretion. To dissect the mechanism by which IL-17-producing MAIT cells contribute to host protection, we analyzed different immune cells that infiltrate the lymph nodes following infection. The absolute number of neutrophils and monocytes in the lymph node in B6.CAST wild-type (WT), B6.CAST MR1^{KO} and B6.CAST IL-17^{KO} mice were not significant **(Figures 7A, C)**. Next, we checked if a deficiency of MAIT cells or IL-17 could impair the functions of neutrophils or monocytes by measuring the production of reactive oxygen species (ROS). We incubated the single cell suspension of the lymph nodes from *K.pneumoniae* infected B6.CAST wild-type (WT), B6.CAST MR1^{KO} and B6.CAST IL-17^{KO} mice with dihydrorodamine 123 (DHR 123). DHR 123 can passively diffuse across membranes where it is oxidized to the cationic rhodamine 123 and exhibits fluorescence that can be detected by flow cytometry. Overall ROS levels as well as ROS levels in the neutrophils and monocytes remained unchanged in all the strains **(Figures 7B and D)**. These results indicate that MAIT cells do not control the influx of infiltrating immune cells into the lymph node.

Altogether, we show that activated MAIT cells in the lymph node are crucial for bacterial pneumoniae infection control. Mechanistically, MAIT cells produce IL-17 that participates in protecting against bacteria.

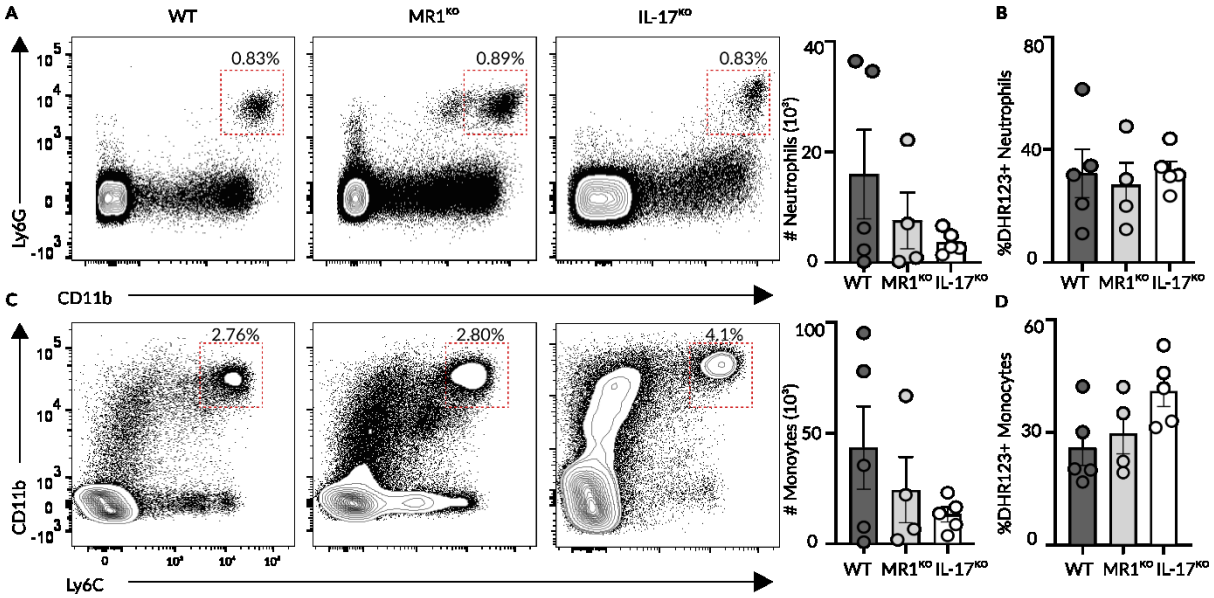


Figure 7. Neutrophils and monocytes upon *K.pneumoniae* infection. (A and C) Flow cytometry plots and absolute number of Neutrophils (A) and monocytes (C) in the mediastinal lymph node of B6-CAST-WT, B6-CAST-MR1^{KO},and B6-CAST IL-17^{KO} mice 3 days post-infection with *K.pneumoniae*. **(B and D)** Percentage of DHR123 staining positive neutrophils (B) and monocytes (D) in the mediastinal lymph node of B6-CAST-WT, B6-CAST-MR1^{KO}, and B6-CAST IL-17^{KO} mice 3 days post-infection with *K.pneumoniae*.

GRAPHICAL ABSTRACTS

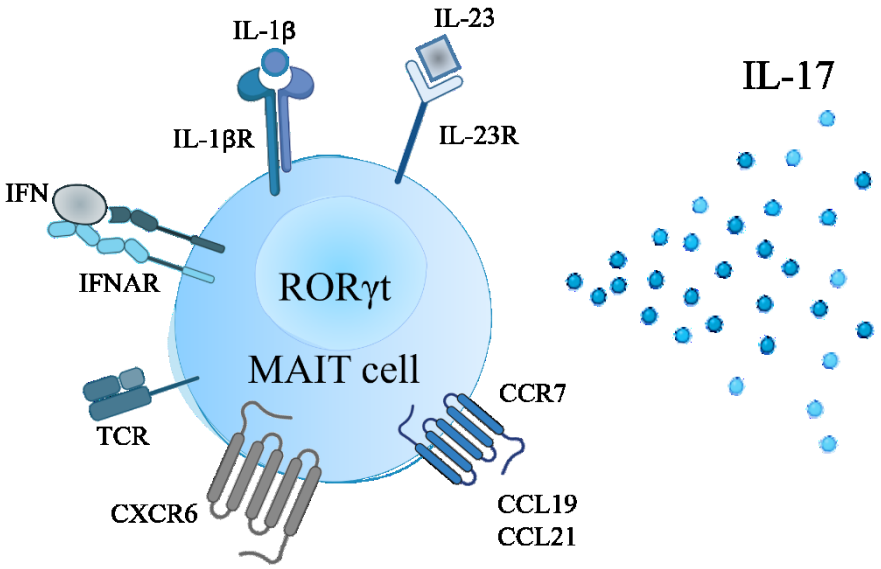


Figure 9. The phenotype of a MAIT cell in the mediastinal lymph node upon respiratory infection in the absence of TCR agonist. RORγt expressing MAIT cell gets activated by interaction of IFNAR receptor with type-I interferon and does not involve MR1-dependent TCR interaction for activation. IL-17 production in a lymph node MAIT cell is triggered by IL-1 and IL-23 receptors signaling synergistically. CXCR6 downregulation and CCR7 upregulation allow their retention in the lymph node.

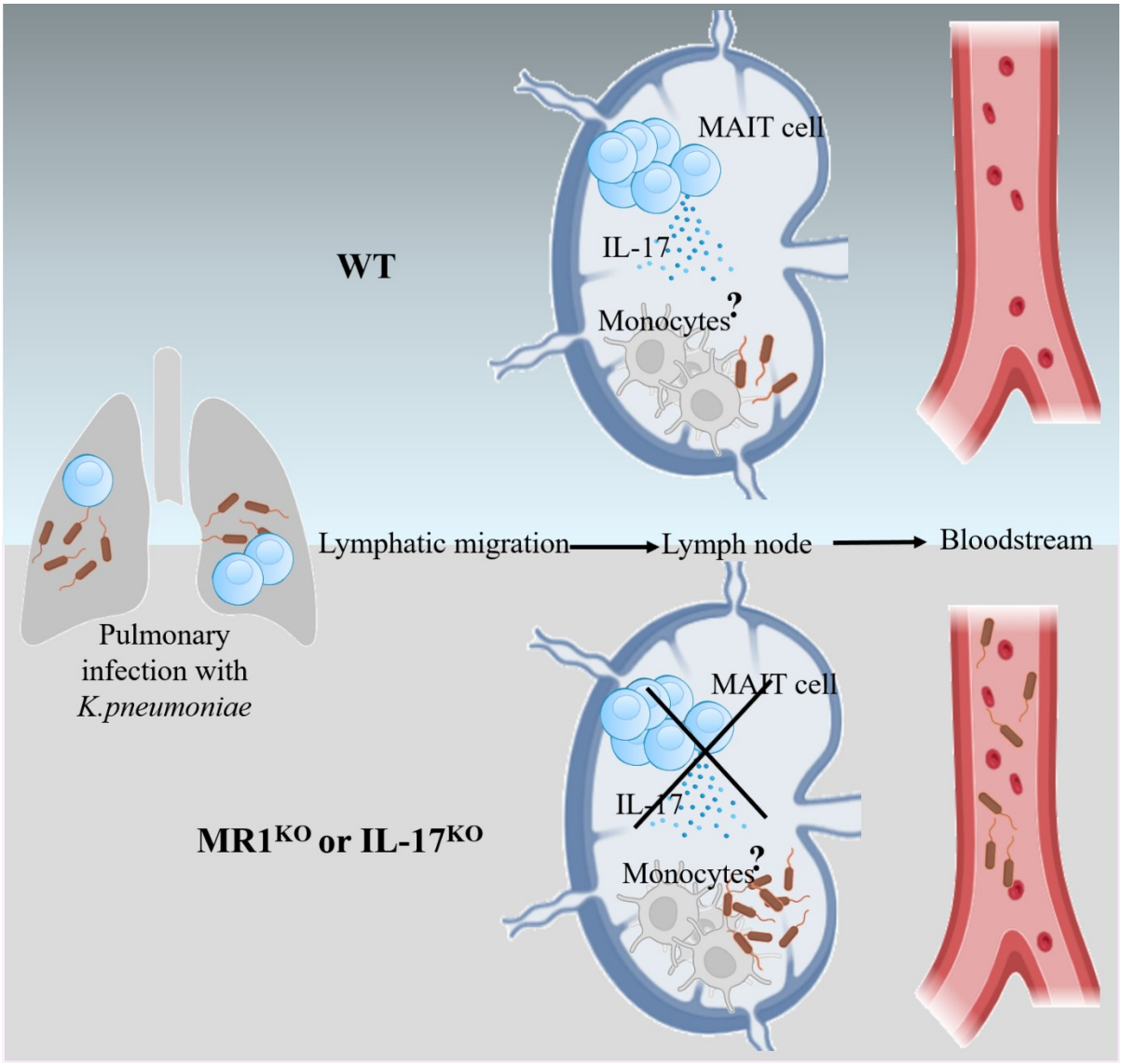


Figure 10. *K.pneumoniae* infection response during respiratory infection in WT, MR1^{KO}, and IL-17^{KO} mice. Lymphatic migration of MAIT cells into the mediastinal lymph node from the lungs upon *K.pneumoniae* infection is followed by IL-17 production. IL-17 secretion helps contain and resolve infection in the lymph node of WT possibly by supporting the phagocytosis of bacteria by monocytes. Incomplete resolution of bacteria in the lymph node in the absence of MAIT cells in MR1^{KO} mice or the absence of IL-17 in IL-17^{KO} mice causes the spread of bacteria into the bloodstream.

DISCUSSION AND PERSPECTIVES

1) MAIT cells at mucosal sites vs in the lymph node, the lymphatic connection

As their name suggests, MAIT cells are particularly enriched at the mucosal sites. Their multifaceted roles at mucosal sites predominantly in the lungs have been broadly described in the context of infection. The novelty of this study is that we report the dynamics of MAIT cells in the lung-draining lymph node that are actively engaged during pulmonary infection by upregulating activation markers (CD69 and CD25) and producing IL-17 as their main effector cytokine. A previous study with the same infection system highlighted that pulmonary MAIT cells drive Th1/cytotoxic effector functions (López-Rodríguez et al., 2023), our study highlights a completely different transcriptomic profile of MAIT cells that enter lymph node via lymphatics from the lung to drive Th17 effector functions by producing IL-17. One may question why MAIT cells in these two organs display different profiles. A possible explanation is that MAIT cells may transition from Th1 to Th17-like T cells upon entering the lymph node as a result of exposure to IL-23 and IL-1 signaling. This plasticity of MAIT cells has been previously observed in the case of human MAIT cells where the exposure to IL-23 and IL-1β drove MAIT cells from producing IFNγ to IL-17 (Kurioka et al., 2017). Indeed, in vivo blocking of IL-23 and IL-1 signaling skewed IL-17 production by MAIT cells in our experimental setting, indicating that similar plasticity may be acquired by murine MAIT cells.

Lymph nodes are a hub of immune cells and the lymphatic course that immune cells from the tissue undertake is an important phenomenon for generating cascades of immune responses. Firstly, this lymphatic migration relays the information from the tissue to the lymph node which is enriched with immune cells like dendritic cells, macrophages, conventional T cells, and B cells that orchestrate the local and systemic immune responses. Secondly, lymph nodes are pathogen-filtering tissue that actively decides the disease progression by controlling entry into the circulation. If the pathogen enters the circulation, it leads to systemic infection (sepsis) which can be fatal for the host. We previously reported that the lymphatic migration of unconventional T cells fine-tunes the immune system to generate a site-specific immune response (Ataide et al., 2022). Therefore, our findings lay the groundwork to thoroughly understand the functions of immune players of the respiratory system by concurrently examining their dynamics within the tissue and the draining lymph nodes.

2) TCR vs cytokine-based MAIT cell activation

We used a unique approach to understand the dynamics of MAIT cells in the lymph node by using multiple stimuli like 5-OP-RU (a known agonist of MAIT cells that directly interacts with the TCR of MAIT cells), Influenza virus, and *K.pneumoniae*. We know that Influenza virus infection results in the activation of MAIT cells in a TCR-independent way as there are no vitamin metabolites produced to allow MR1-based presentation thus the activation is driven by a combination of cytokine signalings that have been previously investigated (Wilgenburg et al., 2018). However, our experiments with the Nur77-GFP transgenic mouse model revealed that MAIT cells during *K.pneumomiae* infection do not get activated via their TCR. This is an interesting finding because theoretically the TCR of MAIT cells should be engaged as these bacteria encode genes involved in the riboflavin synthesis metabolic pathway (*Klebsiella Pneumoniae MNCRE69 Flavin Biosynthesis I (Bacteria and Plants)*, n.d.; Vitreschak et al., 2002). Moreover, pulmonary infection with a mutant *K. pneumoniae* strain with a deleted *ribD* gene (encodes bifunctional enzymes required in the riboflavin pathway by microbes) also resulted in the activation of MAIT cells (López-Rodríguez et al., 2023). Therefore, activation of MAIT cells in *K.pneumoniae* infection occurs through the MR1-independent mechanism. An early study with MAIT cells exposed to BMDC previously infected with a wide range of bacterial (including *K.*pneumoniae) and fungal pathogens permitted the activation of MAIT cells (Le Bourhis et al., 2010). However, in this study, they demonstrated that BMDCs with exposure to viruses did not activate MAIT cells. They concluded that MAIT cells respond "preferentially" to bacteria and yeasts based on this infection setup. In the later years, it was reported that MAIT cells respond to a wide range of viruses including the Influenza virus which reflects the flaws with this study model that drew conclusions based on antigen exposure and not on the fact that MAIT cells can also get activated by cytokines.

One may question why would a MAIT cell opt for cytokine signaling over TCR interaction for activation during bacterial infection. One possible explanation could be that the retention of MR1 molecules in the endoplasmic reticulum may occur which could skew the vitamin metabolite presentation. Indeed, one study demonstrated that compound DB28 (3-[(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)formamido]propanoic acid) and its ester derivative NV18.1 (methyl 3-[(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)formamido]propanoate) can downregulate MR1 presentation in an in vitro model of myeloid cells (THP1 cells) as well as EBV transformed B cells. A similar immune evasion strategy has been found in various

infection models like *Salmonella typhimurium*, *Mycobacterium tuberculosis*, and *Brucella abortus* where the pathogen evades immune detection by inducing downregulation of MHC-II (Ghorpade et al., 2013; Milillo et al., 2017, 2019; Van Parys et al., 2012). The evolution of bacterial species can be responsible for the decision-making which could be addressed by simply measuring the total MR1 transcripts or MR1 on protein level in species known for inducing TCR-based selection like *Legionella longbeachae* versus *K.pneumoniae* in a controlled in vitro system like THP-1 cells infection **(Figure 1)**.

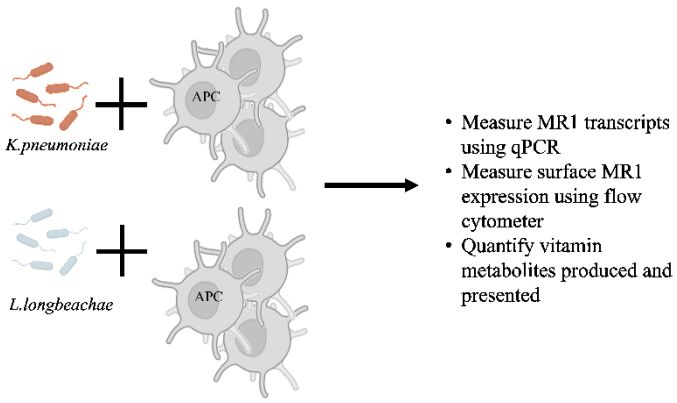


Figure 8. Proposal of an experimental setup to detect downregulation of MR1 during bacterial infection. Infection of Antigen-presenting cells with either *K.pneumoniae* or *L.longbeachae* followed by different quantifying methods.

Another reason why we do not observe the engagement of TCR during *K.pneumoniae* infection could be because the evolutionarily conserved MR1 binding pocket that binds to the vitamin metabolites does not undergo conformational changes due to the novel nature of the ligands associated with this bacteria. A full-scale proteomic analysis of *K.pneumoniae* can be performed to compare the ligands' nature to those bacteria that lead to MR1-dependent activation of MAIT cells.

It may seem like a far-fetched idea but the TCR of MAIT cell could have also gone through minor epigenetic changes along the evolution that make MAIT TCR necessary for selection within the thymus but dispensable for antigen recognition in certain cases when cytokine signaling could act as a rapid and efficient way to get activated. For example, evolutionary pressures from chronic viral infections, such as with Cytomegalovirus (CMV), have led to epigenetic adaptations in memory T cells that exhibit distinct epigenetic landscapes that keep TCR signaling pathways primed for rapid reactivation. These epigenetic changes are thought

to enhance immune memory persistence and efficiency, traits that have evolved to support host survival against long-term infections. One such epigenetic marker is H3K27me3 that is selectively removed from immune effector genes in CMV-specific T cells, allowing these cells to retain high functionality over time despite the antigen's persistence (Kay Chung et al., 2021). Malaria is another instance where the interaction of pathogens with immune cells led to evolutionary pressure on TCRs to enhance their ability to recognize a broader range of antigens. For example, *Plasmodium falciparum* uses PfEMP1 (Plasmodium falciparum erythrocyte membrane protein 1) which displays extensive polymorphisms, allowing the parasite to continually change its surface antigens and evade immune detection (Otto et al., 2019). At the same time, studies suggest that populations heavily exposed to *P. falciparum* malaria have developed a more diverse TCR repertoire, allowing them to recognize and respond to a broader range of *P. falciparum* antigens. This increased TCR diversity and cross-reactivity are thought to be an evolutionary adaptation to the pathogen's antigenic variation (Doolan & Martinez-Alier, 2006).

The treatment with different stimuli (5-OP-RU, Influenza, and *K.pneumoniae*) resulted in imprinting different transcriptomic programs within the lymph node MAIT cells. The TLR signaling varies depending on the nature of the antigens. For example, viral nucleic acids (dsRNA, ssRNA, CpG oligodeoxynucleotides) are detected by the endosomal TLRs (3, 7, 8, 9), whereas components of bacteria (peptidoglycans/lipoproteins, lipopolysaccharides) are detected by the cell surface TLRs (2, 4) (Hedayat et al., 2011; N. Lee et al., 2013). The nature of the MAIT cell transcriptome can depend on the TLRs engaged in the antigen-presenting cell. As we observed a dominant antiviral response oriented type I IFN signature in MAIT cells in the lymph node of Influenza infected mice that is a result of cytokines released from TLR3, TLR7 engaged antigen processing cells (Y. Chen et al., 2021; Saitoh et al., 2017).

3) The functional benefits of MAIT cell localization in the lymph node beyond tissue repair and maintaining homeostasis

An important property of pulmonary MAIT cells that has been broadly described in the context of infection is their ability to kill infected cells by inducing cytotoxicity which occurs primarily via granule exocytosis and relies on the components that enable the destruction of a target cell like perforin and granzymes. In this study, we present MAIT cells with a Th17-type profile with IL-17 as the main effector cytokine in the lymph node. IL-17 cytokine play diverse roles while providing antimicrobial activity. For instance, IL-17A and IL-17F induce chemokines in

epithelial cells that drive neutrophil and monocyte recruitment, contributing to antimicrobial activity against extracellular bacteria (Meller et al., 2015).

IL-17 deficiency in mice reduces mucosal immunity against *Streptococcus pyogenes*, resulting in increased bacterial dissemination to systemic sites (Mills et al., 2024). On the other hand, IL-17 is also implicated in the immune response to *Mycoplasma pneumoniae*, where it contributes to lung inflammation and the pathogenesis of respiratory diseases (Mills et al., 2024). While IL-17 is essential for combating respiratory infections, its dysregulation can lead to excessive inflammation, potentially exacerbating conditions like asthma and chronic obstructive pulmonary disease (COPD). This dual role underscores the complexity of IL-17 in respiratory immunity. In addition to infection, they are also important for maintaining tissue homeostasis while enhancing the secretion of antimicrobial peptides from Paneth cells in the ileum, crucial for maintaining gut microbiota balance and preventing inflammation shows (Brabec et al., 2023). Therefore, a better understanding of IL-17 levels can be focused on their role in pathogenesis as well as in homeostasis. By fine-tuning the activation of local and draining MAIT cells in the affected organs, the levels of IL-17 can be manipulated for therapeutic purposes. For example, intranasal treatment with 5-OP-RU in case of infections where IL-17 provides antimicrobial functions can lead to an infiltration of IL-17-producing MAIT cells.

MAIT cells are often associated with maintaining tissue integrity (du Halgouet et al., 2023). Therefore, the possibility that MAIT cells' arrival could be associated with keeping the tissues (lymph node and lungs) intact during a pathogenic breach is inevitable. Along with triggering antimicrobial activities in the tissues, some MAIT cells could also act as tissue stabilizers by IL-17, IL-22, or amphiregulin production. Indeed, IL-17 has already been shown to induce wound healing by activating HIF1α which drives glycolysis in wound front epithelia (Konieczny et al., 2022). A similar mechanism could be involved for maintaining tissue integrity in case of infection where there is a constant trafficking of immune cells.

Looking at our infection models and previous findings on the functional aspects of IL-17-producing MAIT cells, a transcriptional switching mechanism could be involved that directs these cells towards performing antimicrobial functions instead of tissue healing functions. This could be upstream signal-dependent, organ/niche-dependent, time-dependent, or all of the above. Many signals can modulate Amphiregulin (involved in tissue healing) expression like

IL-23, Tregs, oxidative stress, and hypoxia. In response to this downregulation, MAIT cells might get triggered to produce antimicrobial factors.

4) CXCR6 and CCR7 receptors-based localization of MAIT cells in the lymph node

With the help of scRNA seq data and CXCR6 and CCR7 knock-out models, we showed that the localization of MAIT cells within lymph nodes is CXCR6 and CCR7-dependent. The $CXCR6^{GFP}$ model displayed a significant reduction in the number of MAIT cells in the lymph node followed by a reduced (not significant) number of MAIT cells in the lungs. CXCR6 expression leads to an active mobilization and retention in the lung following respiratory infection in several other immune cells like NKT cells, and ILCs (Chea et al., 2015; Motsinger et al., 2002). Therefore, $CXCR6^{+}$ MAIT cells may be recruited from the lymph node to the lung to perform antimicrobial activities and undergo further activation to enter the lung via circulation in our infection setting. However, in another study, it was demonstrated that CXCR6 supports the infiltration of MAIT cells but not the local proliferation and retention of MAIT cells during pulmonary *Francisella tularensis* infection indicating that other chemokine signaling is responsible for the retention of MAIT cells in the lung (H. Yu et al., 2020c). Factors such as the nature of pathogens and the time point of organ harvest could lead to different observations.

In CCR7 knock-out model, we observe a tendency of reduced MAIT cell number in the lymph node with a minor (not significant) increase in the accumulation of MAIT cells in the lung. However, we cannot draw definite conclusions regarding the role of CCR7 with such a lower sample size (n=3). Repetition of this experiment with a bigger sample size could help us demonstrate the role of CCR7 receptor in this pulmonary infection setting. CCR7, which has been implicated in the retention of T cells in the lymph node (Kobayashi et al., 2017; Laufer et al., 2019; H. Wang & Hogquist, 2018) is absent on the surface of MAIT cells that are in the circulation (Voillet et al., 2018). To better understand the role of CCR7 in the localization of MAIT cells, we aim to compare MAIT cell numbers in the CCR7 knock-out ($CCR7^{KO}$) mouse model and wildtype. A statistically significant decline in the number of MAIT cells in the lymph nodes of $CCR7^{KO}$ mice should indicate the importance of CCR7 in the retention of MAIT cells in the lymph node.

Using confocal imaging on mediastinal lymph nodes of RoRγt-GFP mice, I showed the localization of MAIT cells outside B cell follicle at the site of pathogen entry post-immunization which were diminished in the lymph nodes of NI mice. With the help of our

scRNA seq analysis, I observe an interplay between CXCR6 and CCR7 receptors in the localization of MAIT cells upon infection regarding lymph node residency. A spatio-temporal study with CXCR6 and CCR7 staining could reveal the expression profile of these two markers and if the distribution and localization differ between $CXCR^{+}$/ $CXCR6^{-}$ and $CCR7^{+}$/$CCR7^{-}$ MAIT cells. The proximity of either of these populations to other immune cells like macrophages or non-immune cells like fibroblastic reticular cells could reflect the interactive profile of MAIT cells which conventional methods such as flow cytometry cannot. Intravital microscopy with *Klebsiella pneumoniae* bacteria incorporated with GFP could also help track the localization of bacteria in the respiratory organs.

5) IL-17 as the main effector cytokine of lymph node residing MAIT cell

We found that type I interferon signaling activated MAIT cells during *K.pneumoniae* infection but the aboragtion of this signaling did not affect IL-17 production by MAIT cells. However, this signaling was important for bacteria clearance as $IFNAR^{KO}$ mice displayed enhanced bacterial load in the lymph node. This can be because $IFNAR^{KO}$ mice which cannot control bacteria in the lung, leak or expand the infection into the lymph node. Additionally, our results strongly implicate a synergistic role of IL-1 and IL-23 signaling for the induction of IL-17 production in MAIT cells. Combining these two findings, regarding signals for activation and induction of effector functions, it can be deduced that cytokine receptors of MAIT cells are highly engaged during bacterial infection. We did not investigate the source (or sources) of type I interferons, IL-1 and IL-23 in this infection system. The type I interferon signaling driving MAIT cell activation may occur in the lung parenchyma and the cells with Th1 responses may stay in the lung whereas, MAIT cells with innate Th17 type responses migrate into the lymph node where they are further triggered by IL-1 and IL-23 exposure to produce IL-17. An in vivo tracking system with labeled MAIT cells can be developed to confirm if MAIT cells undergo this trafficking route to finally display effector functions in the lymph node. Moreover, an in vivo depletion of IL-1 and L-23 cytokine drivers can also be performed to further establish the link between MAIT cells with other immune and non-immune cells that are responsible for IL-17 production in the lymph node.

It has been shown that IL-17R signaling in the lung epithelium is critical for establishing chemokine gradients that are essential for mucosal immunity against pulmonary infection with *K. pneumoniae* (K. Chen et al., 2016). Mechanistically, they show that in the absence of IL-17 receptors on lung epithelial cells, neutrophil recruitment is impaired via dampened CXCL5

expression which further leads to defective bacterial clearance. These findings show that IL-17R is important, however in our results using IL-17^{KO} mice where the cytokine is absent, we do not observe any defect in neutrophil recruitment. This striking contrast between their findings and our results can be further dissected by quantifying the chemoattractants involved in neutrophil recruitment during infection with *K.pneumoniae*. While most of the mechanisms described in case of infection are based on Neutrophil recruitment in an IL-17-dependent manner, a study by Pamer and group showed that IL-17 aids the clearance of *K.pneumoniae* by acting on monocytes (Xiong et al., 2016). They demonstrated that an in vitro culture of bone marrow isolated monocytes with *K.pneumoniae* resulted in enhanced phagocytosis and ROS activity by monocytes supplemented with recombinant IL-17 compared to the culture without IL-17. In vivo, the infection model that we described, IL-17^{KO} did not display a reduction in the number of monocytes or ROS production. However, it is possible that the phagocytic activity of the monocytes in IL-17^{KO} mice was obstructed or decreased by the absence of IL-17. To confirm this possibility, I plan to measure the bacterial load in a co-culture of bone marrow cells with *K.pneumoniae* and in the absence or presence of IL-17 using the protocol described by Pamer group (Xiong et al., 2016). Another mechanism that has been described to facilitate bacteria clearance in an IL-17-dependent manner is by enhancing the factors for monocytes/macrophages like G-CSF or TNF-α (Cruz et al., 2015; Karalyan et al., 2016). Indeed, Pamer and group reported a significant reduction of TNFα on mRNA level of IL17^{KO} mice compared to WT in the lungs post-infection. I plan to quantify TNFα produced by monocytes in the lymph nodes of WT and IL-17^{KO} in our infection model of *K.pneumoniae*. A reduction would imply a direct effect of IL-17 on monocytes activation which is probably reduced in MAIT deficient and IL-17^{KO} mice. A more thorough understanding of the mechanism of action implemented by IL-17 during respiratory infection with *K.pneumoniae* can help strategize the therapeutics for early mitigation of infections.

6) Advantage over conventional T cells

This study details the functions of MAIT cells in the lymph nodes that have not been explored before. Due to their abundance, the majority of the work in the field of infectious diseases carried out is centralized to conventional CD4$^+$ and CD8$^+$ T cells that require a series of steps for efficient activation and perform their functions. The advantage that unconventional T cells such as MAIT cells have over conventional T cells is their innate capabilities getting rapidly activated by quickly mobilizing using cargo of cues like surrounding cytokines for exerting effector functions. Therefore, MAIT cells show potential for being targeted for enhancing

protective roles in instances where conventional T cells cannot be relied upon due to their longer activation period.

Of course, these conventional T cells have pivotal functions but the fact that unconventional T cells even in lower frequencies play significant and irreplaceable roles makes them of high importance. For example, we observed that there are no T cells that can compensate for the IL-17 production in the absence of MAIT cells in the lymph nodes post-infection with Influenza, and *K.pneumoniae*. In contrast, MAIT cells, NKT cells, and γδT cells play compensatory roles for IL-17 production in the skin-draining lymph node following infection with *S.aureus* (Ataide et al., 2022). This implies a site-specific, pathogen-oriented function of these unconventional T cells.

LIMITATIONS

In this study, we did not focus on investigating the importance of MAIT cells in rescuing animals from mortality post-infection. However, the work of Wilgenburg et al. with the Influenza virus using a lethal dose (100 pfu) in the respiratory infection system details how MAIT cell-deficient mice show enhanced mortality making their presence vital (Wilgenburg et al., 2018). We used 5 pfu as the infection dose for Influenza virus-related experiments that trigger an efficient immune response without exaggerating the system. Due to ethical constraints, we could not replicate the same set of experiments. Nonetheless, we addressed several outstanding questions that had not been addressed before by implementing different strategies in different immunization models.

Another limitation of this study is that we did not explore how non-immune cells interact like fibroblasts with MAIT cells. Along with antigen-presenting cells, some epithelial cells produce IL-23 (Kim et al., 2024). It would be useful to study MAIT cells along with their niche in different tissues, especially in the context of cancer where the microenvironment plays a major role in deciding the disease progression.

While exploring the functions of IL-17-producing MAIT cells, we did not look into the functional importance of IFN-γ producing MAIT cells in the lung and lymph nodes. The main reason is the scarcity of these cells where we observed the majority of MAIT cells within the lymph node bear Th17-type properties.

Even though all these experiments were carried out in a very controlled environment, the fact that the animal models used are from different sources and crossed with C57BL/6 and CAST backgrounds cannot be dismissed. Therefore, future studies that utilize the findings in this study as a basis of their experimental setup should consider performing similarly with the same mouse models for deducing reliable conclusions.

There are several phenotypes observed in human MAIT cells that are not present in murine MAIT cells (such as the expression of CD161) and vice versa. Thus, it is not possible to deduce if our findings with animal models reflect the mechanism adopted by MAIT cells in humans.

Chapter 3: Role of MAIT Cells in Adaptive Immunity

INTRODUCTION

Adaptive immunity is a tightly regulated interplay between T and B lymphocytes that fine-tunes pathogen-specific immunologic effector pathways and generates immunologic memory. Adaptive immune responses originate in the secondary lymphoid organs such as lymph nodes and the spleen, which capture circulating antigens from the lymph and blood, respectively (Charles A Janeway et al., 2001).

The activation of adaptive immune response is highly dependent on the key players of innate immunity. Antigen-presenting cells sense the environmental antigens and danger signals (ligands for TLR and other systems of pattern-recognition receptors) from barriers such as skin and mucosal sites, where pathogen encounters are most likely (Batista & Harwood, 2009). APCs sample exogenous proteins through phagocytosis, leading to the expression of processed pathogen-derived peptide bound to MHC molecule and migration to the regional lymph nodes, where interaction with T cells can occur. Simultaneous binding to MHC/peptide on the APCs by TCRs and CD4/CD8 brings the cytosolic domains of these molecules into proximity. A subset of the conventional $CD4^+$ T cells residing in the lymph node and the spleen known as T follicular helper cells (T_{FH}) trigger B-cell activation (Charles A Janeway et al., 2001; Iwasaki & Medzhitov, 2010).

For B cells, the first step of activation involves recognition of antigens through the B cell receptor (BCR) on the APCs (Randolph et al., 2005). However, recognition of antigen is not enough to induce the proliferation and differentiation of B cells. Once the BCR is triggered, the BCR-antigen complex is internalized by the cell and delivered to the endosomal compartments by a phenomenon known as antigen processing. Antigen processing allows B cells to upregulate CCR7, which is the receptor for CCL19 and CCL21. These chemokines are present in high levels in the T cell zone, and induce the migration of activated B cells to the B-T cell border (Garside et al., 1998, Reif et al., 2002). At this point, B cells make contact with antigen-specific T_{FH} facilitated by the T cell recognition of MHC/peptide on the surface of B cells (Amigorena et al., 1994). This is the second signal that completes the activation of B cells.

B-T cell interactions trigger CD40 ligand (CD40-L) expression on T cells, which binds to CD40 on B cells, promoting their proliferation. Antigen recognition increases MHCII levels

on B cells, enhancing antigen presentation to T cells. Activation also raises ICAM-1 and LFA-1 levels on B cells, facilitating close contact with T cells for effective collaboration. Co-stimulatory molecules like CD80, CD86, and ICOS also increase, interacting with CD28 and ICOS-L on T cells, supporting B cell proliferation and differentiation into antibody-secreting cells. Additionally, T cells secrete cytokines such as IL-4 and IL-21, crucial for B cell differentiation into effector cells (Horton et al., 2022; Nutt et al., 2015; Shockett & Stavnezer, 1991).

Upon activation, B cells can either differentiate into extrafollicular plasma cells (PC) and produce the first wave of antibodies of the humoral response; mainly low-affinity IgM (MacLennan and Toellner, 2003) or they continue to proliferate forming structures known as germinal centers (GCs). The differentiation of B cells in the GC allows for the production of antibodies that are more efficient in the control of pathogens during infection. The differentiation of some GC B cells also results in the development of adaptive immune memory (Victora and Nussenzweig, 2012). In the germinal center, B cells can switch from producing IgM and IgD to other isotypes like IgG, IgA, and IgE through a process called class-switching. This involves gene rearrangement, where a DNA sequence between the VDJ unit and IgM/IgD genes is cut and joined to a sequence near another immunoglobulin C-region gene. This results in the production of antibodies of different isotypes: IgG, IgA, or IgE (Charles A Janeway et al., 2001). Class-switching is partly controlled by cytokines. For example, IL-4 and IL-13 promote switching to IgE, which can be antagonized by IFN-γ, and IL-10 and TGF-β promote switching to IgA (Horton et al., 2022; Nutt et al., 2015; Shockett & Stavnezer, 1991).

The secreted antibodies amplify the anti-microbial response in case of infection through exertion of different effector functions by building a complement system which is an immune complex formed by binding of antibodies to the Fc receptors of innate immune cells (Vidarsson, Dekkers and Rispens, 2014). The effector functions that are carried by the activation of the complement system are:

- **Phagocytosis**: Neutrophils and macrophages ingest and eliminate pathogens bound by Fc receptors.
- **Antibody-dependent cell-mediated toxicity (ADCC)**: pathogens bound specifically to antibodies are destroyed by innate immune cells such as NK cells, granulocytes and macrophages by secreting granzymes, perforins and lysosomal enzymes (Delves and Roitt, 2000).

- **Degranulation**: Mast cells, neutrophils, eosinophils, and macrophages release inflammatory mediators (Bryceson et al., 2005).

Regulation of B cell responses by MAIT cells

The definition of MAIT cells as innate-like lymphocytes is based on the immediate response that they exert upon pathogenic breach. This was also the case with NKT cells for a very long time until their functions were investigated in the regulation of adaptive immunity.

The crosstalk of MAIT cells during infection with several components of the immune system as described above implies that there are dimensions in which the role of MAIT cells could be of importance but have not been investigated yet. A 2022 study provided evidence that MAIT cells provide B cell help *in vivo* following mucosal challenge with *Vibrio cholerae* by promoting B cell differentiation and production of *V. cholerae* specific IgA antibodies in the serum (Jensen et al., 2022). They described a population of $CXCR5^+$ T follicular helper (T_{FH})-like MAIT cells ($MAIT_{FH}$) which supply IL-21, IL-10, IL-6, and BAFF which are important for B cell differentiation. More recently, another study revealed that priming MAIT cells with 5-A-RU/MG promotes CD40L-dependent activation of lung-associated DCs, expansion of TFH cells, and production of mucosal IgA (Pankhurst et al., 2023). This study provides evidence for the potential role of MAIT cells in vaccine-induced immunity. The limited literature on the relationship between humoral immunity and MAIT cells shows a dire need for thorough research.

RESULT

3.1 MAIT cell deficiency does not affect the differentiation of the Germinal center B cells compartment during respiratory infection with Influenza virus

To explore the role of MAIT cells in adaptive immunity, I compared the B cell responses in B6-MAIT^{CAST} mice (hereafter, referred to as WT) and MR1^{KO} mice, 9 days post-infection with 5 plaque-forming units (PFU) of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain in the lung-draining mediastinal lymph node (mLN). Germinal center B cells were identified as Fas+ (CD95, a marker of rapidly differentiating B cells) and GL-7 (clone of an antibody that recognizes sialylated glycans expressed on the surface of GC B cells) by cytometry analysis. There were no differences in the GC B cells between WT and MR1^{KO} mice 9 days post-infection in mLN **(Figure 1A)**. Moreover, the class-switched IgA, IgM, IgG1, IgG2b, and IgG2c in the GC compartment were not affected by the absence of MAIT cells **(Figure 1B)**. I also evaluated Influenza antigen hemagglutinin (HA) and nucleoprotein (NP) specific GC B cells and found no differences between WT and MR1^{KO} mice. This indicates that MAIT cells do not play significant role in the establishement of the GC compartment **(Figure 1C)**.

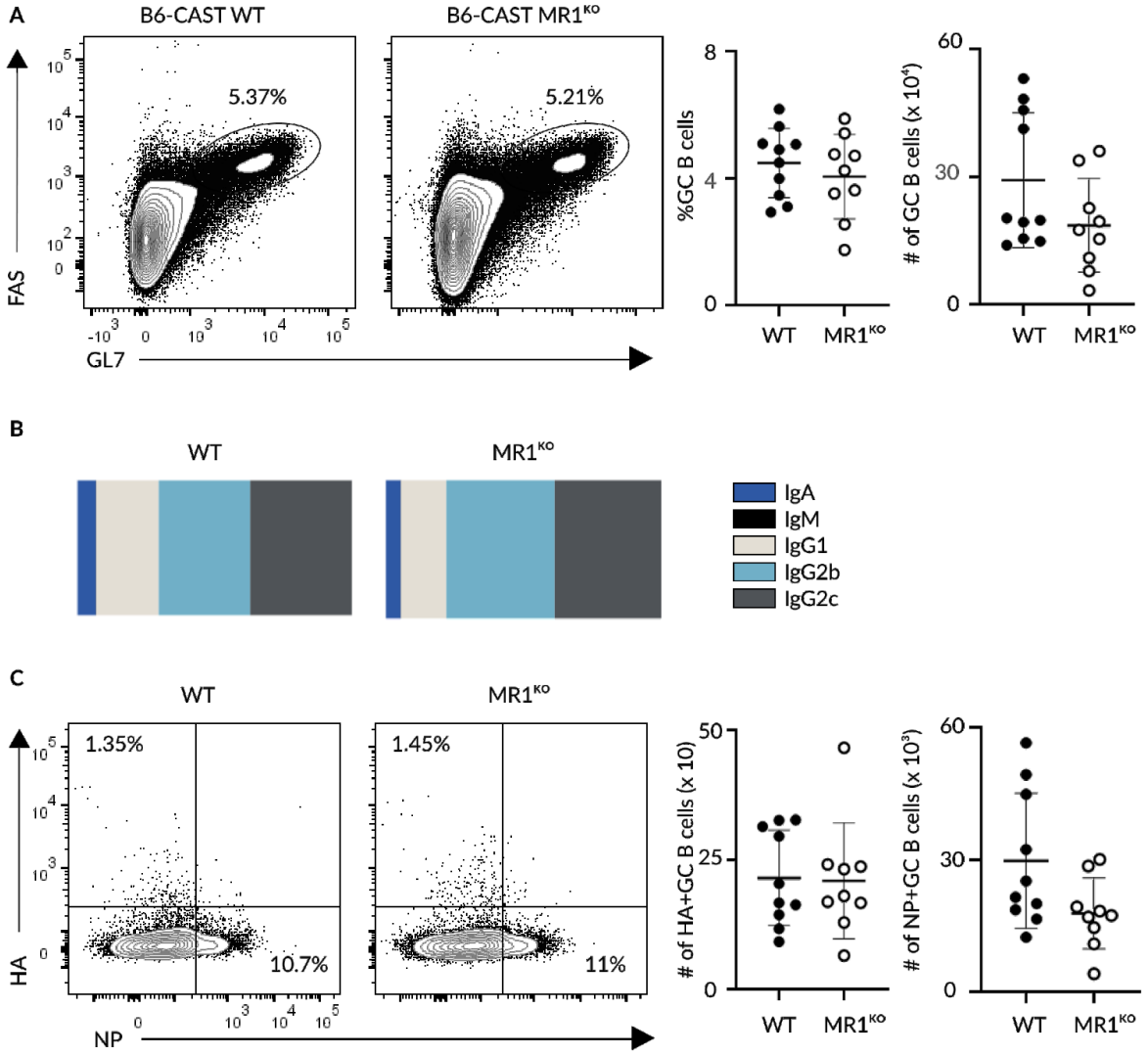


Figure 1. MAIT cells are not required for the regulation of germinal center compartment in the mediastinal lymph node during viral infection. WT and MR1^{KO} mice were infected with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain. Flow cytometry analysis and quantification of **(A)** Germinal center (GC) B cells identified as FAS^{+} GL7^{+} **(B)** Distribution of IgA, IgM, IgG1, IgG2b, and IgG2c class-switched GC B cells in WT and MR1^{KO} mice **(C)** Influenza antigen HA- and NP-specific GC B cells 9 days post-infection in the lung-draining mediastinal lymph node (mLN) of WT and MR1^{KO} mice. In all charts, each dot represents one mouse. Horizontal bars, mean; error bars, SEM. Student's t-test

3.2 MAIT cells regulate Plasmablast differentiation during viral infection

Next, I investigated the highly proliferating plasmablasts in WT and MR1^{KO} mice post-infection. I used CD138 (also known as Syndecan 1, a proteoglycan that is upregulated in mature antibody-secreting cells to avoid apoptosis) and CD93 (reinduced in B cells upon plasma cels differentiation that helps maintain antibodies titer) to identify Plasmablasts in mLN. The cells positive for CD138 marker indicate total Plasmablasts and the cells which are CD138^{+} CD93^{+} represent mature Plasmablasts. The percentage and number of total plasmablasts and mature Plasmablasts in MR1^{KO} were significantly reduced compared to the WT **(Figures 2A and B)**. The reduction in Plasmablasts is not reflected in the lower number of class-switched isotypes in the compartment **(Figure 2C)**. Even though in different organs, these findings are in line with the previous findings where MAIT cells were found to provide B cell help by inducing B cell differentiation from Naïve B cell to plasmablasts following intranasal challenge with *V.cholera (Jensen et al., 2022)*.

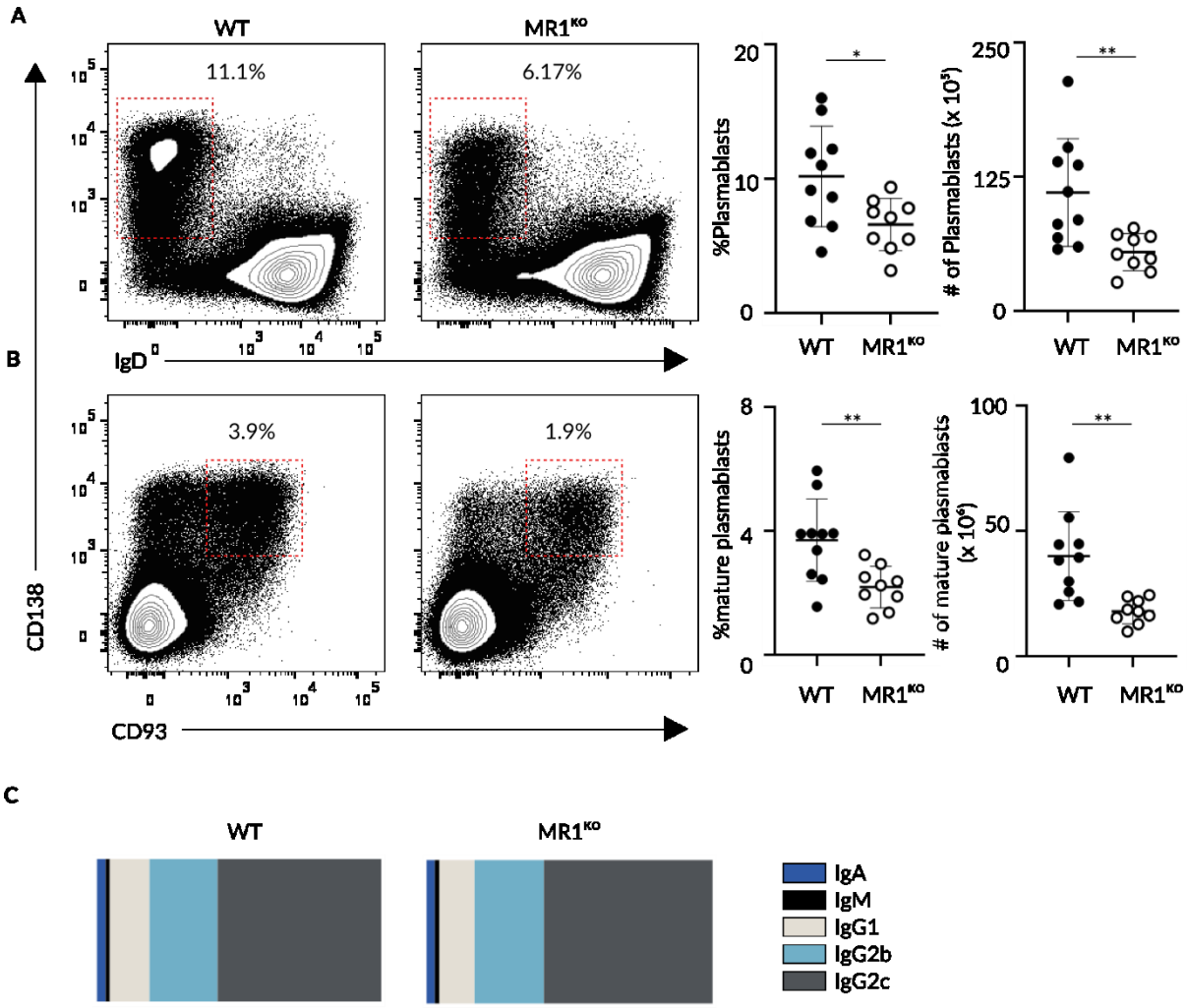


Figure 2. MAIT cells regulate plasmablast differentiation in the mLN upon viral infection. WT and MR1^{KO} mice were infected with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain. Flow cytometry analysis and quantification of **(A)** Total Plasmablasts identified as CD138^{+} IgD^{+} **(B)** Total mature plasmablasts identified as CD138^{+} CD93^{+} **(C)** Distribution of IgA, IgM, IgG1, IgG2b, and IgG2c plasmablasts in WT and MR1^{KO} mice. In all charts, each dot represents one mouse. Horizontal bars, mean; error bars, SEM. Student's t-test

3.3 MAIT cells regulate Influenza-specific IgA antibody in the broncho-alveolar space

Lungs are the primary site of respiratory infection where the pathogen is trapped by the antigen-specific antibodies secreted due to the rapid proliferation of B cells. I quantified Influenza protein NP (Nucleoprotein) specific IgA, IgG, and IgM titer in the broncho-alveolar lavage fluid of WT and MR1^{KO} mice by ELISA. Interestingly, influenza-borne antigen NP-specific IgA was significantly reduced by 4-fold in the BALF of MAIT cell-deficient mice **(Figure 3A)**. At the same time point and same system, IgM and IgG levels were not affected in the BALF of MR1^{KO} mice **(Figure 3A)**. However the total protein concentration in the BALF was unaffected in MR1^{KO} mice **(Figure 3C)**. While not necessarily stronger than other antibodies, IgA is specialized for mucosal protection, making it essential for guarding against lung infections and minimizing inflammation. Its reduced levels in MAIT-deficient mice highlight its key role in lung defense, particularly in the broncho-alveolar space.

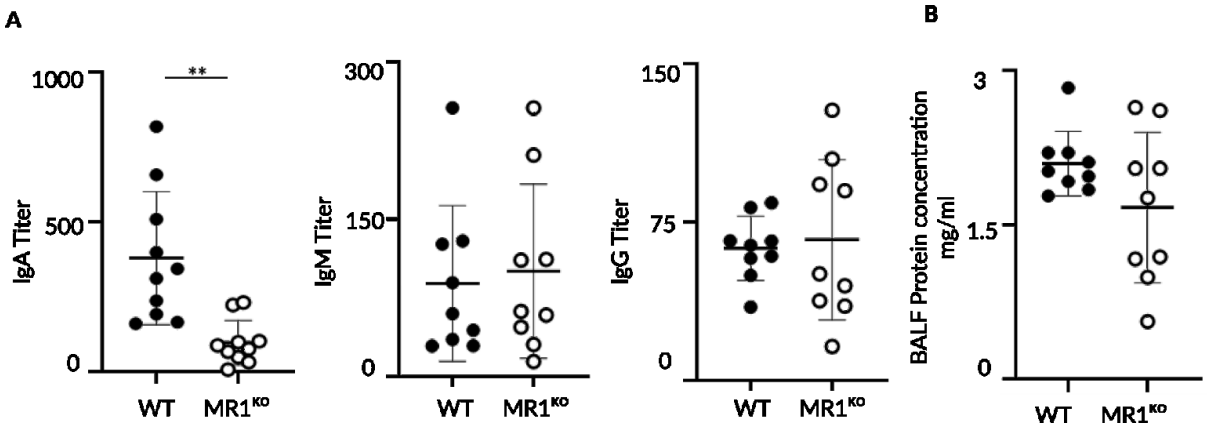


Figure 3. MAIT cells are required for Influenza specific IgA in the broncho-alveolar space during viral infection

WT and MR1^{KO} mice were infected with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain. Broncho-alveolar lavage fluid was collected from the lungs after euthanizing mice

in sterile cold PBS (70-80% recovery rate) after 9 days of infection. Influenza antigen nucleoprotein (NP) specific IgA, IgM, and IgG were measured by ELISA. **(B)** The total protein concentration of BALF was measured by a standard Bradford assay. In all charts, each dot represents one mouse. Horizontal bars, mean; error bars, SEM. Student's t-test

3.4 IL-17 is required for IgA-secretion but not necessary for plasmablast differentiation

In the result section of 'Role of MAIT cells in Innate immunity', I demonstrated that MAIT cells produce an early wave of IL-17 under the regulation IL-23 and IL-1 in mLN post-infection. To discern if the IL-17 produced by MAIT cells was responsible for reduced Plasmablast differentiation and IgA secretion in $MR1^{KO}$ mice, I analyzed PC differentiation and IgA secretion in $IL\text{-}17^{KO}$ mice 9 days post-infection with Influenza. There was no significant difference in the number of germinal center B cells, Plasmablasts and mature plasmablasts between the WT and $IL\text{-}17^{-KO}$ mice in the mLN **(Figures 4A and B)**. However, I observed a significant decrease (2.5-fold) in the NP-specific-IgA titer in BALF whereas IgG levels were unaffected **(Figure 4C)**. Based on these observations, I conclude that plasmablast differentiation is not influenced by IL-17 production. However, IL-17 is crucial for IgA secretion into the broncho-alveolar space.

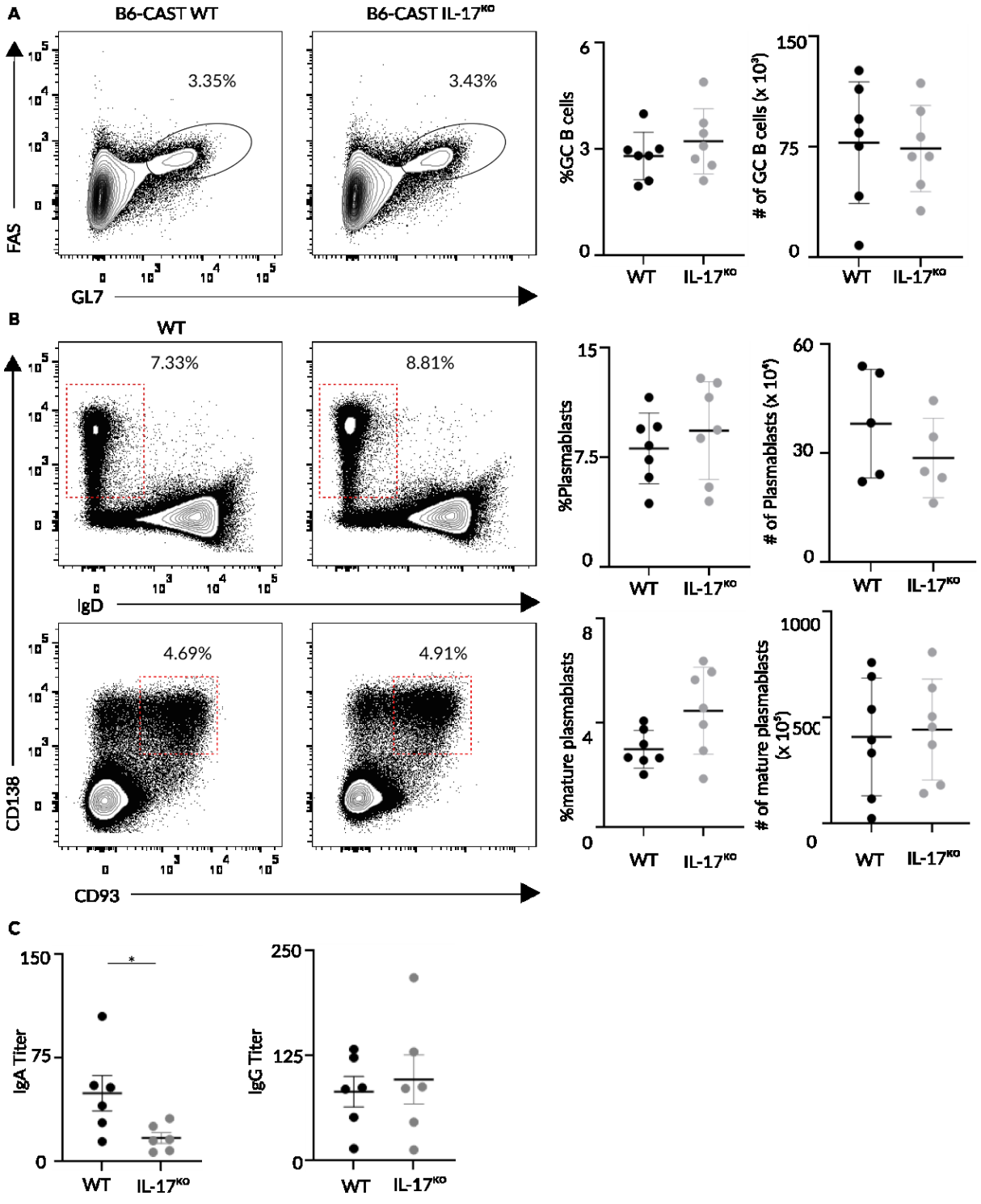


Figure 4. IL-17 is required for Influenza specific IgA in the broncho-alveolar space during viral infection

WT and IL-17^{KO} mice were infected with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain. Flow cytometry analysis and quantification of **(A)** Germinal center (GC) B cells identified as FAS^{+} GL7^{+} **(B)** Total Plasmablasts **(**upper panel**)** identified as CD138^{+} IgD^{+} and Total mature plasmablasts (lower panel) identified as CD138^{+} CD93^{+}. **(C)** Broncho-alveolar

lavage fluid was collected from the lungs after euthanizing mice in sterile cold PBS (70-80% recovery rate) after 9 days of infection. Influenza antigen nucleoprotein (NP) specific IgA and IgG were measured by ELISA. In all charts, each dot represents one mouse. Horizontal bars, mean; error bars, SEM. Student's t-test

3.5 MAIT cells are required for plasmablast differentiation during viral infection but not during bacterial infection

I demonstrated that MAIT cells regulate Plasmablast differentiation in the mLN 9 days post-infection with the Influenza virus and this regulation is IL-17 independent. I questioned if this response of Plasmablast differentiation under MAIT cell regulation was unique to Influenza virus infection. To answer this, I infected WT and $MR1^{KO}$ mice with 2000 pfu of vaccinia virus of western reserve strain (VV) intranasally and observed Plasmablast differentiation in the mLN. It is important to note that the tropism of the vaccinia virus is different from that of the influenza virus, as they belong to separate virus families. However, both viruses have the potential to cause acute lung infections (Hayasaka et al., 2007). The infection with VV did not lead to an effective immune response in 30% of the mice (distribution remained the same in WT and $MR1^{KO}$ mice evidenced by the inability to induce T_{FH} proliferation (identified as $CXCR5^{+}$ $PD\text{-}1^{+}$ $CD4^{+}$ $TCR\beta^{+}$) post-infection compared to the mice **(Figure 5C)**. I analyzed GC and Plasmablast differentiation excluding the subjects that remained unresponsive to the infection. I observed that Plasmablast differentiation was significantly decreased in $MR1^{KO}$ mice compared to WT **(Figure 5B)**. However, the GC compartment was unaffected by MAIT cell deficiency, a response that I also observed in both groups upon Influenza virus infection **(Figure 5A)**. This indicates that MAIT cells regulate Plasmablast differentiation during viral infection regardless of the viral nature. **Figure 5D** shows quantification of GC B cells, plasmablasts and mature plasmablasts in responsive as well as unresponsive mice.

To explore if MAIT cells regulate Plasmablast differentiation during bacterial infection as I observed during viral infection, I analyzed B cell responses in WT and $MR1^{KO}$ mice 9 days post-infection with *Klebsiella pneumoniae* 43816 strain. I observed no significant differences between WT and $MR1^{KO}$ mice in GC and PC compartments **(Figures 6A and B)**. This implies that a possible route of plasmablast regulation that MAIT cells imprint during viral infection differs from bacterial infection.

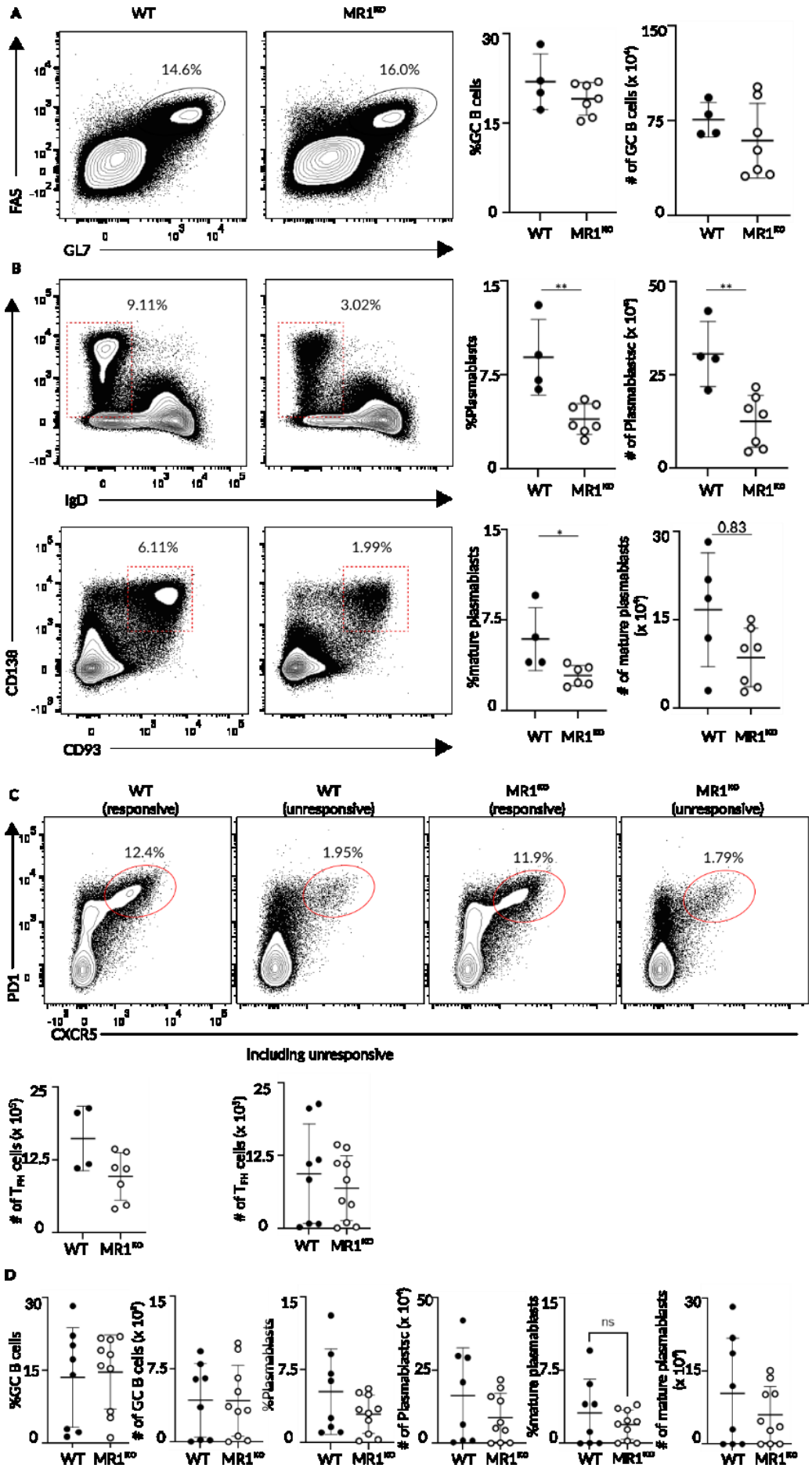

A
WT
MR1KO
14.6%
16.0%
FAS
GL7
%GC B cells
# of GC B cells (x 10⁴)
B
9.11%
3.02%
IgD
%Plasmablasts
# of Plasmablastsc (x 10⁴)
6.11%
1.99%
CD138
CD93
%mature plasmablasts
# of mature plasmablasts (x 10⁴)
0.83
C
WT (responsive)
WT (unresponsive)
MR1KO (responsive)
MR1KO (unresponsive)
12.4%
1.95%
11.9%
1.79%
PD1
CXCR5
Including unresponsive
# of TFH cells (x 10⁴)
D
ns

Figure 5. MAIT cells are required for plasmablast differentiation during viral infection

WT and $MR1^{KO}$ mice were infected with 2000 pfu of Vaccinia virus of Western reserve strain intranasally. Flow cytometry analysis and quantification of **(A)** Germinal center (GC) B cells identified as FAS^+ $GL7^+$ **(B)** Plasmablast (upper panel) identified aa $CD138^+$ and mature plasmablasts (lower panel). **(C)** T follicular helper cells identified as $CXCR5^+$ and $PD1^+$ in $CD4^+$ T cells population in vaccinia virus responsive and unresponsive mice. **(D)** Quantifications including unresponsive subjects. In all charts, each dot represents one mouse. Horizontal bars, mean; error bars, SEM. Student's t-test

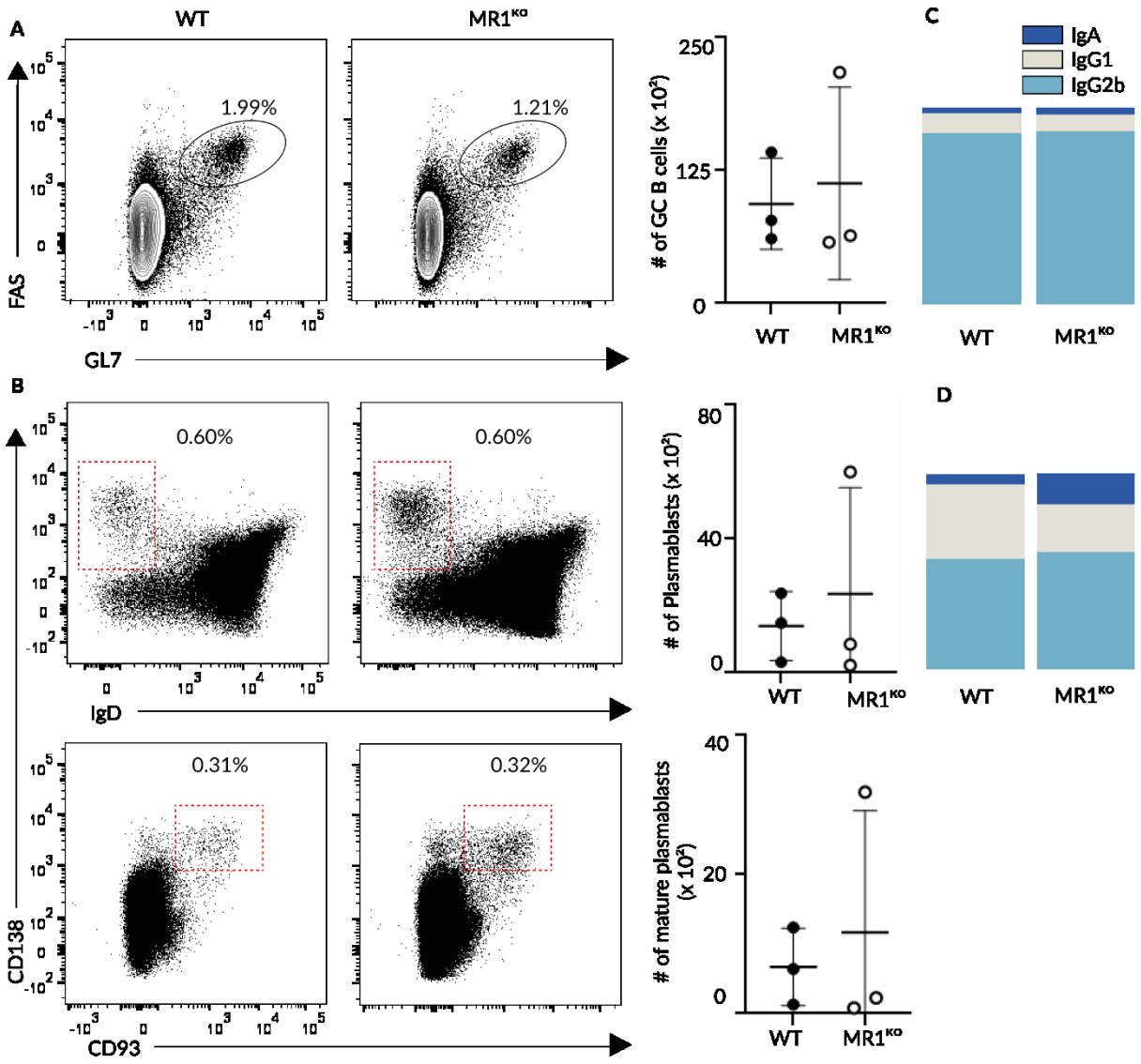


Figure 6. MAIT cells are not important for B cell help during bacterial infection. WT and MR1^{KO} mice were infected with 2 x 10^4 cfu of *Klebsiella pneumoniae* 43816 strain. Flow cytometry analysis and quantification of **(A)** Germinal center (GC) B cells identified as FAS$^+$ GL7$^+$ **(B)** Plasmablast identified as CD138$^+$ and mature plasmablast identified as CD138$^+$CD93$^+$ in mediastinal LN. **(C)** Distribution of IgA$^+$, IgG1$^+$, IgG2b$^+$ GC B cells in WT and MR1^{KO} mice **(D)** Distribution of IgA, IgG1, IgG2b plasmablasts in WT and MR1^{KO} mice. In all charts, each dot represents one mouse. Horizontal bars, mean; error bars, SEM. Student's t-test

3.6 There is no cognate interaction between B cell and MAIT cell during respiratory viral infection

To dissect the mechanism involved in B cell regulation by MAIT cells, we first asked if MAIT cells can act as follicular helper cells by directly providing factors that promote B cell activation followed by activation. A population of $CXCR5^+$ T follicular helper (T_{FH})- like MAIT cells ($MAIT_{FH}$) has been described to provide B cell help in case of *Vibrio cholerae* infection (Jensen et al., 2022). I checked the expression of CXCR5 and PD-1 markers expressed by follicular helper T cells that provide B cell help during infection. Viral infection did not upregulate the expression of CXCR5 and PD-1 indicating the absence of $MAIT_{FH}$ cells as previously reported **(Figure 7A)**. I also checked if MAIT cells indirectly influenced the priming of $CD4^+$ T_{FH} in the mLN by quantifying $CD4^+$ T_{FH} between WT and $MR1^{KO}$ mice post-infection. There were no significant differences in the percentage and number of $CD4^+$ T_{FH} cells **(Figure 7B)**. In order to check if IL-17-producing MAIT cells directly act on the plasmablasts, I checked the expression of IL-17 receptor (IL-17R) on plasmablasts. They cytometry anaysis revealed that plasmablasts do not express IL-17 receptor implying no direct interaction of IL-17 on B cells **(Figure 8)**.

Bone marrow chimera is a powerful tool that is widely used for dissecting the specific role of particular population in a controlled setting. To further assess if MAIT cells interact with B cells in a cognate way, I generated chimeric mice by irradiating µMT animals (mice lacking B cells) and injecting a mixture of 80% µMT bone marrow and 20% of either WT or $MR1^{KO}$ bone marrow. The immune system of the recipient mice (µMT mice in this case) is destroyed as a consequence of irradiation treatment. If MAIT cells are necessary for B cell responses by direct engagement, the repopulation with bone marrow cells from mice devoid of the MAIT cells ($MR1^{KO}$ mice) should result in a poorly established B cell response upon challenge with Influenza compared to the wild-type animals. Therefore, post-reconstitution I challenged the mice with Influenza and analyzed the GC and Plasmablast compartment 9 days after infection **(Figure 9A)**. The number of GC-B cells, plasmablasts, mature plasmablasts, IgA+ plasmablasts were comparable in mice reconstituted with WT and those with $MR1^{KO}$ **(Figures 9B-F).** These observations clearly show that MAIT cells do not directly impact B cells and that other intermediating players relay signals from MAIT cell.

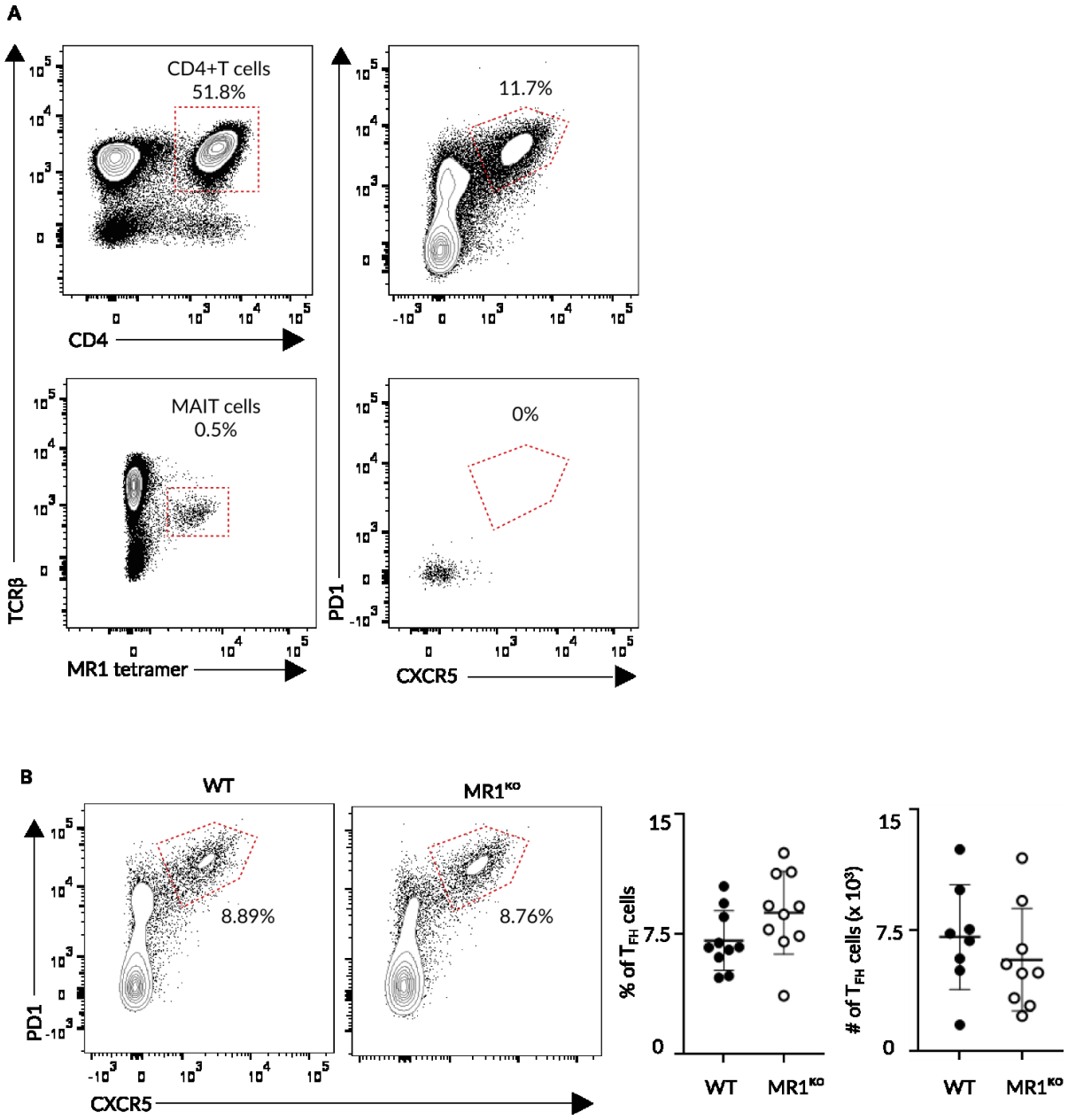


Figure 7. There is no cognate interaction between MAIT cells and B cells. WT and MR1^{KO} mice were infected with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain. **(A)** Expression of CXCR5 and PD-1 in CD4^{+} T_{FH} cells (upper panel) and in MR-1 tetramer^{+} MAIT cells (lower panel) in mLN. **(B)** Flow cytometry analysis and quantification of T_{FH} identified as CXCR5^{+} PD1^{+} in CD4^{+} T cells.

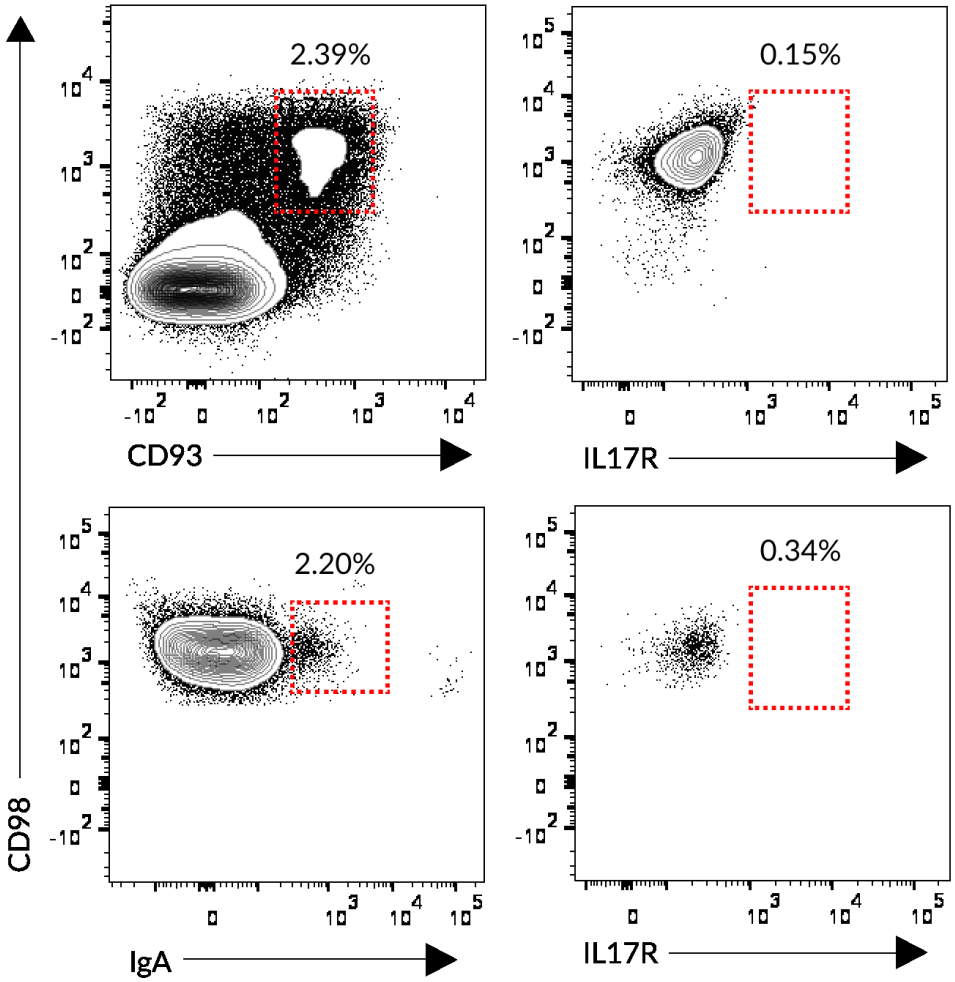


Figure 8. Plasmablasts do not express IL-17R. WT were infected with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain. The expression of IL17R in plasmablasts was identified as $CD93^{+}$ and $CD98^{+}$ (upper panel) in IgA^{+} plasmablast (lower panel) 9 days post-infection in the lymph node.

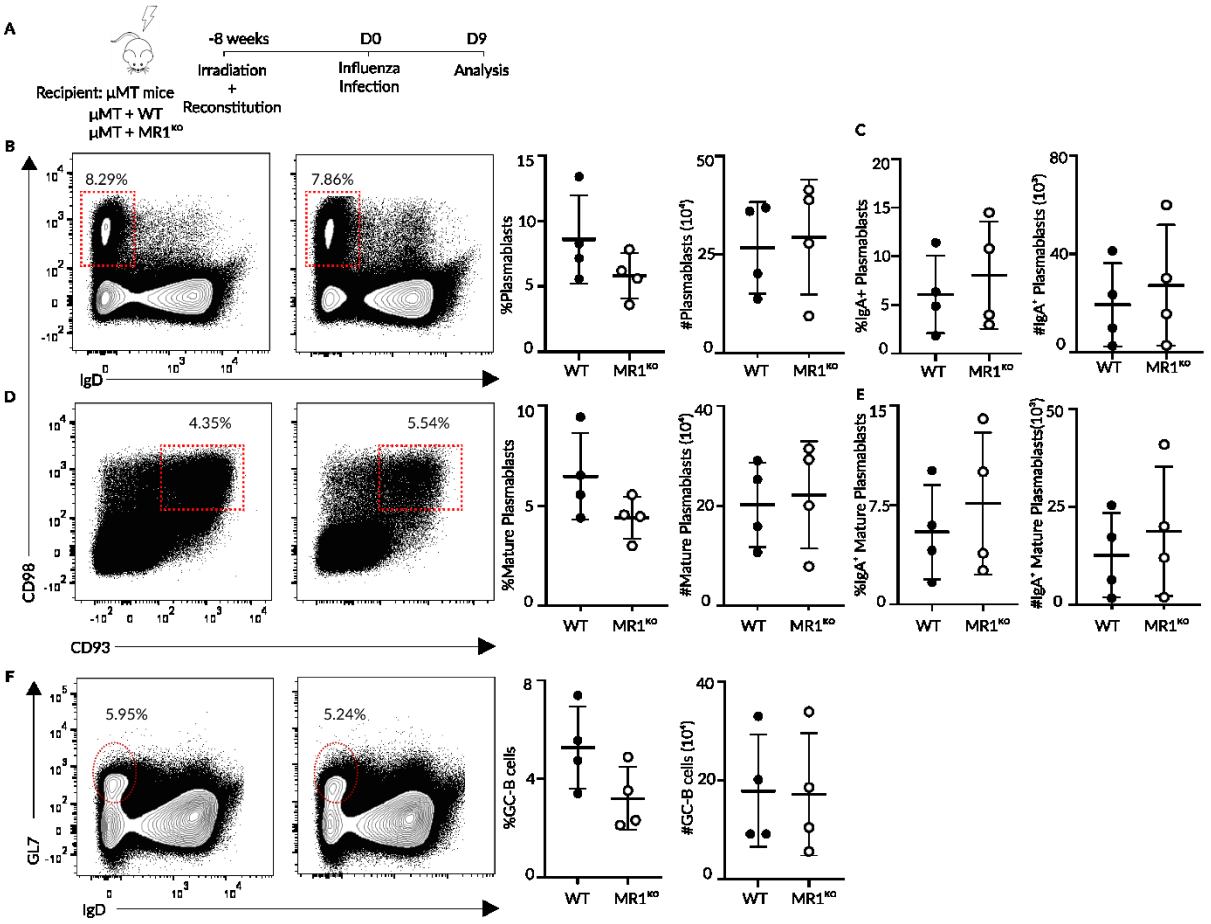


Figure 9. There is no cognate interaction between MAIT cells and B cells. µMT mice were irradiated and reconstituted with 1 x 10^6 bone marrow cells of µMT and B6-MAIT^{CAST} mice (WT) or MR1^{KO} (80-20%). After 8 weeks, mice were infected with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain, and analysis was performed on the mLN after 9 days of infection. Flow cytometry analysis and quantification of **(B)** Plasmablast identified as CD138$^+$ **(C)** % and number of IgA+ Plasmablast **(D)** mature plasmablast identified as CD138$^+$CD93$^+$ **(E)** Percentage and number of IgA+ mature plasmablast **(F)** Germinal center (GC) B cells identified as IgD$^-$ GL7$^+$ in WT and MR1^{KO} mice. In all charts, each dot represents one mouse. Horizontal bars, mean; error bars, SEM. Student's t-test

3.7 Regulation of IL-17-producing MAIT cells on IgA secretion in the broncho-alveolar space is not due to defective transcytosis

The epithelium of secretory organs such as the intestine and lungs are lined with a glycosylated type I membrane protein known as Polymeric immunoglobulin receptor (pIgR) (Brandtzaeg et al., 1988). pIgR receptor is unique to epithelial cells, with the ability to bind both dimeric IgA (dIgA) and pentameric IgM (pIgM) produced by plasma cells in the dermis. This receptor attaches to polymeric immunoglobulins, including dIgA and pIgM, and facilitates their transport through mucosal epithelial cells to the apical surface via transcytosis in a J-chain-dependent manner. Intestinal IgA transcytosis is supported by Th17 cells that help maintain homeostasis by upregulating pIgR (Cao et al., 2012) **(Figure 10)**. I observed that IgA+ PC and other isotype PCs do not express IL-17R **(Figure 8)** in the mLN or the lung. I wondered if the absence of IL-17 could lead to defective transcytosis due to decreased pIgR expression. I measured the soluble pIgR bound to IgA in the BALF of WT and IL-17^{KO} mice 9 post-infection with Influenza. The absence of IL-17 did not affect pIgR levels in the lumen of mice **(Figure 10)**.

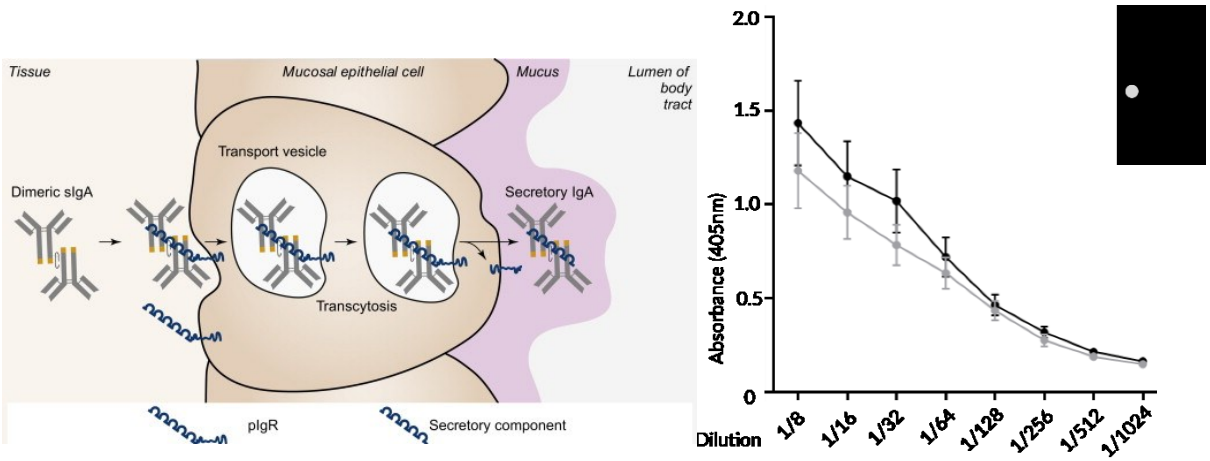


Figure 10. IL-17 does not affect the transcytosis of IgA across lung mucosal epithelium to lumen. Left panel. Schematic diagram of Generation of Secretory IgA by transcytosis and levels of pIgR bound IgA. In this example of a mucosal epithelial cell lining a body tract, dimeric sIgA binds via its J chain to a polymeric Ig receptor (pIgR) expressed on the cell surface. The polymeric sIgA molecule is endocytosed by pIgR, transported across the cell (transcytosis), and released into the lumen of the tract. **Right panel**. Levels of pIgR IgA in the BALF of WT and IL17^{KO} post-infection with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain measured by ELISA.

3.8 MAIT cell deficiency does not affect the metabolic programming of B cell

Majumder et al. showed that IL-17 produced by Th17 cells promotes stromal cell survival in lymph nodes through cell metabolic regulation involving IκBζ-dependent increase in glycolysis and mitochondrial activity in an experimental autoimmune mouse model (Majumder et al., 2019). I questioned if IL-17 in mLN majorly produced by MAIT cells could potentially affect the metabolic programming of B cells by similar mechanism in the mLN. I analyzed the metabolic profiles of B cells using SCENITH (Single Cell ENergetIc metabolism by profilIng Translation inHibition) which allows the study of metabolic responses in multiple cell types in parallel by flow cytometry (Argüello et al., 2020). In other words, this system quantifies the metabolic activity of individual cells by assessing their reliance on key metabolic pathways (glycolysis, oxidative phosphorylation, etc.) through differential protein synthesis under specific inhibitors. Therefore, it helps map metabolic phenotypes in heterogeneous cell populations. There were no differences in the glucose and mitochondrial dependency of Plasmablast, GC or naïve B cells between WT and $MR1^{KO}$ or between WT and $IL\text{-}17^{KO}$ **(Figure 11)**. Interestingly, I observed a tendency for more glucose dependence in PC and higher Glycolytic capacity in GC and switched B cells in $IL17^{KO}$ mice compared to WT and $MR1^{KO}$ mice **(Figure 11 indicated by an arrow)**. These findings reflect that the absence of IL-17 modulates the metabolic profile of B cells. However, there were no differences in the metabolic profiles of B cell populations between WT and $MR1^{KO}$ that could explain the B-cell help provided by MAIT cells.

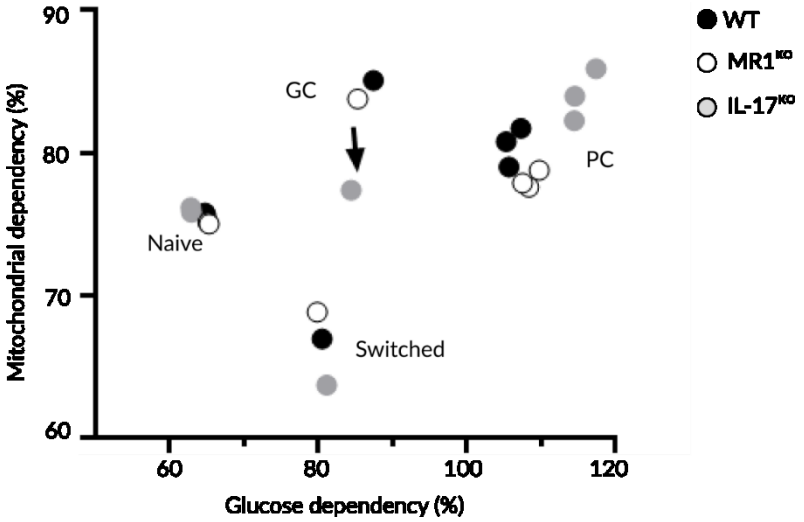


Figure 11. Metabolic profile of B cells is not affected by the absence of MAIT cells

WT, MR1^{KO}, and IL-17^{KO} mice were infected with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain. 9 days post-infection, the metabolic dependency and glucose dependency in Naïve B cells, germinal center (GC) B cells, plasmablasts (PC) and class-switched cells in the mediastinal lymph node were compared between the strains using SCENITH protocol. Arrow represents a statistically significant shift. n=3

3.9 MAIT cells regulate the infiltration of neutrophils in the lung in an IL-17-dependent manner

I report that MAIT cells do not interact with B cells cognitively based on the results above. Hence, B cell help could be a result of intermediate players influenced by the presence of MAIT cells. IL-17 production has been associated with neutrophil recruitment (Meller et al., 2015). I evaluated the infiltration of neutrophils along with monocytes in the lung and mLN of WT and MR1^{KO} 3 days post-infection with the Influenza virus. To distinguish between lung-infiltrated and circulating cells I intravenously injected CD45 antibody 5 minutes before euthanizing mice. I observed a significant reduction in the number of neutrophils in the lungs of MR1$^{-/-}$ compared to the WT in an IL-17-dependent manner since IL-17^{KO} displayed the same defect in neutrophil recruitment upon infection **(Figure 12A)**. Neutrophils play an important role in the clearing of pathogens and these results indicate a new outreach of neutrophils towards mounting an

efficient immune response. In contrast, the number of neutrophils in the mediastinal lymph node of WT and MR1^{KO} and IL-17^{KO} were unchanged following infection **(Figure 12B)**.

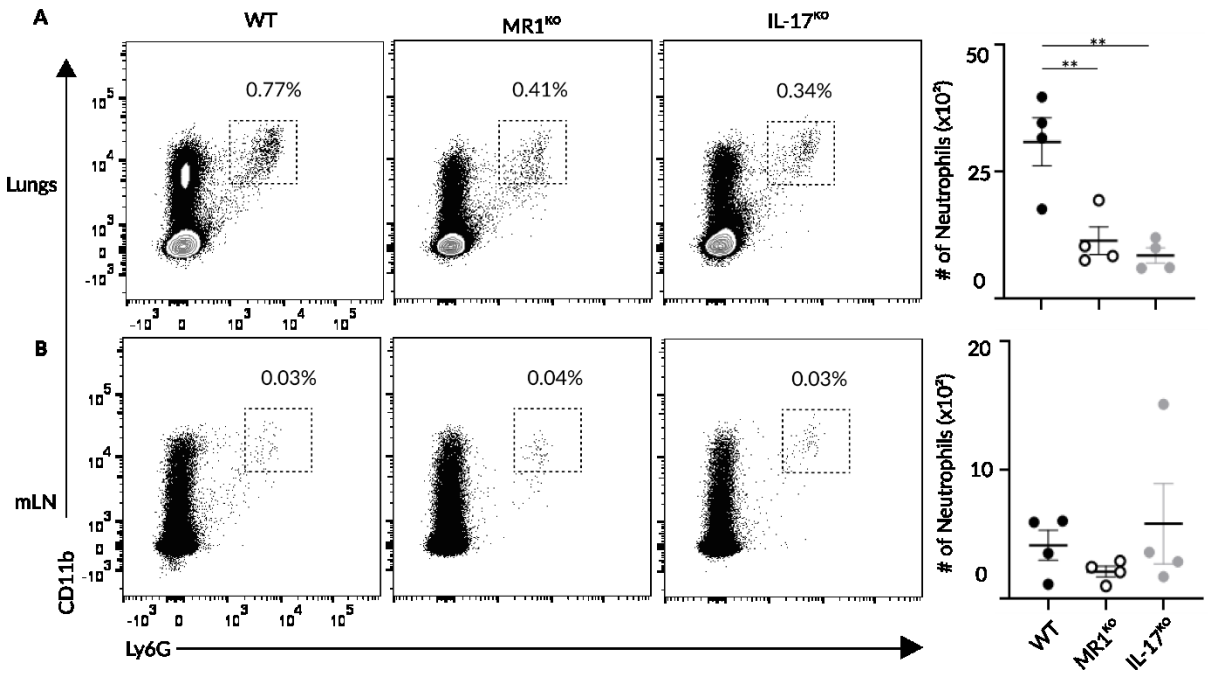


Figure 12. MAIT cells regulate the infiltration of neutrophils in IL-17 dependent manner

WT, MR1^{KO}, and IL-17^{KO} mice were infected with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain. Flow cytometry analysis of Neutrophils identified as CD11b^{+} Ly6G^{+} TCRβ^{-} B220^{-} and i.v. CD45^{-} in the **(A)** lung and **(B)** mediastinal lymph node 3 days post-infection.

2.2.10 Effect of neutrophil depletion on B cell response

So far, I have shown that during viral infection, MAIT cells play a role in innate and adaptive immunity by regulating the recruitment of neutrophils and IgA secretion at the site of infection respectively. I questioned if there is a possibility that the recruitment of neutrophils controls the secretion of IgA in the lungs. In other words, I hypothesize that IL-17-producing MAIT

cells control the recruitment of neutrophils at 3 days post-infection, a phenomenon crucial for IgA secretion at 9 days post-infection. To investigate this, I depleted neutrophils *in vivo* by injecting anti-Ly6G antibody (α-Ly6G) for 9 days post-infection with Influenza and observed differences in the B cell responses with and without depletion treatment **(Figure 13A)**. The depletion protocol was efficient as I did not observe any Neutrophils in the lungs and lymph node of α-Ly6G mice at day 9 post-infection **(Figure 13B)**. The primary experiment was performed with B6-MAIT^{CAST} mice in which I observed a decline in the number of IgA+ plasma cells and IgG2b+ plasma cells in the lung parenchyma of neutrophil-depleted mice **(Figure 13E)**. However, there were no significant differences in the total number of plasmablasts, the different isotypes-specific plasmablasts, and T_{FH} post-depletion **(Figures 13D and F)**. Due to the lack of B6-MAIT^{CAST} mice, I used C57BL/6 mice to repeat this experiment of depleting neutrophils to explore the correlation between neutrophils and IgA. I observed a trend of decreased Influenza specific IgA antibody in BALF of neutrophil-depleted subjects however, the difference was not statistically significant **(Figure 13G)**. These findings show a new pathway for the establishment of an anti-viral response under an indirect regulation of MAIT cells.

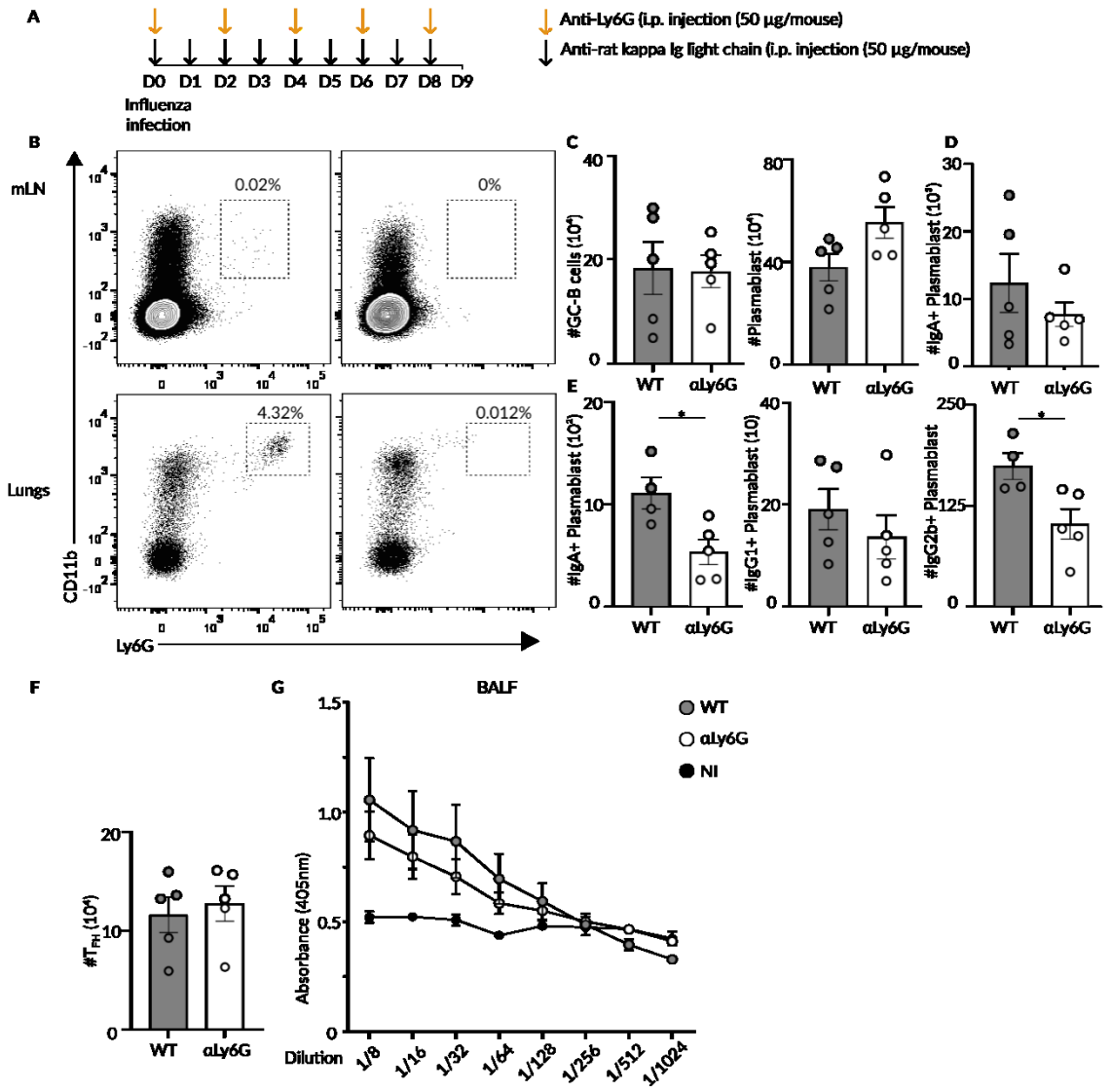


Figure 13. Infiltration of neutrophils is important for pulmonary IgA and IgG2b plasmablast in response to viral infection. (A) Experimental setup: WT mice were infected on D0 with 5 pfu of influenza A virus H1N1 Puerto Rico/8/34 (PR8) strain and neutrophils were depleted by intra-peritoneal injection of Anti-Ly6G (αLy6G) 50µg/mouse from the day of infection (D0) for 9 days along with intra-peritoneal injection of Anti-Rat Kappa Immunoglobulin light chain 50µg/mouse on alternate days (D0, D2, D4, D6, and D8) and organs were analyzed on D9. **(B)** Flow cytometry plots of mediastinal lymph node (mL) (upper panel) and lung (lower panel) in WT mice (left) and mice with neutrophil depletion (αL6yG) (right). **(C, D, and F)** Total number of Germinal center (GC) B cells and plasmablasts in the mLN (C), IgA+ plasmablasts (D), and T follicular helper cells in the mLN of WT mice and neutrophil-depleted mice (αL6yG). **(E)** Total IgA, IgG1, and IgG2b Plasmablasts in the lungs

of WT mice and neutrophil-depleted mice (αL6yG). **(G)** Influenza antigen nucleoprotein (NP) specific IgA in the Broncho-alveolar lavage fluid (BALF) of WT mice, neutrophil-depleted mice (αL6yG), and non-immunized mice (NI) measured by ELISA. In all charts, each dot represents one mouse. Horizontal bars, mean; error bars, SEM. Student's t-test.

GRAPHICAL ABSTRACT

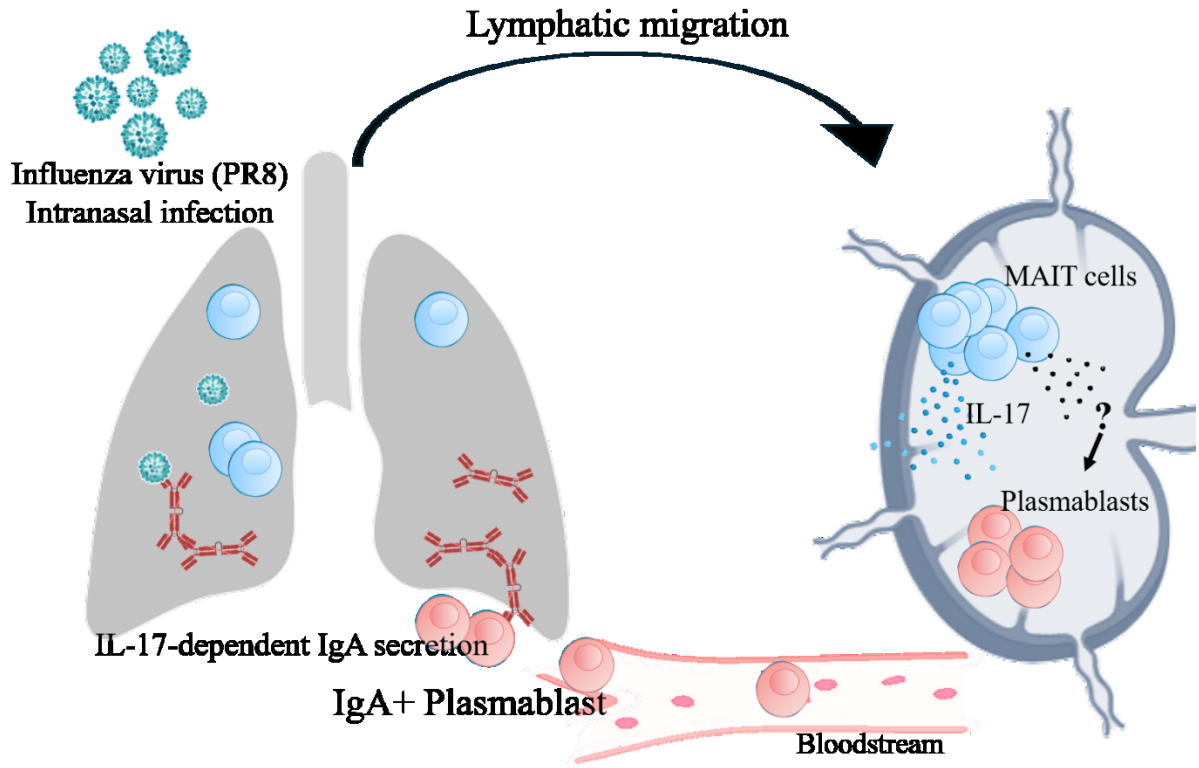


Figure 14. Graphical abstract- Regulation of adaptive immune responses by MAIT cells during pulmonary viral infection. Upon infection with Influenza virus (PR8), MAIT cells migrate into the lymph node via lymphatics and produce an early wave of IL-17 at day 3. MAIT cells regulate the differentiation of Plasmablasts in the lymph node via unknown pathway and it is IL-17 independent at day 9 post-infection. MAIT cells support the migration of IgA+ Plasmablasts resulting in IgA secretion in the bronchoalveolar space.

DISCUSSION AND PERSPECTIVES

In the previous section (see: Discussion and perspective section of 'MAIT cells and innate immunity') I discussed the importance of MAIT cells' positioning in the lung-draining lymph node, their effector functions in innate immunity, the importance of Th17-type profile in the context of infection, the different mechanism involved in the regulation of immune responses by MAIT cells, role of different chemokines in the recruitment of MAIT cells, signaling responsible for the activation and induction of IL-17 by MAIT cells. In this section, I will discuss the importance of MAIT cell role in adaptive immunity which is in the results section of this chapter followed by different factors that affect MAIT cells' number and functions.

I demonstrated that IL-17-producing MAIT cells help regulate IgA secretion. These findings are important for the respiratory infection field because IgA is the most abundant antibody found in mucosal tissues, including the lungs, gastrointestinal tract, and other surfaces exposed to the external environment (Cerutti, 2008). It is predominantly present in secretions like mucus, saliva, and tears, and it forms the first line of defense at these barrier surfaces. IgA operates in the respiratory tract by neutralizing pathogens at the mucosal surface before they can enter deeper tissues or the bloodstream (Cerutti, 2008; Joseph, 2022). This "immune exclusion" is highly effective because IgA prevents pathogens from adhering to and invading the epithelial cells lining the respiratory tract. IgA is not necessarily "stronger" than other antibodies, but it is more specialized and abundant at mucosal surfaces like the lungs, making it uniquely suited to protect against pulmonary infections. Its ability to act locally in the mucosa, combined with its non-inflammatory properties, makes it crucial for maintaining lung health during infections without causing excessive tissue damage. Therefore, the decrease in IgA levels in MAIT deficient mice unequivocally establishes a major role played by MAIT cells in specific secretion of only IgA and not other isotypes in the broncho-alveolar space.

IL-17-producing MAIT cells help influenza-specific IgA secretion into the bronchoalveolar space. But, we do not know the functional relevance of this finding at 9 days post-infection. If the Influenza specific-IgA antibodies have longer survival in the wild-type mice compared to the MAIT deficient mice at later time points like 35 days or 70 days post-infection, it could reveal the significance of MAIT cells in making IgA antibodies available during a rechallenge.

Besides IL-17, MAIT cells produce several other factors that traditional methods like scRNA seq and cytometry overlook. It would be worth exploring the whole cytokine profile of MAIT cells using a combination of techniques at different time points. IL-17-producing MAIT cells at day 3 post-infection can undergo an inert phase and later secrete other cytokines like IL-22, TGFβ, and more for B cell help.

Another hypothesis worth exploring is that MAIT cells indirectly provide B cell help by regulating the infiltration of neutrophils in case of viral infection with Influenza virus. The first experiment showed a significant decrease of IgA+ Plasmablast in the lung post-depletion of neutrophils. Replicating these results would prove that neutrophil recruitment is responsible for IgA^+ plasmablast positioning in the lung parenchyma and further boosts IgA secretion. Additionally, it has been shown that there is a population called 'B helper neutrophils' that aid the secretion of IgM by Marginal zone B cells and favor class-switching to IgG2 and IgA (Harwood et al., 2012). These B helper neutrophils also secrete APRIL, BAFF, and IL-21 which are crucial for efficient B cell activation.

In this thesis, I focus on the innate and adaptive immune response generated at day 3 and day 9 post-infection respectively. I found that IgA secretion and plasmablast differentiation is supported by MAIT cells. However, I did not explore the importance of MAIT cells during a secondary challenge. If the IgA+ plasma cells display prolonged positioning in the parenchyma of the lung of WT compared to the MAIT cell-deficient (>35 or 70 days), it would imply that MAIT cells could also support memory B cell responses which is crucial during secondary infection (Gregoire et al., 2022). Some studies have hinted that MAIT cells can boost the memory B cell compartment (Pankhurst et al., 2023; Rahman et al., 2020). I did not investigate the role of MAIT cells in the memory B cells' differentiation. This is something that can be explored by rechallenging wild-type and MAIT-deficient mice. If MAIT cells confer protection with the secondary challenge of a lethal dose of Influenza virus (or with a different strain of Influenza virus to mimic natural reinfection), this could establish a strong link between MAIT cells and B cells that can be harnessed for the development of mucosa based vaccination strategies.

I observe a defect in the plasmablast differentiation during viral infection with Influenza and Vaccinia. In contrast, I did not observe the same defect upon bacterial challenge with *K.pneumoniae* even though the level of IL-17 production during viral and bacterial infection is

the same. Moreover, IL-17 knocked-out mice did not have plasmablast differentiation defect which I observed in MAIT-deficient mice upon viral challenge ruling out the possibility of IL-17 mediated plasmablast expansion. It can be hypothesized that the transcriptomic profile of MAIT cells at day 9 post viral and bacterial infection are different resulting in aiding B cell responses. MAIT cells may imprint distinct transcriptomic programming in the plasmablast that supports their proliferation (while simultaneously impacting the IgA secretion). Since there were no differences in the B cell responses between wild-type and MAIT-deficient mice during bacterial infection, it indicates the prevalence of a specific interaction between MAIT cells and B cells during viral infection that is absent during bacterial infection. My scRNA seq data analysis on MAIT cells in the Influenza infection setting displayed enhanced regulation of genes directly related to anti-viral functions whereas those in the *K.pneumoniae* infection displayed more homeostasis regulation-related genes upregulation in MAIT cells. One can hypothesize that a diverse transcriptional profile of MAIT cells in different infection settings could further lead to varying downstream functions affecting responses generated by other immune and non-immune cells.

The findings mentioned in this thesis related to innate and adaptive immune regulation by MAIT cells show that there are several participants of the immune system that orchestrate an effective immune response. Antigen-presenting cells that provide Type-I interferon, IL-23, and IL-1 for the activation and IL-17 secretion by MAIT cells, innate immune cells that respond to factors released by MAIT cells, B cell help by IgA secretion and plasmablast differentiation by MAIT cells. Therefore, MAIT cells in the lymph node participate in mucosal immunity along with those present in the lung and other barrier tissues.

MATERIALS AND METHODS

Mice and cells

8 to12 weeks old mice were used in all the experiments. All the mice used in the 'Role of MAIT cells in adaptive immunity' section were females. All the mice used in the 'Role of MAIT cells in Innate Immunity' were a mix of males and females. B6-MAIT^{CAST}, MR1^{KO}, RoRγt-GFP mice were obtained from Olivier Lantz, Institute Cure, Paris. IL-17^{KO}, IL-17^{GFP} mice were obtained from Jackson laboratories. IL-18^{KO} mice were obtained from Marc Bajenoff, CIML, France. IFNAR^{KO} mice were obtained from Elena Tomasello, CIML, France. Unless specified otherwise, all mice mentioned above were introgressed into the B6-MAIT^{CAST} background.

C57BL/6 from Janvier Labs were used as controls with CXCR6-GFP mice (on C57BL/6 background) obtained from Rachel Golub, Institute Pasteur, France and CCR7^{KO} mice were obtained from Elena Tomasello, CIML, France. µMT mice were obtained from Stéphane Mancini, Centre de Recherche en Cancérologie de Marseille, France.

For the generation of mixed bone marrow chimeras, µMT mice of 6-8 weeks of age were irradiated with 2 doses of 4.75 Gy, 4 hours apart. Bone marrow cells from B6-MAIT^{CAST} and MR1^{KO} were injected *i.v.* in isoflurane-anesthetized recipient animals (1x10^6 cells in total) on the following day in an 80:20 ratio (recipient strain: donor strain). Mice were kept on Bactrim containing water for 3 days prior and 3 weeks post-irradiation. Chimeras were used after 8 weeks of reconstitution.

All experiments were conducted in an accredited animal facility by the French veterinarian department following ethical guidelines approved by the relevant ethical committee (APAFIS no. 16708-2018091116493528).

Viral and bacterial infections, 5-OP-RU immunization, and injections

Mice were anesthetized i.p. with Ketamine/Xylazine (100mg/kg body) and intranasally infected with 5 PFU of Influenza virus A/Puerto Rico/8/1938 (PR8) H1N1 strain or 2 x 10^4 CFU of *Klebsiella pneumoniae* 52.145 or 2 x 10^4 CFU of *Klebsiella pneumoniae* 43816 strain or 0.4 nmoles of 5-OP-RU (obtained from Olivier Lantz, Institute Curie, Paris), 2000 pfu of

Vaccinia virus Western reserve strain in 20µl of PBS for intranasal treatment. *Klebsiella pneumoniae* 52.145 strain and 43816 strain cultures were grown in Luria-Bertani (LB) broth without antibiotics at 37°C overnight with shaking at 180 rpm. For infection, overnight-grown bacteria were inoculated in LB media for 2-3 hours of culture until the OD_{590} reached 0.6. The bacterial pellet was washed twice with PBS. The final suspension of 2 x 10^4 CFU per 20ul of bacteria was prepared in PBS. Experiments with Influenza, Vaccinia, and *K.pneumoniae* were conducted in the biosafety level 2 laboratory of CIML, France. For in vivo labeling of immune cells in circulation, mice were anesthetized with isoflurane, and 3 µg of biotinylated anti-CD45 antibody (diluted in PBS) was injected i.v. 5 minutes before sacrificing the animals.

In vivo blocking with α-IL-1R and α-IL-23 antibodies was performed by intraperitoneal injection on the day of infection (D0) with 300 µg/mouse and with 100 µg/mouse on days 1 (D1) and 2 (D2) post-infection.

In vivo depletion of neutrophils was performed using the protocol validated before (Boivin et al., 2020). In brief, each mouse was injected i.p. with 50 µg of anti-mouse α-Ly6G monoclonal antibody (BE0075-1, BioXCell) from the day of injection until the day before euthanizing mice) along with alternate days i.p. treatment with 50 µg of Anti-rat kappa Immunoglobulin light chain antibody (BE0122, BioXCell).

Flow cytometry

Mice were euthanized by cervical dislocation, and mediastinal lymph nodes and lungs were harvested in cold PBS. Single-cell suspension of mediastinal lymph nodes was prepared by smashing on a 70 µm nylon mesh cell filter with a plastic plunge in PBS containing 2% FCS. For lungs, single-cell suspensions were obtained by enzymatic digestion with 1mg/ml of DNAse I and 7 mg/ml of collagenase II in 3 ml of wash buffer (RPMI, 100 µg/mL of Penicillin/Streptomycin, 25 mM HEPES, 2% FCS). The tissue digestion was performed with a gentleMACS octo dissociator (Miltenyi Biotec) following the manufacturer's instructions. The lung cell suspension was further separated using an 80%/40% percoll gradient (Fisher Scientific, 17089101). Cell suspensions were further incubated with red blood lysis buffer for 5 minutes before washing. To block nonspecific antibody binding, cell suspensions were incubated with hybridoma supernatant of 2.4G2, diluted 1/4 in PBS 2%FCS for 20 minutes at

4°C. For surface marker labeling, cells were stained with indicated antibodies at 4°C (except for CXCR6 and CCR7 staining that was performed at 37°C) in brilliant staining buffer (BD). HA and NP proteins (Sino Biologicals) were labeled with PE or APC conjugation kit (Abcam) according to the manufacturer's protocol. For cytokines staining, cell suspension was incubated with cell stimulation (Thermo Fisher Scientific 00-4980-03) and protein transport inhibitor (Thermo Fisher Scientific 00-4970-03) cocktail for 4 hours in complete RPMI 1640 media (10% FCS, 100 µg/mL Penicillin/Streptomycin, 10 mM Hepes, 1 mM Sodium Pyruvate, 1X Non-essential Amino Acid, 50 µM β-mercaptoethanol) at 37°C, permeabilized with BD fix/perm kit, and intracellular staining was performed at 4°C. Fortessa X20, LSR II UV, Symphony, and LSR-II 561 (BD) were used for acquisition. FlowJo software was used for the analysis. For unbiased clustering of the cells from the IL-17^{GFP} and ROR^{GFP} lineages, data from all samples were concatenated and analyzed using OMIQ software.

Tissue processing for bacterial load

Mice were euthanized by cervical dislocation, blood was drawn immediately by a puncture to the heart in heparin bead tubes. Mediastinal lymph nodes and lungs were collected in cold PBS. Both the organs were smashed on a 70 µm nylon filter with PBS, serially diluted, plated on LB agar plates without antibiotics, and incubated for 24 hours at 37°C.

Plaque assay for PR8 viral load

Mediastinal lymph nodes and lungs were collected in cold PBS. mLNs were smashed on 70 µm nylon filter with PBS. Lungs were processed with tissue homogenizer to prepare lysate in PBS. MDCK cells were grown in 6-well plates in complete RPMI 1640 media and incubated with lysate for 1 hour at 37°C in RPMI media supplemented with 2% FCS without antibiotics. The wells were overlaid with Leibovitz's L-15 medium, 1% agarose, and 1 µg/ml TPCK trypsin (Fisher Scientific) and incubated for 3 days at 37°C. Plates were fixed with 4% formaldehyde, agarose was removed, washed with PBS and plaques were visualized with the staining of crystal violet. ImageJ software was used to count the plaques.

MAIT cell culture and adoptive transfer

Thymus from 4 to 6-week-old B6-MAIT^{CAST} or B6-CAST IL17^{KO} mice were processed by mechanical dissociation through a 70 µm cell strainer in FACS buffer (1% PBS, 2% BSA, 2mM EDTA). Cell suspension was incubated with MR1:6FP tetramer to avoid unspecific staining followed by staining with MR1:5-OP-RU-PE tetramer at room temperature. Anti-PE microbeads (Miltenyi) were used for positive selection on LS columns (Miltenyi). 2 x 10^6 cells/mL were plated in complete RPMI 1640 media (10% FCS, 100 µg/mL Penicillin/Streptomycin, 10 mM Hepes, 1 mM Sodium Pyruvate, 1X Non-essential Amino Acid, 50 µM β-mercaptoethanol) and stimulated with 150 nM 5-OP-RU and 10 ng/mL of rmIL-2 (Peprotech). MAIT cells were expanded for 9 days with the addition of fresh rmIL-2 (every 2 days). 8 x 10^5 cells in PBS were intravenously injected in C57BL/6 mice through the retro-orbital sinus route.

scRNA seq and data analysis

Single-cell suspension of lymph nodes was prepared and incubated with hashtag oligonucleotides (HTO) to multiplex 4 conditions from 16 individual mice. Live, CD19$^-$, RoRGFP$^+$ cells were sorted using BD FACSMelody cell sorter. 30,000 cells were loaded onto a chromium 3' chip following the Single Cell 5' Kit V2 (10X Genomics). 10x 5' scRNA-seq libraries were prepared according to the manufacturer's instructions with modifications for generating the TCR-seq libraries. Following cDNA amplification, SPRI select beads were used to separate the large cDNA fraction derived from cellular mRNAs (retained on beads) from the HTO-containing fraction. For the cDNA fraction derived from mRNAs, 50ng was used to generate transcriptome library, and around 5ng was used for TCR library construction. Gene expression libraries were prepared according to the manufacturer's instructions. For MAIT-TCR libraries, cDNA were amplified by two rounds of PCR (12 cycles) using a combination of external primer with MAIT-TCR specific primer (TTAGCTTGGTCCCAGAGCCCCAGATC*A*A where * indicates sites of Phosphorothioate inclusion from IDT) recommended by 10x Genomics, and 800 pg of purified amplified cDNA was tagmented using Nextera XT DNA sample Preparation kit (Illumina) and amplified for 12 cycles using the SI-PCR forward primer (10x Genomics) and a Nextera i7

reverse primer (Illumina). For the HTO-containing fraction, 5ng was used to generate the HTO library. The resulting libraries were pooled and sequenced on an Illumina NextSeq550 of the CIML genomics platform.

Dataset analysis was performed using R package Seurat (V.5.0.3). Variable genes (n=2000) were used for dimensionality reduction with vst algorith. MAIT+ and non-MAIT/MAIT- or 'other cells' were distinguished by the expression of Trav1. Cells with >0.5 expression were assigned as MAIT+ and the others as MAIT-. Visualization was done with the RunUMAP method using 20 Pcs. Clustering was performed using FindNeighbors and FindClusters methods with 20 PC for SNN graph build and the Leiden clustering method with a resolution of 0.3. Marker genes between clusters were identified using the FindAllMarkers method of Seurat package using the Wilcoxon Rank. These genes were used to prepare the heatmap with the DoHeatmap function showing their scaled expression in each cell sorted by treatment. For pathway enrichment analysis, the ontological enrichment was performed from the list of marker genes with R/Bioconductor package clusterProfiler (p-value<0.05; Benjamini-Hochberg q-value<0.2; minGSSize = 30; maxGSSize = 500). For MdsPlot analysis, the pseudobulk was created by the sum of gene expression within the cells belonging to the same treatment. The variance of the genes was calculated and Mdsplot was created by selecting the top 200 most variable genes. For visualization of MdsPlot, ggplot(v.3.5.0) package was used.

Microscopy

Lymph nodes were harvested in PBS and briefly blotted on tissue before overnight incubation with PE-labeled 5-OP-RU loaded MR1 tetramer in 2% FCS in 1X PBS at 4°C. The next day, lymph nodes were washed with PBS, fixed with 4% PFA for 1 hour, cryopreserved and dehydrated with 30% sucrose for 3 hours, and snap-frozen in liquid nitrogen in an OCT-filled mounting plastic cryomold. Solidified OCT was removed from the plastic cryomold and stored at -80ºC till sectioning. 20 µm cryosections of LNs were cut at -20ºC using a Bright Cryostat OTF 5000 and placed on glass slides. Slides were stored at -80ºC until use. For immunofluorescence staining, tissue sections were thawed and dehydrated over silica beads for 20 minutes and washed 3 times with PBS. Slides were blocked and permeabilized with PBS containing 0.5% saponin, 1% goat serum, 1% FCS, and 2% BSA for 30 minutes at room temperature. Slides were incubated for 3 hours at room temperature with Anti-GFP chicken

monoclonal antibody (Abcam, 13970) and anti-mouse B220 antibody diluted in 1/500 and 1/100 diluted respectively in blocking solution. Slides were then washed 3 times with PBS, and incubated with Goat anti-chicken AF 488 secondary antibody (Life Technologies, A11039). Glass coverslips were mounted onto tissue sections using Fluomount-G. All the slides were kept in the dark till confocal microscopy imaging. Confocal microscopy images were acquired with an inverted Zeiss Axiovert LSM 780 microscope. Objectives used were 20x, 40x, and 63x oil immersion objectives. Images were recorded with ZEN black software and processed using ZEN and ImageJ software.

ELISA

ELISA (enzyme-linked immunosorbent assays) were used to quantify the antigen-specific antibodies and pIgR-bound IgA. BALF was collected from mice euthanized by cervical dislocation. In brief, 1mL of sterile cold PBS was injected into the broncho-alveolar space with the blunt end of a 25G needle and insulin syringe. Samples with clear liquid (with no blood contamination) and a 60-80% recovery rate were used for ELISA. To measure PR8- NP-specific antibodies, plates were coated with 1µg/ml of NP (ELJ842 from Interchim) in PBS overnight at 4ºC. Plates were blocked with 2% BSA in 1XPBS solution for 2 hours at room temperature and then incubated for 2 hours with serial dilutions of BALF collected at 9 days post-influenza infection. Plates were probed for 1 hour using biotinylated antibodies against mouse IgA, IgM, or IgG (1:1000) diluted in a blocking buffer. All ELISA plates were developed using p-nitrophenylphosphate. 405nm absorbance was detected using a SPECTRAmax190 plate reader (Molecular Device).

SCENITH Experiments

SCENITH kit containing all reagents (including anti-puromycin, clone R4743L-E8) and protocols were obtained from R. Arguello team at CIML (www.scenith.com). Briefly, single-cell suspension of lymph nodes was plated in complete medium in a 96-well plate and treated with Puromycin (10 µg/mL) and DMSO (Co), 2-deoxy-glucose (2DG; 100 mM), oligomycin (O; 1 µM), a combination of 2DG and oligomycin (DGO) or harringtonine (H; 2 µg/mL) for

40 min at 37°C, 5 % CO_2. Cells were washed in cold FACS buffer and stained with surface markers for 30 minutes at 4°C. Cells were further fixed and permeabilized with the Foxp3 staining kit according to the manufacturer's instructions (ThermoFisher Scientific). Intracellular staining with anti-puromycin was performed by incubating cells for 1 hour at 4°C in FOXP3-permeabilization Buffer (ThermoFisher Scientific).

Statistical analysis and figure preparation

For each experiment, the number of independent experiments, replicates, and the statistical tests used are indicated in the figure legends. The following statistical tests were used and calculated by GraphPad Prism (GraphPad software): Mann-Whitney, Wilcoxon, unpaired t-test, paired t-test, one-way Anova, and two-way Anova. In figures, asterisks stand for: *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001, and "ns" or no symbol was included if the comparison was non-significant. Figures were prepared using Biorender, Servier, MS Presentation, and Figma.

List of antibodies for cytometry, imaging, in vivo treatment and tetramers

5-OP-RU loaded MR1 tetramer conjugated with PE and BV421, 6-FP MR1 tetramer conjugated with PE and BV421, and PBS 57 loaded CD1d tetramer conjugated with APC were supplied by the National Institutes of Health (NIH) tetramer core facility (Emory University).

InVivoPlus anti-mouse Ly6G (Catalog number BE0075-1), InVivoMAb anti-mouse IL-1 R (CD121a) (Catalog number BE0256), InVivoMAb anti-mouse IL-23 (p19) (Catalog number BE0313) and their isotype controls were purchased from BioXCell.

Target	Fluorochrome	Clone	Catalog Number	Manufacturer
CD103	BV711	2E7	121435	Biolegend
CD11b	Alexa700	M1/70	101222	Biolegend
CD11b	APC-Cy7	M1/70	101226	Biolegend
CD11b	BV421	M1/70	101235	Biolegend
CD11c	Alexa488	N418	117311	Ozyme

CD11c	Pacific Blue	N418	117321	Biolegend
CD11c	PerCP Cy5-5	N418	117327	Ozyme
CD138	BV510	281-2	142521	Biolegend
CD138	BV711	281-2	142519	Biolegend
CD138	BV785	281-2	142534	Biolegend
CD138	PE	281-2	142504	Ozyme
CD169	Alexa647	3D6.112	142408	Biolegend
CD185 (CXCR5)	Alexa647	L138D7	145532	Biolegend
CD19	Alexa594	6D5	115552	Biologend
CD19	Alexa647	6D5	115522	Biolegend
CD19	APC-Cy7	6D5	115530	Biolegend
CD19	BV750	6D5	115561	Ozyme
CD19	PECy5	6D5	115510	Ozyme
CD1d (CD1.1; Ly-38)	Alexa647	1B1	123512	Biolegend
CD25	Alexa647	PC61	102019	Biolegend
CD25	PE-CF594	PC61	562694	Becton
CD3	FITC	17A2	100203	Ozyme
CD38	BUV395	90	740245	BD
CD38	Pacific Blue	90	102720	Biolegend
CD38	PECy7	90	102718	Biolegend
CD3e	Alexa700	17A2	100216	Biolegend
CD3e	PerCP Cy5-5	17A2	100218	Ozyme
CD4	BV605	GK1-5	100451	Biolegend
CD4	PECy7	RM4-5	100528	Biolegend
CD44	BUV737	IM7	612799	Becton
CD45	BV510	30-F11	563891	Becton
CD45	FITC	30-F11	103108	Ozyme
CD45	PE	30-F11	103106	Ozyme
CD45	APC-Cy7	30-F11	103116	Biolegend
CD45	BV510	30-F11	103138	Biolegend
B220	Alexa594	RA3-6B2	103254	Biologend
B220	Alexa647	RA3-6B2	103226	Biolegend
B220	BUV395	RA3-6B2	563793	BD
B220	BUV737	RA3-6B2	612839	BD
B220	BV510	RA3-6B2	103247	Biolegend
B220	eFluor570	RA3-6B2	41-0452-80	Life technologies
B220	eFluor615	RA3-6B2	42-0452-80	Thermo Fisher
B220	FITC	RA3-6B2	103206	Ozyme
B220	Pacific Blue	RA3-6B2	103227	Biolegend
B220	PE	RA3-6B2	103208	Ozyme
CD5	Alexa700	53-7.3	100636	Biolegend
CD64	PE	X54-5/7.1	139303	Ozyme
CD69	PECy7	H1.2F3	104512	Biolegend
CD80	PECy5	16-10A1	104711	Ozyme
CD8a	APC-Cy7	53-6.7	100714	Biolegend

CD8a	PE	53-6.7	100708	Ozyme
CD8a	V500	53-6.7	560776	Becton
CD8a	BV711	53-6.7	563046	BD
CD93	BV786	AA4.1	740941	BD
CD93	BUV737	AA4.1	741800	BD
CD93	BV650	R139	740548	Becton
CD95	BV510	Jo2	563646	BD
CD95	BV605	SA367H8	152612	Biolegend
CD95	BV605	Jo2	740367	BD
CD95	FITC	SA367H8	152606	Biolegend
CD98	APC	RL388	128212	Biolegend
CD98	PE	RL388	128208	Ozyme
CD98	PECy7	4F2	128214	Biolegend
class II IA-IE	Alexa647	M5/114.15.2	107618	Biolegend
F4/80	Alexa700	BM8	123130	BD
F4/80	APC-Cy7	BM8	123117	Biolegend
F4/80	BV421	BM8	123437	Biolegend
GL7	Alexa647	GL7	144606	Biolegend
GL7	Pacific Blue	GL7	144613	Biolegend
GL7	PECy7	GL7	144620	Biolegend
Interferon-γ	PECy7	XMG1.2	505826	Biolegend
IgA	Alexa647	polyclonal	1040-31	Clinisciences
IgD	BV786	11-26c.2a	563618	BD
IgG1	V450	A85-1	562107	BD
IgG1	PE	A85-1	562027	BD
IgG2a	APC/Fire750	RMG2a-62	407123	Biolegend
IgG2b	Alexa488	RMG2b-1	406718	Ozyme
IgG2b	BV421	R12-3	743174	BD
IgG2c	FITC		97254	abcam
IgG3	BV650	R40-82	744136	Becton
IgM	PECy7	R6-60.2	552867	BD
IgM	PerCP Cy5-5	RMM-1	BLE406512	Ozyme
IL17A	APC	TC11-18H10.1	506915	Biolegend
IL17A	FITC	TC11-18H10.1	BLE506908	Ozyme
IL-17Ra (CD217)	PE	PAJ-17R	15299299	Fisher Scientific
IL4	BV421	11B11	504127	Biolegend
IL4	PE	11B11	504104	Ozyme
Ly6C	Alexa647	HK1-4	128010	Biolegend
Ly6G	Alexa488	1A8	127626	Ozyme
Ly6G	Alexa647	1A8	127610	Biolegend
LY6G	PECy7	1A8	127618	Biolegend
TCRβ	BV650	H57-597	109251	Biolegend
TCRβ	FITC	H57-597	109205	Ozyme
TCRγδ	APC/Fire750	GL3	118136	Biolegend

CXCR6 (CD186)	PE/Dazzle 594	SA051D1	151117	Biolegend
CCR7 (CD197)	PECy5	4B12	BLE120113	Biolegend
CD69	APC-Cy7	FN50	561240	BD
CD45	biotinylated	30-F11	103104	Biolegend
anti-GFP	purified		13970	abcam
Streptavidin Alexa fluor 488	Alexa 488		405235	Ozyme

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