RECENT ADVANCES IN MAIT CELLS BIOLOGY

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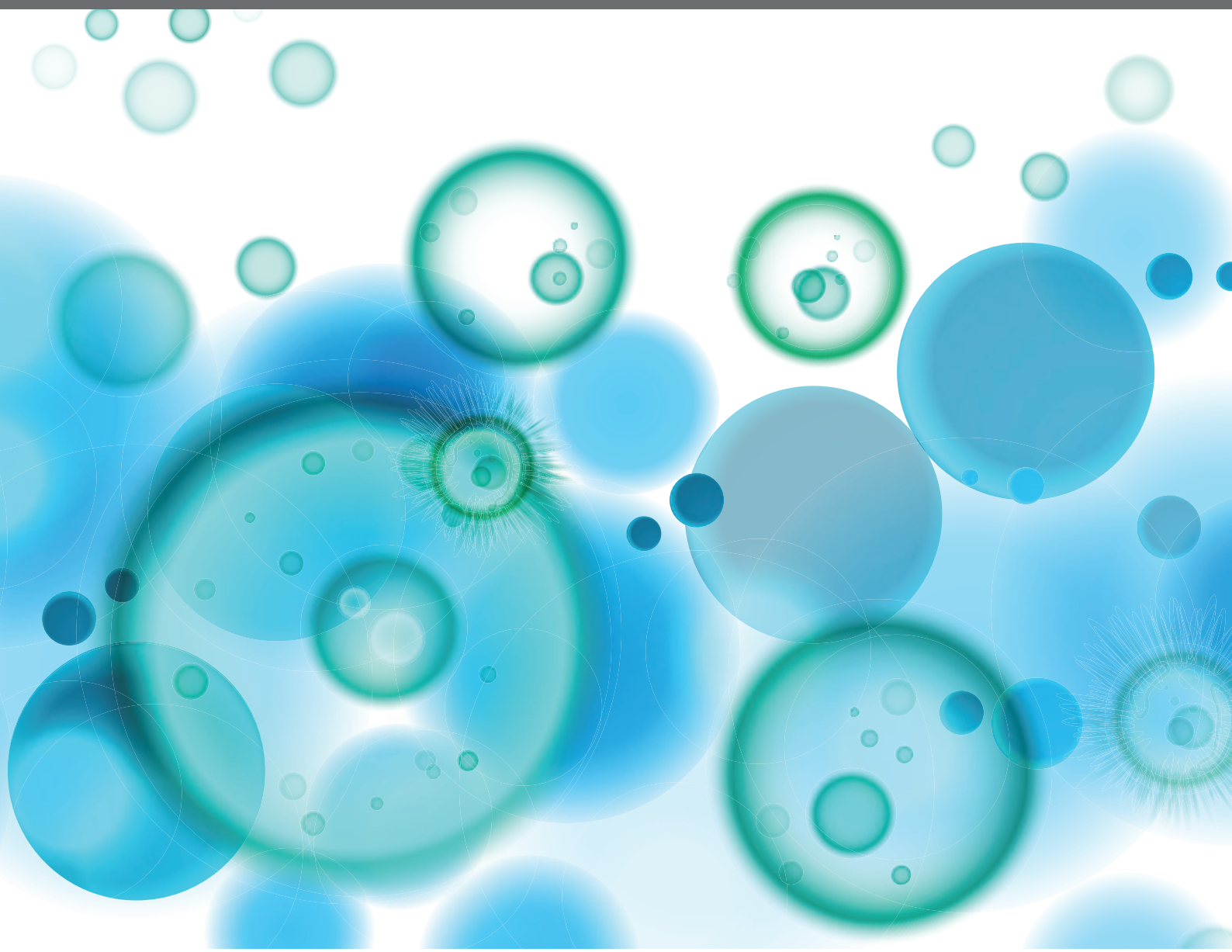

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RECENT ADVANCES IN MAIT CELLS BIOLOGY

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MINI REVIEW
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MAIT Cell Activation and Functions

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Mucosal associated invariant T (MAIT) cells are striking in their abundance and their strict conservation across 150 million years of mammalian evolution, implying they must fulfill critical immunological function(s). MAIT cells are defined by their expression of a semi-invariant αβ TCR which recognizes biosynthetic derivatives of riboflavin synthesis presented on MR1. Initial studies focused on their role in detecting predominantly intracellular bacterial and mycobacterial infections. However, it is now recognized that there are several modes of MAIT cell activation and these are related to activation of distinct transcriptional programmes, each associated with distinct functional roles. In this minireview, we summarize current knowledge from human and animal studies of MAIT cell activation induced (1) in an MR1-TCR dependent manner in the context of inflammatory danger signals and associated with antibacterial host defense; (2) in an MR1-TCR independent manner by the cytokines interleukin(IL)-12/-15/-18 and type I interferon, which is associated with antiviral responses; and (3) a recently-described TCR-dependent "tissue repair" programme which is associated with accelerated wound healing in the context of commensal microbiota. Because of this capability for diverse functional responses in diverse immunological contexts, these intriguing cells now appear to be multifunctional effectors central to the interface of innate and adaptive immunity.

Keywords: mucosal-associated invariant T cell, activation, innate, T cells, human, mouse, review

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INTRODUCTION

Mucosal-associated invariant T (MAIT) cells are innate-like T cells defined by their semi-invariant αβ T cell receptor (TCR) which recognizes small-molecule biosynthetic derivatives of riboflavin synthesis (1–3) presented on the restriction molecule major histocompatibility complex (MHC)-related protein-1 (MR1) (4). MAIT cells were described in 1999 (5) based on their TCR comprising a semi-invariant TCR-α chain—usually Vα7.2–Jα33/12/20 in humans, Vα19–Jα33 in mice—predominantly associated with the β-chains Vβ2/Vβ13 in humans and Vβ6/Vβ8 in mice (5, 7).

MAIT cells contrast with conventional T cells which have highly variable TCRs, capable of targeting a vast array of peptide epitopes produced by viruses, bacteria and malignant cells. Conventional T cells therefore have exquisite specificity for individual peptides, and individual clones may undergo massive expansion, to provide T cell memory. However, at the first encounter with a pathogen the frequency of any individual peptide-specific T cell will be very low. In contrast, the MAIT cell TCR provides an innate capacity to respond to a specific set of ligands without the need for expansion.

A key discovery was the identification of these ligands presented on MR1, which include the potent MAIT cell ligands 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) (2, 8) produced by a wide variety of bacteria, mycobacteria and yeasts during riboflavin (vitamin B2) synthesis (3, 9–12). This pathway is absent from mammals; therefore, its immune detection allows effective host-pathogen discrimination.

Several properties of MAIT cells imply fundamental roles in mammalian immunity. First, MAIT cells have an intrinsic effector-memory phenotype, usually $CD45RA^{-}CD45RO^{+}$ $CD95^{Hi}CD62L^{Lo}CD44^{Hi}$ (4, 13–15), with capacity for rapid secretion of several pro-inflammatory cytokines (13, 15). Second, MAIT cells are remarkably abundant in human tissues, typically comprising 1–4% of all T cells in peripheral blood (16, 17) and up to 10% of airway T cells (18, 19) and 20–40% of liver T cells (13, 20). Moreover, as each TCR recognizes the same ligand, early in an immune response, MAIT cells will markedly exceed the numbers of conventional antigen-specific T cells responding to cognate antigens. Third, the MR1-MAIT cell axis is strikingly conserved across 150 million years of mammalian evolution (21), with ∼90% sequence homology for MR1 between mouse and human (21), implying a strong evolutionary pressure maintaining the MAIT cell repertoire. Nonetheless identifying the critical functional role(s) played by these cells has not proved straightforward, perhaps because these cells perform not a single, but several distinct functions. Indeed, several new MAIT cell functions have recently been discovered, representing distinct transcriptional programs which can be triggered via distinct activation pathways.

Here we review both the mechanisms and requirements for MAIT cell activation, and our current understanding of their diverse functional roles, including response to bacterial infections, viral infections and tissue repair. Consequently, these diverse roles suggest the potential to harness MAIT cells in protection against infectious disease, in vaccine design and in promoting wound healing.

MAIT CELL ACTIVATION

TCR Mediated Activation

MAIT cells can be activated in response to TCR ligation by riboflavin intermediates presented on MR1, under co-stimulatory signals from specific cytokines or toll-like receptors (TLR) (22). Activated cells expand substantially inducing a rapid innate-like immune response and effector functions including anti-microbial cytotoxic products, inflammatory chemokines, and cytokines.

During riboflavin biosynthesis, the pyrimidines 5-OP-RU and 5-OE-RU are generated from the precursor 5-amino-6-D-ribitylaminouracil (5-A-RU) by non-enzymatic condensation with methylglyoxal and glyoxal, respectively (1, 2, 23). RibD is a key gene in this pathway, encoding a pyrimidine deaminase/reductase that generates 5-A-RU. RibD expression correlates with MAIT cell response (24). Thus, bacteria possessing this pathway, for instance, *Escherichia*, *Lactobacillus*, *Staphylococcus* (9), *Shigella Flexneri* (25), *Salmonella*, *Mycobacteria,* and *Clostridioides* species (3, 9, 26) besides *Mycobacteria* (27) or fungi equipped with riboflavin synthesis, such as *Saccharomyces* (9), *Candida* (9, 28), and *Aspergillus* (29) can activate MAIT cells in an MR1-dependent manner, while other bacterial species lacking the full riboflavin pathway such as *Enterococcus faecalis* (9) and *Listeria monocytogenes* (27) are not.

Unlike conventional T cells which recognize peptide antigen presented by MHC molecules, MAIT cells are restricted by MR1, a non-polymorphic, β2-microglobulin-associated antigen-presenting molecule, widely expressed in multiple tissues (30, 31). Unlike class 1 and class 2 MHC, MR1 does not constitutively present self-ligands. Generally, MR1 molecules reside in the endoplasmic reticulum (ER) in an incompletely folded ligand-receptive conformation, free of β2 microglobulin. Riboflavin metabolites are transported to the ER, bind MR1 via formation of a Schiff base, followed by a complete folding and association with β2-microglobulin. The ternary complex then traffics through the ER and the Golgi to the cell membrane (32). Although recycling of the MR1 molecule can occur (33), most MR1 molecules are degraded and reinternalized intracellularly, which contributes to MR1's rapid presentation of extracellular riboflavin antigens and MAIT cells' rapid activation (32). Moreover, NF-κB signaling is necessary for MR1 signal transduction (34). Either bone marrow-derived antigen-presenting cell (APC) such as dendritic cells (27, 35), monocytes (9, 13), macrophages (35), B cells (36, 37), or non-bone marrow-derived epithelial cells (27, 37) can activate MAIT cells via MR1 (38).

As with conventional T cells, MR1-TCR signaling alone is insufficient to fully activate MAIT cells which also require co-stimulation (22, 38–40) by CD28 (39), TLR agonists, bacterial products or cytokines (22). Such cytokines include interleukin (IL)-7 (41, 42), tumor necrosis factor (TNF) (43), type-I interferons (IFNs) (44), IL-1β and/or IL-23 (38, 41). MAIT cells express several cytokine receptors including IL-7R, IL-12R, IL-15R, IL-18R, and IL-23R (9, 13, 38). IL-7 enhances MAIT cell responses to bacteria and promotes cytotoxicity (42). IL-12 and IL-18 potentiate MR1-dependent bacterial MAIT cell activation (34, 45). Agonists of the pathogen recognition receptors TLR1, TLR2 and TLR6 in humans (34), and TLR3, TLR4, TLR6/2, and TLR9 in mice (22, 46) promote MAIT cell activation mainly in an indirect way through the activation of APCs via enhancement of MR1 presentation, stimulation of cytotoxic molecules and inflammatory cytokines or up-regulation of co-stimulatory ligands (22, 34, 47, 48). In addition, inducible T cell co-stimulator (ICOS), highly expressed by MAIT cells is also essential for optimal activation and maintenance of retinoic acid-related orphan receptor γt (RORγt) expression (38).

Whilst the MAIT cell TCR-α chain is usually Vα7.2–Jα33/12/20 in humans, the β chain is more diverse: Vβ2

Abbreviations: 5-OE-RU, 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil; 5-OP-RU, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil; MAIT, mucosal associated invariant T; MHC, major histocompatibility complex; MR1, major histocompatibility complex-related protein-1; TCR, T cell receptor.

and Vβ13 are the most common. Some data suggest certain Vβ segments are associated with decreased TCR-dependent MAIT cell responses (49). Recently non-cognate TCR-dependent MAIT cell activation has been described for several bacterial superantigens. Although group A streptococci lack the riboflavin pathway, three molecules they produce bind specifically to Vβ2 leading to rapid TNF-dominated MAIT cell activation, likely contributing to the cytokine storm in streptococcal toxic shock syndrome (50). Likewise, in toxic shock induced by *S. aureus*, the staphylococcal enterotoxin B acts as a superantigen preferentially ligating MAIT cell Vβ13.2 with MHCII (51).

TCR-Independent Activation

In the absence of TCR-mediated antigen recognition, MAIT cells can also be partially activated by cytokines, such as IL-7, IL-12, IL-15, IL-18, and type-I IFNs, broadening the potential range of pathogens to which MAIT cells can respond to include viruses (35, 52–54). IL-7 can induce expression of cytolytic effector molecules (42). IL-12 or IL-15 together with IL-18, produced from APCs in response to TLR ligands, can directly stimulate MAIT cells to produce IFN-γ (35, 48, 55) and release granzyme B and perforin (45). IFN-α/β alone can activate MAIT cells but not induce cytokine production or upregulation of co-stimulatory molecules (44). Type-I IFNs induce significant IFN-γ and granzyme B only when combined with IL-12 or IL-18 (35). Likewise, the gut-associated pro-inflammatory cytokine, TNF-like protein 1A (TL1A/TNFSF15) activate MAIT cells in combination with IL-12 and IL-18 (56).

TCR-mediated and -independent activation work synergistically in optimal MAIT cell activation (**Figure 1**). Upon stimulation, there is increased expression of activation markers CD69 and CD25, degranulation marker CD107a (9, 25, 57), production of cytotoxic substances such as perforin and granzyme B (13, 42, 58), secretion of pro-inflammatory cytokines including IFN-γ, TNF, IL-17, and colony stimulating factor 2 (CSF2/GM-CSF) (9, 12, 13) and release of chemokines such as XCL1, CCL3, CCL4, and CXCL16 (40, 46). MAIT cells exert antimicrobial activity not only by direct recognition and killing of infected cells, but also indirectly, for example by recruiting neutrophils (59), increasing bactericidal activity of phagocytes (60), promoting the production of IFN-γ from DCs (35), and promoting monocyte to DC differentiation (61). Despite the considerable overlap in transcriptional and functional profiles, there are differences between these two modes of activation. TCR stimulation generally results in a more rapid immune response and multiple pro-inflammatory cytokines and chemokines production than cytokine stimulation alone. Elevated expression of RORγt, and the cytokines IL-17A, TNF, and CSF2 are seen with TCR-mediated activation, consistent with a Tc17-like phenotype (46, 59, 62), whilst TCR-independent activation is dominated by IFN-γ, perforin and granzyme B under the control of promyelocytic leukemia zinc finger (PLZF) (46) and T-bet (59), consistent with a Tc1-like phenotype. Importantly it is TCR-dependent activation which induces a tissue-repair programme (46, 56, 59). Knowing TCR-dependent and -independent MAIT cell responses are distinct, there remain many unanswered questions. For example, do MAIT cells shift between Tc17-like phenotype and Tc1-like phenotype? If such plasticity exists, what conditions are required and what are the influences?

MAIT CELL FUNCTIONS IN INFECTION

As a specific population of innate-like lymphoid cells, MAIT cells are involved in early immunity against infection in peripheral tissue, with a more rapid response to pathogens and shorter time to effector function *in vivo* than conventional MHC-restricted T cells in infectious disease (63). Next, we review MAIT cell functions antibacterial and antiviral host defense. The pathogens, activation elements, and effectors responses are summarized in **Supplementary Table 1**.

Antibacterial Host Defense

A wide range of bacterial, mycobacterial, and fungal pathogens have been shown to activate MAIT cells *in vitro* (**Figure 2**). These pathogens all express the riboflavin pathway and activation is via TCR-dependent activation. MAIT cells co-cultured with bacterially-infected monocytes (9, 13, 35, 51) or *M. tuberculosis*-infected lung epithelial cell lines (27) release IFN-γ in an MR-dependent manner. MAIT cell TCR-transgenic mice were better protected against infection by *E. coli* or *M. abscessus* than $Mr1^{-/-}$ MAIT cell TCR-transgenic mice (9). Furthermore, bacterially-activated MAIT cells express perforin, undergo degranulation and can directly kill *E. coli*-infected human epithelial cell lines (HeLa-MR1) modified to over-express MR1 (25). With the intracellularly-invasive *Shigella*, this cell killing may occur in parental HeLa cells, suggesting a predilection for MAIT cells to respond to intracellular bacteria (25). Thus, there is potent inhibition of *Mycobacterium bovis* BCG growth within macrophages when co-cultured with MAIT cells (12). Consistent with this, *Streptococcus pneumoniae* has a poor capacity to activate MAIT cells when the APC is a monocyte or monocyte cell line, and this activation is dependent on cytokines rather than MR1 (64), perhaps because of monocytes' poor phagocytic and antigen-presenting capacity (65, 66). Conversely, *S. pneumoniae* does induce MR1-dependent activation in the presence of monocyte-derived macrophages, which have a greater phagocytic capacity (64). Likewise, *S. pneumoniae* activates MAIT cells in the presence of human monocyte-derived dendritic cells, in an MR1-dependent manner, with the extent of activation correlating with the activity of the RibD operon (24). In co-culture, MAIT cells reduce growth of *S. pneumoniae* within infected primary bronchial epithelial cells.

To date, few pathogens have been found to induce MAIT cell expansion *in vivo*, and interestingly those that have are also predominantly intracellular pathogens. In a murine model, using a live vaccine strain (LVS) of the rare opportunistic intracellular pathogen *Francisella tularensis,* pulmonary MAIT cells expand markedly during acute intranasal infection (67). These cells express IFN-γ, IL-17, TNF, and inducible nitric oxide synthase (67) as well as GM-CSF which enhances pulmonary recruitment of inflammatory monocytes (61). There is impaired protection against infection by pulmonary *F. tularensis* LVS in $CD4^{+}CD8^{+}$-depleted $MR1^{-/-}$ mice in

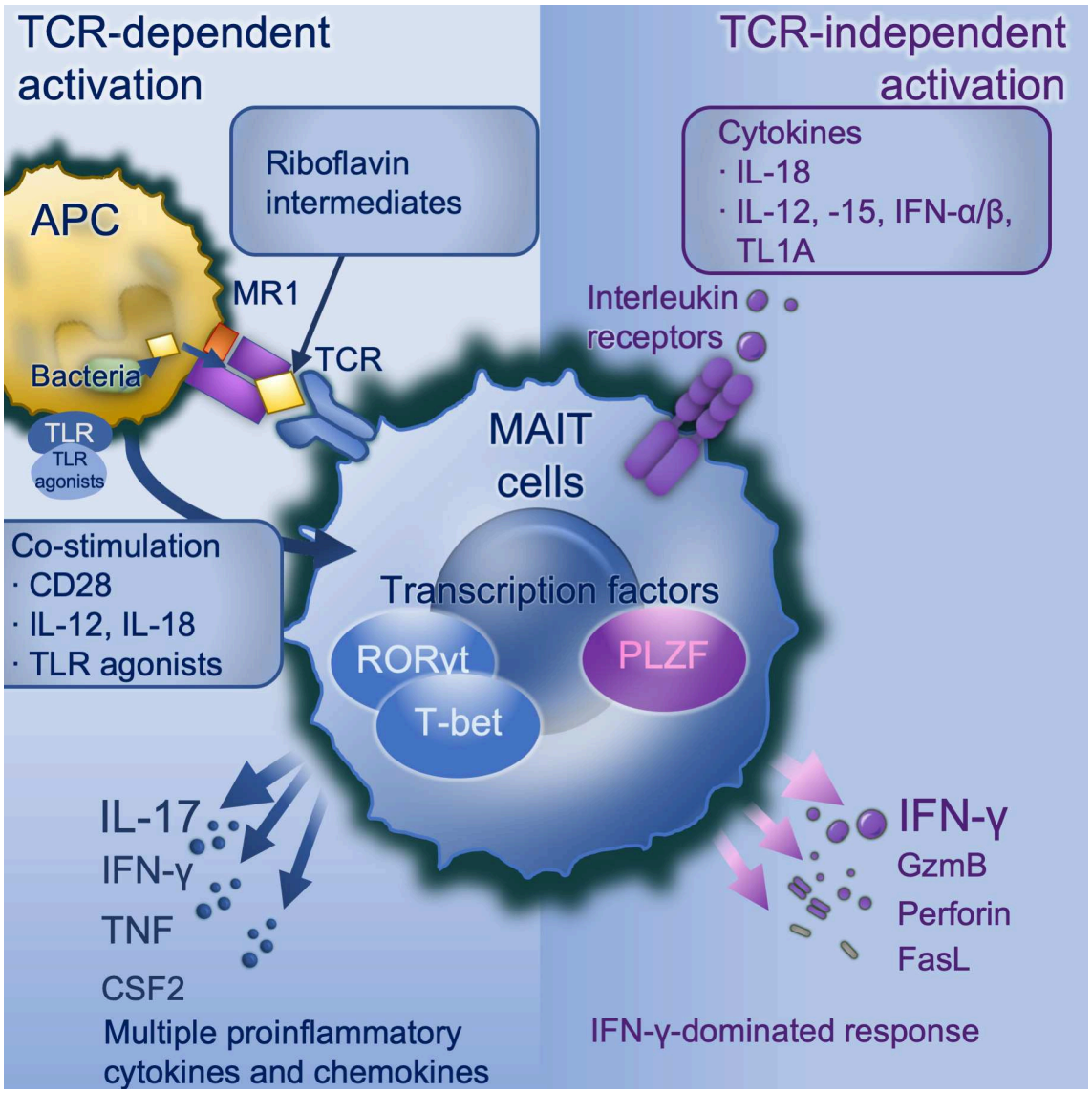

FIGURE 1 | MAIT cell activation. TCR-dependent activation requires microbially-derived riboflavin intermediates such as 5-OP-RU to be presented on MR1 to a MAIT cell TCR in conjunction with co-stimulation. Co-stimulation may include CD28 and/or cytokines particularly IL-12/-18, and can be induced by danger signals including TLR agonists (in mice TLRs 3, 4, 6/2, or 9, in human TLRs 1, 2, or 6). TCR dependent activation induces a strong and broad production of pro-inflammatory cytokines and chemokines, dominated by IL-17A under the control of the transcription factor RORγt. TCR-independent activation is driven by IL-18 in synergy with IL-12, or−15, or IFN-α or -β, and potentiated by TL1A. This activates a more modest cytokine response dominated by IFN-γ under the control of PLZF. APC, antigen-presenting cell; CD, cluster of differentiation; CSF2, colony stimulating factor 2 (GM-CSF); GzmB, granzyme B; IFN, interferon; IL, interleukin; MAIT, mucosal associated invariant T; MR1, major histocompatibility complex-related protein-1; PLZF, Promyelocytic leukaemia zinc finger protein; RORγt, retinoic acid-related orphan receptor gamma; TCR, T cell receptor; TL1A, tumor necrosis factor (TNF)-like protein 1A (TNFSF15); TLR, toll-like receptor.

contrast to their wild-type counterpart, which demonstrates MAIT cells' significance in mucosal immunity in absence of $CD4^+$ and $CD8^+$ T cells. In a second model of intracellular pulmonary bacterial infection, intranasal *S.* Typhimurium induced rapid, MR1-dependent expansion of pulmonary MAIT cells frequencies which persisted as an expanded population long-term comprising 35–50% of all pulmonary αβ T cells (22).

In the *S.* Typhimurium model, bacterial clearance was not dependent on MAIT cells, due to other redundant mechanisms including conventional $CD4^+$ and $CD8^+$ T cells (22). Impaired control of microbial growth *in vivo* has been observed with *F. tularensis* (61, 67) and the mycobacteria *M. abscessus* (9) and *M. bovis* BCG (12) in $MR1^{-/-}$ mice which have an absolute deficiency of MAIT cells. In a murine model of Legionnaire's disease, two clinically-important strains of

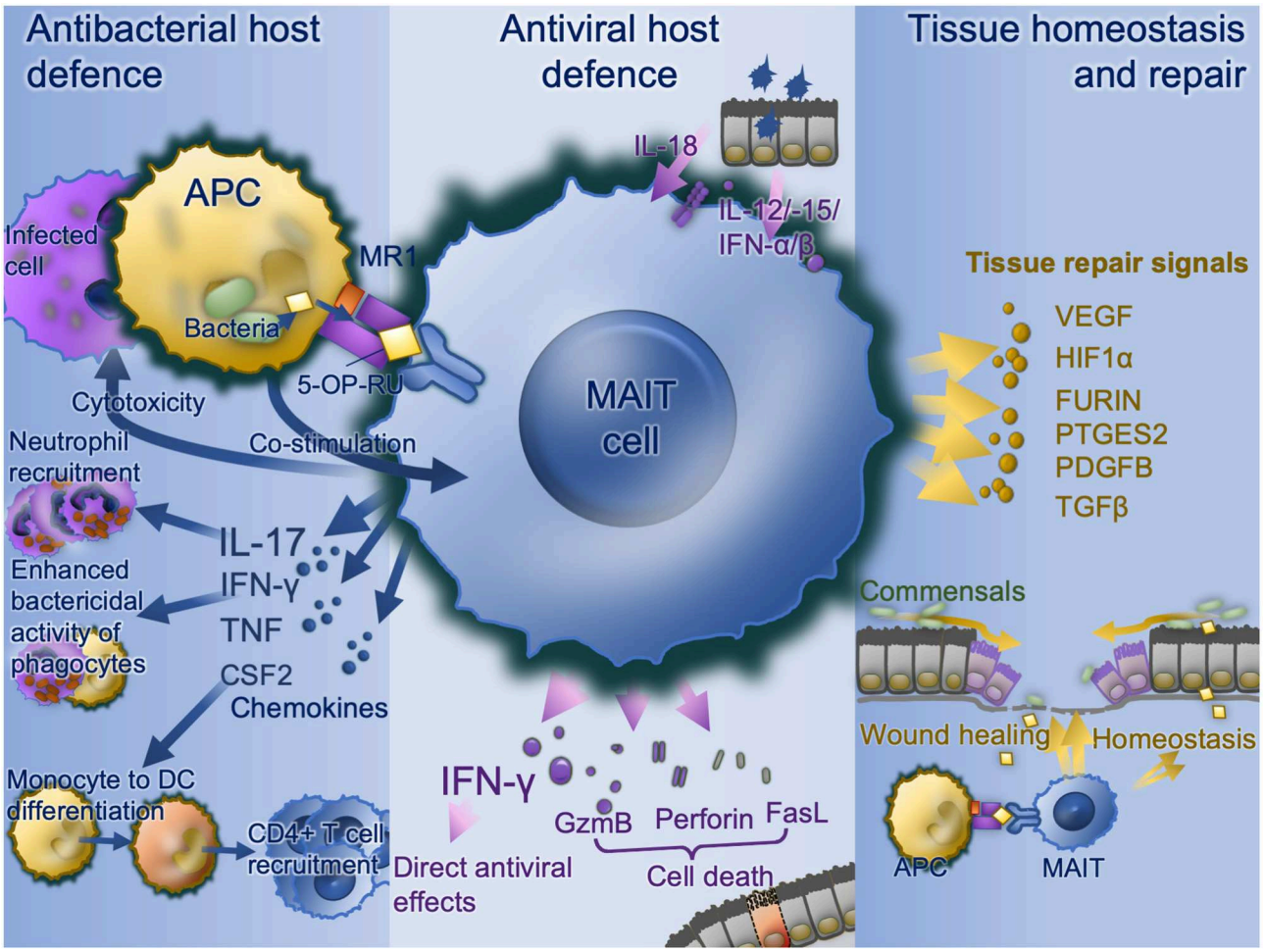

FIGURE 2 | MAIT cell functions. Bacterially-infected cells present MAIT cell ligands on MR1 with co-stimulation and activate MAIT cell antibacterial defense functions including IFN-γ enhancement of antibacterial function in phagocytes, recruitment of neutrophils via IL-17 and differentiation of inflammatory monocytes to DCs in turn driving CD4 T cell recruitment. Virally infected cells produce IL-18, IL-12, IL-15, and type I interferons which upregulates IFN-γ with direct antiviral effects as well as direct cytotoxic MAIT cell functions via granzyme B, perforin, and Fas ligand. Commensal derived MAIT cell ligands, in the absence of co-stimulation stimulate MAIT cells to promote tissue homeostasis. Where a mucosal barrier is damaged these signals are enhanced and accompanied by damage signals to drive accelerated tissue repair signals. APC, antigen-presenting cell; CSF2, colony stimulating factor 2 (GM-CSF); DC, dendritic cell; FasL, Fas cell surface death receptor ligand; GzmB, granzyme B; IFN, interferon; IL, interleukin; MAIT, mucosal associated invariant T; MR1, major histocompatibility complex-related protein-1; TCR, T cell receptor.

Legionella—pneumophila and *longbeachae*—activated human MAIT cells *in vitro* (62). *L. longbeachae* induced rapid pulmonary MAIT cell expansion, by up to 580-fold over 7 days, which persisted long-term, with a similar effect observed with *L. pneumophila*. 5-bromo-2′-deoxyuridine incorporation showed this was due to early, local MAIT cell proliferation in the lung and draining lymph node. MR1$^{-/-}$ mice had delayed bacterial clearance at days 10–14 post-infection, although in both models this effect was small, due to immunological redundancy. To remove these additional layers of immunological redundancy, we adoptively transferred MR1-5-OP-RU positive MAIT cells into Rag2$^{-/-}\gamma C^{-/-}$ mice which lack T, B and NK cells. MAIT cells were sufficient to rescue mice completely from fatal *L. longbeachae* infection (62).

How do MAIT cells provide this protection? Using adoptive transfer from mice deficient in various signaling pathways, we showed this immune protection depended predominantly on MAIT cell IFN-γ, and to a lesser extent on GM-CSF, but not, with this organism, on perforin or granzyme (62). *Legionella* infects inflammatory cells, particularly neutrophils and macrophages, rather than epithelia and it is likely MAIT-derived IFN-γ limits intracellular growth of bacteria in these cells through multiple mechanisms including enhancement of oxidative burst, nitric oxide production, antigen presentation, phagocytosis and upregulation of CD80/86 co-stimulation, cytokines and chemokines (68). IFN-γ is also critical for protection against mycobacterial disease including *M. tuberculosis*, so this early production of MAIT cell-derived IFN-γ may likely be an important and non-redundant component of protection against mycobacteria. Indeed, polymorphism in MR1 is associated with susceptibility to *M. tuberculosis* (69).

Might MAIT cells be important in more common respiratory diseases? Persistent infection with *Haemophilus influenzae,* which can persist intracellularly (70), can drive inflammation in chronic obstructive pulmonary disease (COPD) (71) and severe neutrophilic asthma (72, 73) and enhance susceptibility to virally-induced inflammation (74, 75). MAIT cells are abundant in the normal airway mucosa, but we have shown frequencies are markedly reduced by therapeutic corticosteroids in both asthma (18) and COPD (19). *In vitro H. influenzae* enhances surface expression of MR1 on human pulmonary macrophages and induces MAIT cell IFN-γ, but both functions are also inhibited by corticosteroids, which may explain the enhanced susceptibility to bacterial pneumonias in airways diseases (19), suggesting a potential clinical benefit from more judicious use of inhaled steroids or MAIT-cell enhancing strategies.

MAIT cells are therefore capable of providing TCR-dependent rapid immune defense against a range of pathogenic intracellular bacteria. However, paradoxically, despite evidence of strong evolutionary pressure to maintain MAIT cell populations, it has been hard to identify a profound, non-redundant phenotype of pure MAIT cell deficiency either in the clinic or in animal models. The explanation may be that in addition to this undoubtedly important antibacterial role, it is now clear that MAIT cells perform additional roles in mucosal immunology, including antiviral host defense and tissue repair.

Antiviral Host Defense

Conventional T cells are critical for eventual clearance of most viruses by producing a peptide-specific cell-mediated immune response, which takes 4–7 days to evolve. Given their capacity for cytokine-mediated, TCR-independent activation, do MAIT cells play a role in anti-viral host-defense? Virus-induced activation of $CD161^{+}V\alpha7.2^{+}$ MAIT cells was observed *in vivo* in human peripheral blood MAIT cells during clinical infection of dengue, hepatitis C and influenza A (35), as well as at the peak viremia stage during acute infection by human immunodeficiency virus (76). Activation, measured by CD38 or granzyme B upregulation, increased during each infection *in vivo* whilst also *in vitro* MAIT cells upregulated CD69 and IFN-γ in the presence of virally-infected APCs. Similar findings were observed in a separate report of patients hospitalized with the H7N9 strain of influenza A virus, and interestingly higher MAIT cell frequencies were associated with subsequent recovery (53). Indeed cytokine-activated MAIT cells could reduce replication of hepatitis C *in vitro* in an IFN-γ dependent manner (35). However, the correlative clinical observations cannot prove causality, and neither study addressed whether the consequences of virus-induced MAIT cell activation in the intact host would be a beneficial contribution to antiviral defense, or conversely a worsening of immune-pathology, perhaps even augmentation of the cytokine-storm which can prove fatal in acute influenza infection.

To address this question we compared survival in mice infected with H1N1 influenza A virus in the presence or absence of an intact MR1: MAIT cell axis (52). MR1-tetramer^{+} MAIT cells accumulated in the lungs and were activated, with MAIT cell frequencies and CD25, CD69, and granzyme B upregulation peaking at day 5 post-infection, at least 48 h earlier than peak frequencies of conventional $CD8^{+}$ T cells (defined as $TCR\beta^{+}$ $CD45.2^{+}$ $CD19^{-}$ MR1-5-OP-RU tetramer^{-} $CD8^{+}$cells). Activation was dependent predominantly on IL-12 and to a lesser extent on IL-15, IL-18, and IFN-α. MAIT cell-deficient $MR1^{-/-}$ mice showed enhanced weight loss and mortality which was ameliorated by prior adoptive transfer of pulmonary MAIT cells in both immunocompetent and immunodeficient $Rag2^{-/-}\gamma C^{-/-}$ mice. This confirmed that MAIT cells did not worsen immunopathology, but rather they contributed to immune protection. Moreover, current yearly influenza vaccines show limited efficacy and little heterologous protection between strains, whereas the TCR-independent nature of the antiviral MAIT cell response and the adoptive transfer experiments suggest the possibility of clinical benefit from therapeutic enhancement of airway MAIT cell numbers, such as a pre-exposure boosting using inhaled MAIT cell ligands. Such an approach would require clinical trials.

MAIT CELL FUNCTION IN TISSUE REPAIR AND HOMEOSTASIS

Recently a third, distinct function has been discovered for MAIT cells. Using a transcriptomic approach on sorted MR1-tetramer^{+} cells, we sought to define the transcriptome of an activated MAIT cell in both human and mice. In addition to the strong expression of pro-inflammatory cytokines, we discovered in both species TCR-mediated activation-induced expression of a tissue-repair programme (46). The genes upregulated in both species included TNF, CSF2, HIF1A, FURIN, VEGFB, PTGES2, PDGFB, TGFB1, MMP25, and HMGB1. These genes had previously been identified as a geneset expressed by skin-homing Tc17 cells induced by commensal flora and able to accelerate repair of an epithelial wound in mice (77). Such Tc17 cells were restricted by another MHC class 1b molecule H2-M3, but this molecule is absent in humans and given their commensal dependence and capacity for IL-17 production it seemed likely MAIT cells might share this programme. Indeed in a comparative transcriptomic analysis of different T cell subsets in the ImmGen database (78) activated MAIT cells shared the greatest similarity with these commensal-induced epithelial Tc17 cells (46). This tissue repair programme is observed in MAIT cells stimulated by TCR ligands but not by cytokine-mediated stimulation alone (56, 59). Supernatants from TCR-activated MAIT cells accelerated wound closure in an intestinal epithelial cell line system (56).

Further studies *in vivo* have shown that skin colonization of germ-free mice with a common skin-commensal *Staphylococcus epidermidis* induced expansion of cutaneous MAIT cell populations and upregulation of this tissue repair programme in cutaneous MAIT cells (79). These MAIT cells are predominantly localized in the dermis

near the dermal-epidermal junction and their MAIT cell expansion depended on 5-OP-RU and MR1. Moreover, these MAIT cells could accelerate the closure of a punch-biopsy induced skin wound, which was enhanced by application of topical 5-OP-RU.

Commensal organisms play a fundamental role in the development, function and homeostasis of the host immune system. Maintenance of the optimal symbiotic relationship between commensal microbiota and the immune system allows protective immune responses to occasional invasive pathogens (80, 81). Since MAIT cells are predominantly located in tissues colonized by commensal microbes, with broad antimicrobial specificity and tissue-repair function, MAIT cells' response to commensal organisms and tissue damage and infections may be as important for the restoration of homeostasis as their role in protection against pathogen invasion. Furthermore, it seems likely that, depending on the distinct tissue microenvironment, MAIT cells may express both antibacterial and also tissue repair functions at different stages in the evolution of an infectious or physical injury.

MAIT CELL FUNCTION IN AUTOIMMUNITY AND INFLAMMATION

Conventional T cells are implicated as effectors in many organ-specific autoimmune diseases such as type-1 diabetes or multiple sclerosis, but strong HLA associations in a range of systemic autoimmune diseases imply a pathogenic role in these diseases as well. What role might MAIT cells play in these diseases? As the MAIT cell ligands are not synthesized by human cells, MAIT cell activation in these conditions would be presumed to be via the cytokine-mediated TCR-independent pathway. Changes in MAIT cell frequencies and phenotype are observed in a range of autoimmune conditions.

Blood MAIT cells are decreased in children with type 1 diabetes mellitus. In the non-obese diabetic mouse $MR1^{-/-}$ mice have accelerated diabetes and increased gut permeability, suggesting MAIT cells may be protective against diabetes by supporting intestinal mucosal integrity, although the data are complicated by evidence in mice and humans of MAIT cell activation, exhaustion and capacity for islet-cell killing (82). Similarly, in type 2 diabetes peripheral blood MAIT cells are reduced in frequency, associated with increased caspase-3-dependent apoptosis (83).

In rheumatoid arthritis MAIT cell frequencies are increased in synovial tissue and so may contribute to maturation and cross-differentiation of T cells locally (84). Consistent with a pathogenic role, inflammation is reduced in murine collagen-induced arthritis in $MR1^{-/-}$ mice (85).

MAIT cells are increased in inflammatory lesions in human multiple sclerosis (86), although data from animal models suggest they may play a protective role in the lesions as inflammation and pathology are reduced by adoptive transfer and exaggerated in $MR1^{-/-}$ animals (87). Likewise, inflammation is suppressed by inhibitory MAIT cell ligands in an animal model of systemic lupus erythematosus (88).

In the gut, MAIT cells are found in proximity to *Helicobacter pylori* in human gastric mucosa, and in mice, MAIT cells were associated with accelerated *H. pylori* gastritis (89). In inflammatory bowel disease, several studies show decreases in peripheral blood MAIT cell frequencies and generally an increase in intestinal tissue (90–93), with increased IL-17 and IL-22 production by blood MAIT cells, although it remains to be seen if these changes are causally linked and whether they are pathogenic or protective by restoring mucosal integrity (94, 95).

POTENTIAL CLINICAL TRANSLATION OF MAIT CELL BIOLOGY

Several characteristics of MAIT cells—their abundancy, their intrinsic effector memory phenotype (13), their mucosal distribution and the invariant nature of their receptor—make them excellent candidates to harness in the development of vaccines. In a therapeutic vaccine, for instance, for a respiratory infection, MAIT cell frequencies might be rapidly increased by stimulation with a MAIT cell ligand and co-stimulation, as has been shown in mice (22, 46, 62). More likely if more stable MAIT cell-activating ligands could be developed, these could be added to B cell or T cell prophylactic vaccines to enhance biological adjuvancy (96).

In autoimmune diseases in which MAIT cells had a predominantly pathological role, it would be possible to inhibit MAIT cells in a highly-selective manner using potent, inhibitory MAIT cell ligands such as acetyl-6-formylpterin (97). As orally-active small molecules, these could be attractive targets.

These cells' recently discovered role in promoting wound healing is perhaps the most exciting, from a translational perspective. Healing can be slow in chronic skin wounds such as leg ulcers, sacral pressure sores or burns, and could potentially be accelerated either by local wound re-colonization with riboflavin-synthesizing commensals, or through topical application of synthetic MAIT cell ligands. The latter approach has already been tested in a proof-of-principle murine study (79), and could very easily be translated into large-scale clinical trials.

In summary, the conservation and abundance of MAIT cells is likely explained by their broad range of functionality attributable to different modes of activation, each triggering a distinct transcriptomic programme. Because of their capability for diverse functional responses in diverse immunological contexts, these intriguing cells now appear to be multifunctional effectors central to the interface of innate and adaptive immunity. Already three major functions—antibacterial host defense, antiviral host defense, tissue repair and homeostasis—have been described for

these intriguing cells, but it is likely other functions remain to be discovered.

AUTHOR CONTRIBUTIONS

TH and X-WZ jointly conceived the article, conducted the literature review, and drafted the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01014/full#supplementary-material

Supplementary Table 1 | Effects on MAIT cell activation and functions of various bacterial pathogens.

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Mucosal-Associated Invariant T Cells Develop an Innate-Like Transcriptomic Program in Anti-mycobacterial Responses

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Conventional T cells exhibit a delayed response to the initial priming of peptide antigens presented by major histocompatibility complex (MHC) proteins. Unlike conventional T cells, mucosal-associated invariant T (MAIT) cells quickly respond to non-peptidic metabolite antigens presented by MHC-related protein 1 (MR1). To elucidate the MR1-dependent activation program of MAIT cells in response to mycobacterial infections, we determined the surface markers, transcriptomic profiles, and effector responses of activated human MAIT cells. Results revealed that mycobacterial-incubated antigen-presenting cells stimulated abundant human $CD8^+$ MAIT cells to upregulate the co-expression of CD69 and CD26, as a combinatorial activation marker. Further transcriptomic analyses demonstrated that $CD69^+CD26^{++}$ $CD8^+$MAIT cells highly expressed numerous genes for mediating anti-mycobacterial immune responses, including pro-inflammatory cytokines, cytolytic molecules, NK cell receptors, and transcription factors, in contrast to inactivated counterparts $CD69^{+/-}CD26^{+/-}$ $CD8^+$MAIT cells. Gene co-expression, enrichment, and pathway analyses yielded high statistical significance to strongly support that activated $CD8^+$ MAIT cells shared gene expression and numerous pathways with NK and $CD8^+$ T cells in activation, cytokine production, cytokine signaling, and effector functions. Flow cytometry detected that activated $CD8^+$MAIT cells produced TNFα, IFNγ, and granulysin to inhibit mycobacterial growth and fight mycobacterial infection. Together, results strongly support that the combinatorial activation marker $CD69^+CD26^{++}$ labels the activated $CD8^+$MAIT cells that develop an innate-like activation program in anti-mycobacterial immune responses. We speculate that the rapid production of anti-mycobacterial effector molecules facilitates MAIT cells to fight early mycobacterial infection in humans.

Keywords: mucosal-associated invariant T (MAIT) cells, transcriptome, MHC-related protein 1 (MR1), *Mycobacterium tuberculosis*, innate-like activation

INTRODUCTION

Innate and adaptive immune systems have framed a classical dichotomy of immune responses in health and diseases. Innate immune cells rapidly recognize and respond to microbial and endogenous ligands with germline-encoded innate receptors (1). In contrast, conventional naïve T cells undergo a prolonged clonal expansion in responses to major histocompatibility complex (MHC)-presented peptide antigens (1, 2). It has been demonstrated that $CD4^+$ and $CD8^+$ T cells are crucial in combating mycobacterial infections in T cell-deficiency and adoptive transfer mouse models (3, 4). In humans, $CD4^+$ T cells are essential in fighting mycobacterial infections evidenced by a much higher risk of active tuberculosis in HIV coinfection with defective $CD4^+$ T cell responses (5) and conventional $CD8^+$ T cells suppress mycobacterial growth (4). However, a large number of mycobacterial-reactive $CD8^+$ T cells in humans are activated by non-peptidic antigens presented by MHC class I-related protein I (MR1) and are identified as mucosal-associated invariant T (MAIT) cells (6–12). Therefore, labeling the mycobacterial-reactive MAIT cells and defining their activation program regarding its similarities to conventional T cell or innate cells will facilitate the understanding of their unique activation pathways and effector functions in anti-mycobacterial responses.

Previous observations support that MAIT cell response is "innate-like" upon the stimulation with mouse (8–12) and bacterial antigens (6–8), as different from conventional T cells. The fundamental explanation of this "innate-like" MAIT cell activation initially bases on the evidence that MAIT cells express an invariant TCRα chain to interact with a conserved metabolite antigen presented by a monomorphic MR1 protein (12–17). This conservation interestingly contrasts to the tri-molecular interaction for conventional T cell activation using heterogeneous TCRs to recognize variable peptide antigens presented by heterogeneous HLA molecules (1, 2). In a new bacterial infection, MAIT cells can be instantly activated by MR1-mediated antigen presentation bypassing a long priming stage for conventional T cells (8–12). Recently, MAIT cells were demonstrated to protect mice against mycobacterial infections (8, 18) and respond to mycobacterial-infected human cells (7, 8). Surprisingly, abundant MAIT cells in uninfected and latently infected individuals exhibit strong reactivity to mycobacteria and potentially complement conventional T cells in anti-mycobacterial defenses (7, 8). Moreover, reduced frequency of MAIT cells in active tuberculosis (7, 8, 19, 20) seriously demands the elucidation of MAIT cell activation programs and effector responses in mycobacterial infections. Although riboflavin precursor metabolites have been identified from multiple non-tuberculous bacteria (13, 16, 17) and the general activation program of MAIT cells has been defined using *E.coli*, cytokine, or anti-CD3 stimulation (21–26), it remains uncertain whether mycobacteria efficiently activate human MAIT cells and which surface markers specifically label activated MAIT cells in mycobacterial infection. Moreover, whether activated human MAIT cells show an innate-like transcriptomic program to elicit strong anti-mycobacterial immune responses is unknown. Therefore, applying MAIT cell-specific stimulants, defining the activation program, and determining the effector responses of human MAIT cells in mycobacterial infections will be highly translational to clinically label MAIT cells and mechanistically understand the anti-mycobacterial immune responses of MAIT cells in diseases.

In this study, we elucidated the activation programs of MAIT cells in mycobacterial infections to determine their similarity to $CD8^+$ T and natural killer (NK) cells. Specifically, we activated the dominant $CD8^+$MAIT cell population with mycobacterial stimulation and used the combinatory marker $CD69^+CD26^{++}$ to separate activated MAIT cells for transcriptomic analyses. Results revealed that $CD69^+CD26^{++}$ $CD8^+$MAIT cells shared key gene expression and activation pathways with conventional $CD8^+$ T cells and NK cells, supporting a unique "innate-like" activation program to induce early anti-mycobacterial responses.

MATERIALS AND METHODS

Preparation of Human Monocyte-Derived Dendritic Cells and MR1-Overexpressing Cells

Blood samples free of detectable infectious or non-infectious diseases were obtained from healthy donors with written informed consent at the Hoxworth Blood Center in the University of Cincinnati. De-identified blood samples were processed according to the protocols approved by the Institutional Review Board of the University of Cincinnati. We isolated human peripheral blood mononuclear cells (PBMCs) and prepared human monocyte-derived dendritic cells (MoDCs) as performed (27, 28). Briefly, upon the isolation of PBMCs using Ficoll-paque gradient (GE Healthcare), we enriched monocytes by plastic adherence and differentiated monocytes using human recombinant granulocyte-macrophage colony-stimulating factor (300 U/ml) and interleukin 4 (200 U/ml) for 5 days (27, 28). The matured MoDCs were further activated with lipopolysaccharide (LPS) at 50 ng/ml for 24 h or incubated with bacteria. We also overexpressed the human MR1 protein by transducing the wild type human MR1 gene with the retroviral vector pMXIP (12) into human HLA-defective myeloid cell line K562 (K562.hMR1) (29) as we reported (11). The expression of the recombinant human MR1 protein was confirmed with flow cytometry, and its function was tested by MAIT cell line activation in bacterial incubation.

Abbreviations: MAIT, mucosal-associated invariant T; MHC, major histocompatibility complex; MR1, MHC-related protein 1; CD, cluster of differentiation; NK, natural killer; NKT, natural killer T cells; TNFα, tumor necrosis factor α; IFNγ, interferon γ; TCR, T cell receptor; HLA, human leukocyte antigens; PBMCs, peripheral blood mononuclear cells; MoDCs, monocyte-derived dendritic cells; LPS, lipopolysaccharide; BCG, Bacille Calmette-Guerin; *M. bovis, Mycobacterium bovis; M. tuberculosis, Mycobacterium tuberculosis; E. coli, Escherichia coli; L. monocytogenes, Listeria monocytogenes;* ELISPOT, enzyme-linked immunospot; Vα7.2, variable segment 7.2 of the T cell receptor alpha chain; APC/Cy7, Allophycocyanin/Cyanine7; PE, phycoerythrin; DEGs, differentially expressed genes; FDR, false discovery rate; Tbet, T-box transcription factor; Eomes, omesodermin.

Bacterial Incubation of Antigen-Presenting Cells

MoDCs and K562.hMR1 cells were incubated with *Listeria monocytogenes (L. monocytogenes* strain J0161, Bei resources*)*, *Escherichia coli (E. coli* strain BL21, New England BioLabs), *Mycobacterium bovis-* (*M. bovis-*) derived Bacille Calmette-Guerin (BCG) vaccine (Pasteur strain) (30), and avirulent *Mycobacterium tuberculosis* (*M. tuberculosis*, strain H37Ra, Colorado State University, Fort Collins, CO) (31). Both mycobacterial strains BCG and H37Ra were cultured for 5–6 days in middlebrook 7H9 complete medium at 37°C using an orbital shaker with a speed setting at 270 rpm. *E.coli* and *L. monocytogenes* were cultured overnight at 37°C in the Luria-Bertani broth using an orbital shaker at 100 rpm. Bacteria were harvested at a log-growing phase, washed with phosphate buffer saline (PBS), and measured for their absorbance (optical density at wavelength 600 nanometres, OD_{600}) according to the report (32). OD_{600} provides a semi-quantitative method to estimate bacterial cell numbers sufficient for MAIT cell activation (32). Human MoDCs or K562.hMR1 cells were incubated with *E.coli* in an estimated cell to bacteria ratio of 1:5 and 1:40 and with BCG in a ratio of 1:0 and 1:100. The blockage of activation was performed with an anti-MR1 antibody (clone 26.5, mouse IgG2a, at 2 μg/ml) that blocks MR1-dependent MAIT cell activation (10–12). Anti-HLAI antibody (clone W6/32, mouse IgG2a, Biolegend, at 2 μg/ml) was used as an isotype control for the anti-MR1 antibody and was also used to block the irrelevant effect of MHC class I proteins with similar structures as MR1 (33). Moreover, the chemical inhibitor cyclosporine A (CsA), mainly blocking TCR-mediated calcium signaling pathway for T cell activation (34, 35), was applied at 0.5 μg/ml.

Enzyme-Linked Immunospot

Upon incubation with bacteria overnight, MoDCs and K562.hMR1 cells were washed and incubated with the MAIT cell line (D466F5) (7) in a ratio of 5:1 and 1:4, respectively, by considering the estimated sizes of these cell types for optimal cell contact. The enzyme-linked immunospot (ELISPOT) assay was performed, as we reported (27). Briefly, both bacterial-incubated antigen-presenting cells and MAIT cells were co-cultured for 5 or 15 h on the multiscreen filter plate (Millipore) coated with anti-human IFNγ antibody (Mabtech). $IFN\gamma^+$ MAIT cell spots were then developed with an indirect immunostain approach using a biotinylated anti-human IFNγ antibody (Mabtech), ExtraAvidin conjugated by alkaline phosphatase (Sigma), and substrates BCIP/NBT (Sigma). We used CTL-ImmunoSpot S6 Micro Analyzer to visualize and quantify $IFN\gamma^+$ MAIT cell spots. Directional differences between bacterial-incubated and non-incubated conditions and between without and with anti-MR1 blockage were statistically analyzed using a paired *t*-test.

Isolation and Activation of Primary Human MAIT Cells

Upon removing adhered monocytes, human PBMCs were incubated with anti-Vα7.2 antibody (3C10) conjugated with PE (Biolegend) and followed by a positive selection with anti-PE antibody-conjugated magnetic beads (MACS, Miltenyi Biotec), according to the manufacturer's instructions. Bacterial-incubated K562.hMR1 cells were washed and further incubated with anti-Vα7.2-enriched PBMCs with abundant primary human MAIT cells in a ratio of 1:4 for 15 h with the presence of an anti-CD28 (clone CD28.2) antibody at 2 μg/ml. In addition to the stimulation with bacterial-incubated MR1-expressing K562 cells, anti-Vα7.2-enriched (MAIT cell-enriched) and anti-Vα7.2-depleted (conventional T cell-enriched) PBMCs were also activated with pre-coated anti-CD3 (clone UCHT1) at 1 μg/ml and anti-CD28 (clone CD28.2) at 2 μg/ml as positive controls. Hence, anti-CD28 (clone CD28.2) at 2 μg/ml was used for all K562.hMR1- and anti-CD3-mediated stimulation of T cells. For intracellular cytokine staining, Brefeldin A (10 μg/ml), which inhibits protein transport from the endoplasmic reticulum to the Golgi complexes, was added 2 h prior to cell harvesting for intracellular staining.

Antibodies and Flow Cytometry for Human MAIT Cells

Upon the co-culture for 15 h, surface markers, intracellular cytokines, and transcription factors of MAIT cells were stained following the manufacturer's instructions (Biolegend unless noted). In brief, cells were washed twice with staining buffer (PBS with 2% FBS) then blocked with anti-human Fc receptor antibodies, including anti-CD64 (mouse IgG1, clone 10.1), CD32 (mouse IgG2b, clone FUN-2), CD16 (mouse IgG1, clone 3G8), and additional Fc receptor blocking solution human TruStain FcX. For surface staining, cells were incubated with the combination of monoclonal antibodies, including phycoerythrin (PE)-Vα7.2, biotin-CD4 (OKT4) and streptavidin-conjugated quantum dot 525 or brilliant violet 510-CD4, brilliant violet 711-CD8α (RPA-T8), Allophycocyanin/Cyanine7 (APC/Cy7)-CD161 (HP-3G10), brilliant violet 605 or 421-CD69 (FN50), and PE/Cy5-CD26 (BA5b), for 30 min at 4°C in dark. PE-CD3 (OKT3) was used to replace PE-Vα7.2 for Vα7.2-depleted T cells. The MR1 tetramer loaded with the *E.coli* metabolite 5-amino-6-D-ribitylaminouracil (5-A-RU) (16, 36) and labeled with brilliant violet 421 was obtained from the NIH tetramer facility. For the staining of intracellular cytokines and transcription factors, cells were first incubated with antibodies against surface markers. Then, cells were fixed and permeabilized using the Fix/Perm Kit (Biolegend) and further stained in the 1 x Perm buffer for 30 min on ice with anti-cytokine and anti-transcription factor antibodies, including PE/Cy7-TNF-α (MAb11), APC-IFNγ (4S.B3), Alexa fluor 647-granulysin (DH2), PE/Cy7-Tbet (4B10), and Alexa fluor 488-Eomes (644730, R&D systems). Flow cytometry used BD Fortessa and Millipore Guava EasyCyte 12 channel high throughput flow cytometer according to the manufacturer's instructions. Flow cytometry data were further compensated and analyzed using Millipore Guava incyte and FlowJo software programs. Directional differences between mycobacterial stimulation and Listeria control was statistically tested using a paired *t*-test.

RNA-Seq of Human MAIT Cells

Anti-Vα7.2-enriched cells were co-cultured with BCG-incubated K562.hMR1 cells or stimulated with anti-CD3 antibody plus an anti-CD28 antibody for inducing co-stimulation in all conditions. Upon co-culture for 15 h, cells were first gated on $V\alpha7.2^{+}CD161^{+}CD4^{-}CD8^{+}$ to enrich this major MAIT cell subset and further sorted based on $CD69^{+}CD26^{++}$ and $CD69^{+/-}CD26^{+/-}$ to represent stimulated MAIT cells and non-stimulated MAIT-enriched cells. Similar to our report (27, 28), around one thousand cells were collected and lysed in the Lysis Buffer for total RNA extraction using mirVana kit (ThermoFisher, Grand Island, NY). RNA integrity was measured by a Bioanalyzer using Agilent RNA 6000 Pico Kit (Agilent, Santa Clara, CA) and showed a high quality of samples. Next, NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs, Ipswich, MA) was used for polyA RNA purification. The library for RNA-seq was prepared by using NEBNext Ultra Directional RNA Library Prep kit (New England BioLabs, Ipswich, MA). During the second cDNA synthesis, dUTP was incorporated to maintain strand specificity. The library was enriched and indexed via 15 cycles of PCR. The amplified libraries, together with the negative control, were cleaned up for Bioanalyzer QC analysis. To study differential gene expression, individually indexed and compatible libraries at 15 pM total were proportionally pooled for clustering on single-read flow cells in cBot system (Illumina, San Diego, CA). The clustered libraries were sequenced to generate 51 bp reads at ∼25 million per sample. RNA-seq data can be accessed at the GEO database (accession number GSE124381).

Bioinformatic Analysis of Differentially Expressed Genes (DEGs)

Similar to our previous report (27), transcriptomic data were analyzed to identify DEGs between $CD69^{+}CD26^{++}$ $CD8^{+}$MAIT cells and $CD69^{+/-}CD26^{+/-}$ $CD8^{+}$MAIT-enriched cells representing activated MAIT cells and inactivated MAIT-enriched cells. The list of DEGs of MAIT cells were analyzed for the gene co-expression, enrichment, and activation pathways in comparison to innate immune cells and conventional T cells. Sequence reads were first aligned to the genome and converted to intensity counts. Resulted intensity counts were compared between $CD69^{+}CD26^{++}$ and $CD69^{+/-}CD26^{+/-}$ cells from three donors using the edgeR program on the Bioconductor R platform to identify DEGs based on the absolute fold change (>2 folds) and p-values (<0.05). DEGs were shown with volcano plots, and representative altered genes were shown with a heatmap generated with edgeR and ggplot2 programs on the R platform. To predict the co-expression and shared functional clusters of DEGs with innate immune cells and conventional T cells, we used a ToppCluster software package to search DEGs against several databases, including KEGG and REACTOME, and obtained multiple clusters of genes overlapped with other activated immune cells at a statistically significant level. ToppCluster uses a hypergeometric test to obtain functional enrichment (https://toppcluster.cchmc.org/) (37). Enrichment analyses were performed using the Gene Set Enrichment Analysis (GESA) software by searching multiple available expression databases according to the instruction (38). The shared gene clusters between MAIT cells and other immune cells were further input to software Cytoscape Version 3.3.0 (www.cytoscape.org/), a broadly used open-source software platform for visualizing complex networks. We also applied Cytoscape to search various databases, including PANTHER, MSigDB, KEGG, NCI Pathway, and Reactome databases, to identify comprehensive pathways for the activation of conventional T cells and NK cells. The DEGs were annotated using the representative pathways of T cell and NK cell activation.

Mycobacterial Growth Inhibition in Antigen-Presenting Cells

The mCherry-labeled BCG was obtained from Dr. Russell's lab (39) and used to incubate MoDCs and K562.hMR1 cells at a ratio of 50:1 for 2 h. BCG-incubated cells were washed for 4 times and further co-cultured with rested or anti-CD3/CD28-activated primary MAIT cells upon magnetic enrichment with anti-Vα7.2 antibody. After overnight co-culture, we measure the remained mCherry-labeled BCG with antigen-presenting cells (% $mCherry^{+}$ cells).

RESULTS

Rapid Activation of a Human MAIT Cell Line by Dendritic Cells and MR1-Expressing Cells

Different from published monocytes (8, 24) and a lung epithelial cell line (7), antigen-presenting cells in this study used a human MR1-overexpressed human hematopoietic cell line K562 (K562.hMR1) with defective MHC expression (12, 40) and monocyte-derived dendritic cells (MoDCs) with strong co-stimulation. K562.hMR1 cells allow us to specifically detect bacterial-activated human MAIT cells and delineate MR1-dependent MAIT responses to bacterial infection, as different from bacterial-irrelevant cell activation by anti-CD3 antibody, cytokines, or autologous monocytes (22–24). MoDCs were differentiated using granulocyte-macrophage colony-stimulating factor and interleukin 4 as we reported (27, 28). Prior to the co-culture with T cells, MoDCs were overnight incubated with live *E.coli* or *M. bovis* (BCG) at indicated ratios of cell:bacteria. The bacterial-incubated MoDCs were then washed and co-cultured with a human MAIT cell line (D466F5), which was derived from MAIT cells of an active tuberculosis patient (7). As measured in an Enzyme-linked Immunospot (ELISPOT) assay, the number of $IFN\gamma^{+}$MAIT cell spots significantly enhanced upon the overnight stimulation (15 h of co-culture) of BCG- and *E.coli*-incubated MoDCs, to an extent much higher than the non-infected condition or the background response of MoDCs (**Figures 1A,B**). An anti-MR1 antibody (11) significantly blocked MR1-mediated MAIT cell activation, to an extent comparable to the blockage of cyclosporine that inhibits calcium-mediated signaling cascades in T cell receptor-mediated pathways (35). Statistical analyses support MAIT cell activation

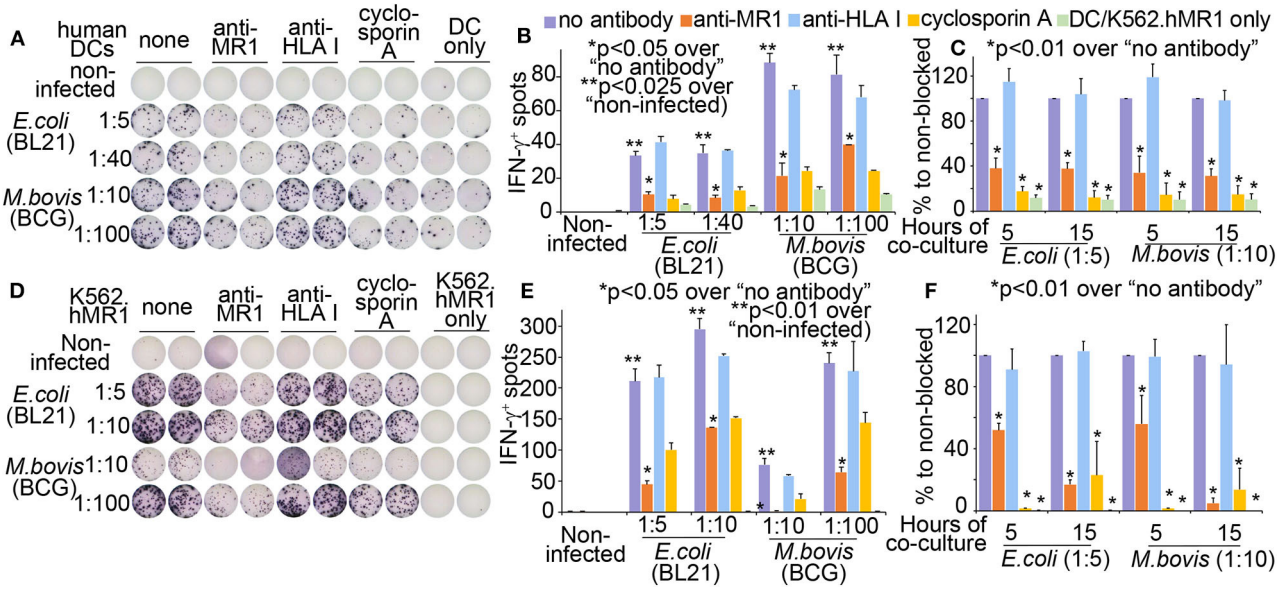

FIGURE 1 | Monocyte-derived dendritic cells (MoDCs) and human MR1-overexpressed K562 cells (K562.hMR1) to activate a human MAIT cell line. Differentiated MoDCs were incubated with *E.coli* and *M. bovis* strains overnight. Bacterial cell number was estimated by OD600 absorbance and ratios of bacteria to DCs were shown. Bacterial-incubated MoDCs were washed and further co-cultured with the MAIT cell line (D466F5) (7) for 15 h. Anti-MR1 (clone 26.5, 2 μg/ml), Anti-HLAI antibody (clone W6/32 at 2 μg/ml), cyclosporine A (0.5 μg/ml), and MoDC only were used as controls. IFNγ$^+$ MAIT cells measured by an ELISPOT assay were shown from one independent assay with MoDCs from one donor **(A)**. Means from technical duplicates in panel A are shown with standard errors and statistically analyzed using a paired *t*-test **(B)**. Three independent assays of DC-MAIT cell co-culture were performed with antibody blockage for 5 or 15 h. Data were normalized to non-blocked condition, shown with standard errors, and statistically analyzed using a paired *t*-test **(C)**. At the same setting to MoDCs, bacterial-incubated K562.hMR1 cells were co-cultured with the MAIT cell line (D466F5) to stimulate IFNγ$^+$ spots **(D)**. IFNγ$^+$ MAIT spots in technical duplicates were similarly analyzed **(E)**. Three independent assays were similarly performed and analyzed **(F)**.

upon overnight co-culture and its dependence on MR1-mediated antigen presentation by MoDCs (**Figure 1B**). Results from three independent assays with a short (5 h) and overnight (15 h) co-culture and anti-MR1 antibody blockage confirmed the activation of MAIT cells and MR1 dependence (**Figure 1C**). These data demonstrated that MAIT cells were rapidly activated by bacterial-incubated MoDCs within hours in a manner partially dependent on MR1-mediated antigen presentation. However, T cell activation using autologous MoDCs or monocytes is potentially confounded by autoreactivity, as shown with a high CD69 expression of MAIT cells irrelevant to bacterial infection (24). Using bacterial-incubated K562.hMR1 cells, we showed that the human MAIT cell line was also activated in a manner largely blocked by an anti-MR1 antibody and the chemical inhibitor cyclosporine (**Figures 1D,E**). Three independent assays similarly demonstrated that bacteria-incubated K562.hMR1 rapidly activated MAIT cells with 5 h of co-culture (**Figure 1F**). Although the MAIT cell line D466F5 was derived from an active tuberculosis patient, its TCR (*TRAV1-2/TRBV6*) was not evidenced being biased to *M. tuberculosis* antigens in this assay (**Figure 1**) or in other reports (7, 41). Together, these results established a rapid assay of MR1-dependent human MAIT cell activation using K562.hMR1 cells, allowing us to identify surface markers and activation pathways of primary human MAIT cells in the following assays.

CD69$^+$CD26^{++} Labels Activated Human CD8$^+$MAIT Cells

To date, it remains unclear which surface markers specifically detect and separate activated MAIT cells. The discovery of these markers is crucial for the translational detection of MAIT cells and allows further functional or mechanistic characterization of MAIT cells in bacterial infections and various diseases. Previous studies show that the early T cell activation marker CD69 expresses on stimulated MAIT cells upon bacterial infection; however, CD69 also upregulates in uninfected conditions (8, 12, 24). Moreover, dipeptidyl peptidase (DPP4) named CD26 upregulates on MAIT cells in mycobacterial infection (42). However, CD26 also expresses on MAIT cells in the absence of stimulation (42) and on NK cells (43). Therefore, CD69 or CD26 alone appears unable to separate stimulated MAIT cells from the non-stimulated cells completely. To quantify and define activated MAIT cells, we showed that the co-expression of CD69 and CD26 as a combinatorial marker CD69$^+$CD26^{++} separated the activated CD8$^+$MAIT cells from the inactivated CD69$^{+/-}$CD26$^{+/-}$ CD8$^+$MAIT-enriched cells (**Figure 2**). The latter population was defined based on the remaining subset in the conditions without bacterium or with the negative control *Listeria* (**Figure 2A**). In this assay, we co-cultured Vα7.2-enriched human PBMCs with bacterial-incubated K562.hMR1 cells and an anti-CD28 antibody. Upon overnight co-culture, cells were gated at Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$ or

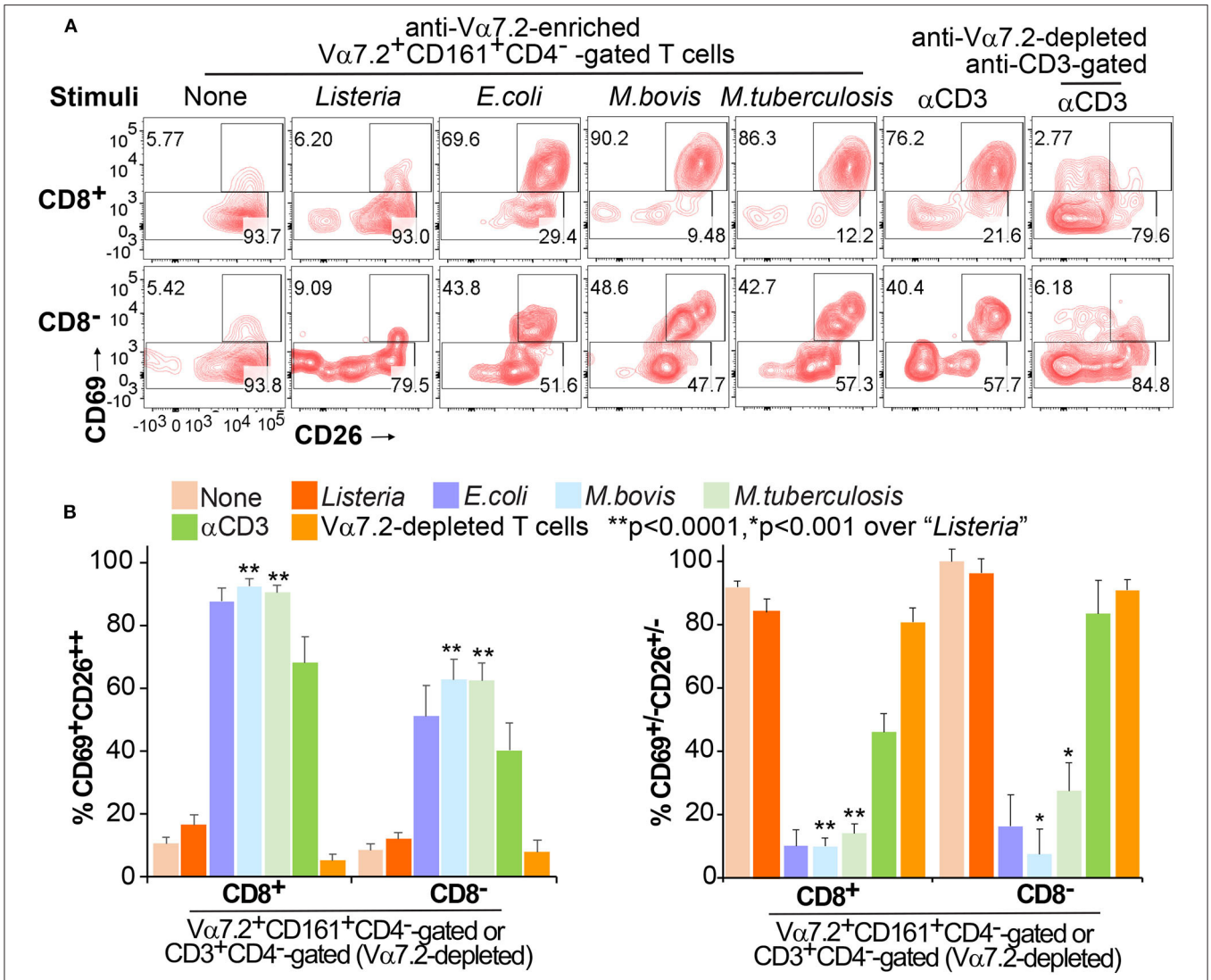

FIGURE 2 | Differentiation of Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$CD69$^+$CD26^{++} MAIT cells. Anti-Vα7.2-enriched PBMCs were co-cultured with bacterial-incubated K562.hMR1 cells and anti-Vα7.2-depleted PBMCs were stimulated with anti-CD3 for 15 h. All groups contained an anti-CD28 antibody for co-stimulation. Flow cytometry gated Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$ (or CD8$^-$) in anti-Vα7.2-enriched PBMCs and CD3$^+$CD4$^-$CD8$^+$ (or CD8$^-$) in anti-Vα7.2-depleted PBMCs. % CD69$^+$CD26^{++} MAIT cells (upper) and CD69$^{+/-}$CD26$^{+/-}$ cells (bottom) are annotated. Contour plots show data from one independent assay using PBMCs from one healthy donor **(A)**. Data from four independent assays were plotted with standard errors and analyzed with paired *t*-tests **(B)**.

Vα7.2$^+$CD161$^+$CD4$^-$CD8$^-$ to separate CD69$^+$CD26^{++} cells from CD69$^{+/-}$CD26$^{+/-}$ cells (**Figure S1**), as Vα7.2$^+$CD161$^+$ gating has been used in multiple studies to detect MAIT cells (8, 42, 44–46), especially bacterial-activated MAIT cells (7, 8, 23). Notably, the percentage of CD69$^+$CD26^{++} MAIT cells was dramatically upregulated through co-culture with MR1-expressing cells, which were pre-incubated with *E.coli, M. bovis (BCG)*, and avirulent *M. tuberculosis* (H37Ra) (**Figure 2**). Moreover, CD69$^+$CD26^{++} MAIT cells were clearly separated from CD69$^{+/-}$CD26$^{+/-}$ CD8$^+$MAIT-enriched cells. The latter subset remained in non-optimal stimulatory conditions or non-stimulated conditions, such as conditions without bacteria or with non-antigenic *Listeria monocytogenes* (13, 16). Direct stimulation of Vα7.2-enriched cells with anti-CD3/CD28 antibodies as a positive control also upregulated CD69$^+$CD26^{++} CD8$^+$MAIT cells (**Figure 2**). Similarly, in the Vα7.2$^+$CD161$^+$CD4$^-$CD8$^-$ population, CD69$^+$CD26^{++} cells were also upregulated upon various bacterial-stimulations (**Figure 2**). Moreover, these CD69$^+$CD26^{++} MAIT cells nearly disappeared upon anti-MR1 blockage in contrast to the isotype control using an anti-HLA class I antibody (**Figure S2**), since stimulation using K562.hMR1 cells likely skewed to the induction of MR1-dependent MAIT cell activation. Thus, mycobacterial stimulation consistently upregulates the CD69$^+$CD26^{++} CD8$^+$MAIT cells dependent on the MR1 molecule, while reversely reduce the CD69$^{+/-}$CD26$^{+/-}$ CD8$^+$MAIT-enriched

cells (**Figure 2B**), strongly supporting that $CD69^{+}CD26^{++}$ is a specific and sensitive marker to label activated MAITs. These results support that the MR1-dependent MAIT cell activation labeled with a combinatorial marker $CD69^{+}CD26^{++}$ can be broadly applied as a promising translational measurement to detect the activated MAIT cells in human.

Recently, the MR1 tetramer loaded with the *E.coli* metabolite 5-amino-6-D-ribitylaminouracil (5-A-RU) (16, 36) was used to stain non-stimulated or metabolite antigen-stimulated MAIT cells from healthy donors or patients (45, 47–50). However, whether the MR1-5-A-RU tetramer also serves as a reliable tool to stain activated MAIT cells in bacterial infection is unknown. To explore this possibility, we co-cultured fresh peripheral blood mononuclear cells (PBMCs) without anti-Vα7.2 and magnetic enrichment with bacterial-incubated K562.hMR1 cells for 15 h. Results showed that the MR1-5-A-RU tetramer was able to stain non-stimulated MAIT cells in PBMCs without bacterial incubation or with *Listeria* incubation. We also showed $V\alpha7.2^{+}$MR1-5-A-RU^{+} PBMCs were mostly $V\alpha7.2^{+}CD161^{++}$ (**Figure S3A**), as consistent with previous reports (24, 45, 47, 48). Minimal background staining of the MR1-5-A-RU tetramer in $V\alpha7.2^{-}$ PBMCs was also similar to that in reports (24, 48). However, MR1-5-A-RU tetramer staining was surprisingly weak or negative for bacterial-stimulated MAIT cells (**Figure S3A**). Expended gating showed that $CD69^{+}CD26^{++}$ cells re-emerged from $V\alpha7.2^{+/-}$MR1-5-A-$RU^{+/-}$ population (**Figure S3B**), indicating that mycobacterial-activated MAIT cells downregulated Vα7.2 expression and MR1-5-A-RU staining signals consistent to the report (51). Regardless, activated MAIT cells in the same PBMC samples were still feasibly recognized by Vα7.2 and CD161 antibodies, as eventually labeled by the combinatorial marker of $CD69^{+}CD26^{++}$ (**Figure S3C**) at a similarly high percentage to the anti-Vα7.2-enriched and rested MAIT cells (**Figure 2**). Therefore, the combinatorial marker $CD69^{+}CD26^{++}$ is able to detect activated MAIT cells dependent on MR1-mediated antigen-presentation from $V\alpha7.2^{+}CD161^{+}$ population in bacterial infections. Importantly, $CD69^{+}CD26^{++}$ can define human MAIT cells based on MR1 dependence, similar to the definition of CD1-restricted T cells (52, 53) and previous definition of MAIT cells by MR1-dependent activation (7, 8, 10–12).

$CD69^{+}CD26^{++}$ Human $CD8^{+}$MAIT Cells Display a Transcriptome With Multiple Upregulated Activation Markers

Our finding of the activation marker $CD69^{+}CD26^{++}$ allows us to separate activated MAIT cells ($CD69^{+}CD26^{++}$) unprecedentedly from inactivated MAIT-enriched cells ($CD69^{+/-}CD26^{+/-}$) in mycobacterial infection or in disease conditions to elucidate the activation program of $V\alpha7.2^{+}CD161^{+}CD4^{-}CD8^{+}$ MAIT cells. Recent publications used a MAIT cell antigen (21), anti-CD3 antibody (22), *E.coli* infection (23), and MR1-independent stimulants (22, 23) to determine the transcriptomes of activated MAIT cells. Different from these activation conditions, we would determine the transcriptomic program of abundant human $CD8^{+}$ MAIT cells in response to the initial priming of mycobacterial infection in an MR1-dependent manner. MR1-dependent activation also allow us to specifically define MAIT cells, as the similar definition used for CD1-restricted T cells (52, 53) and in early studies of MAIT cells (7, 8, 10–12). Anti-Vα7.2-enriched human PBMCs ($n = 3$ donors) were co-cultured with BCG-incubated K562.hMR1 cells or stimulated with anti-CD3 antibody for 15 h similarly using anti-CD28 for co-stimulation in all conditions. BCG is the only licensed vaccine against tuberculosis in children and partially protects adults (54). BCG vaccination and *M. tuberculosis* infection enhance MAIT cell frequency in primates, similar to BCG vaccination in humans (55, 56). BCG stimulation also allows us to sort activated primary human MAIT cells through the core facility at an allowed biosafety level for transcriptomic analyses, which inform the MAIT cell activation program in mycobacterial infection. As in **Figure S1**, we sorted $CD69^{+}CD26^{++}$ and $CD69^{+/-}CD26^{+/-}$ cells for transcriptomic analyses. We further isolated polyA RNA from sorted cells and performed quality control (27, 28). Then polyA RNA was reversed transcribed, barcoded, and sequenced using the Illumina Hiseq approach as we reported (27, 28). After the determination of gene identities and quantification of intensity counts, we deposited the transcriptomic data to the GEO database (accession number GSE124381) and normalized transcriptomes using the edgeR program. As a result, $CD8^{+}$ MAIT cell transcriptomes detected a total of 14,077 transcripts with minimally 1 count per million (cpm) from at least one out of three donors. Differentially expressed genes (DEGs) were defined with more than 2-fold difference of intensity counts between $CD69^{+}CD26^{++}$ activated $CD8^{+}$ MAIT cells and $CD69^{+/-}CD26^{+/-}$ inactivated $CD8^{+}$ MAIT-enriched cells. We used a p-value (<0.05) to test different gene expression between activated and inactivated $V\alpha7.2^{+}CD161^{+}CD4^{-}CD8^{+}$ cells from three donors by considering the variability in different donors, *in vitro* bacterial stimulation, T cell activation, cell sorting, and RNA preparation in different assays. To validate the results, we used a heatmap to list multiple clusters of representative genes relevant to the activation of MAIT cells, conventional T cells, and NK cells, as referred below, validating our transcriptomic datasets of the dominantly activated $CD69^{+}CD26^{++}$ $CD8^{+}$MAIT cells (**Figure 3A**). For example, these signature genes encode surface markers *CD69, CD8A, DPP4 (CD26), CD3Z*, and *KLRB1* (CD161); cytokine and receptors *TNF* (TNFα), *IFNGR1, IL18R1* (IL-18Rα) and *IL21R;* cytolytic effector molecules *GNLY* (granulysin), *PRF1* (perforin) (44), and *FAS* (CD95) (57), signaling molecules *SLA, SLAMF1,* and *NFKB1;* transcription factors *TBX21* (Tbet), *RORC* (RORγt) (46), *IKZF2* (Helios), *RUNX2*, and *ZBTB16* (PLZF) (24, 58) (**Figure 3A**).

$CD69^{+}CD26^{++}$ Human $CD8^{+}$MAIT Cells Co-express Genes and Share Functional Pathways With Activated T Cells and Innate Immune Cells

Although MAIT cell transcriptomes had been shown different from other T cells or between MAIT cell subsets (21, 59), we would address whether mycobacterial infection

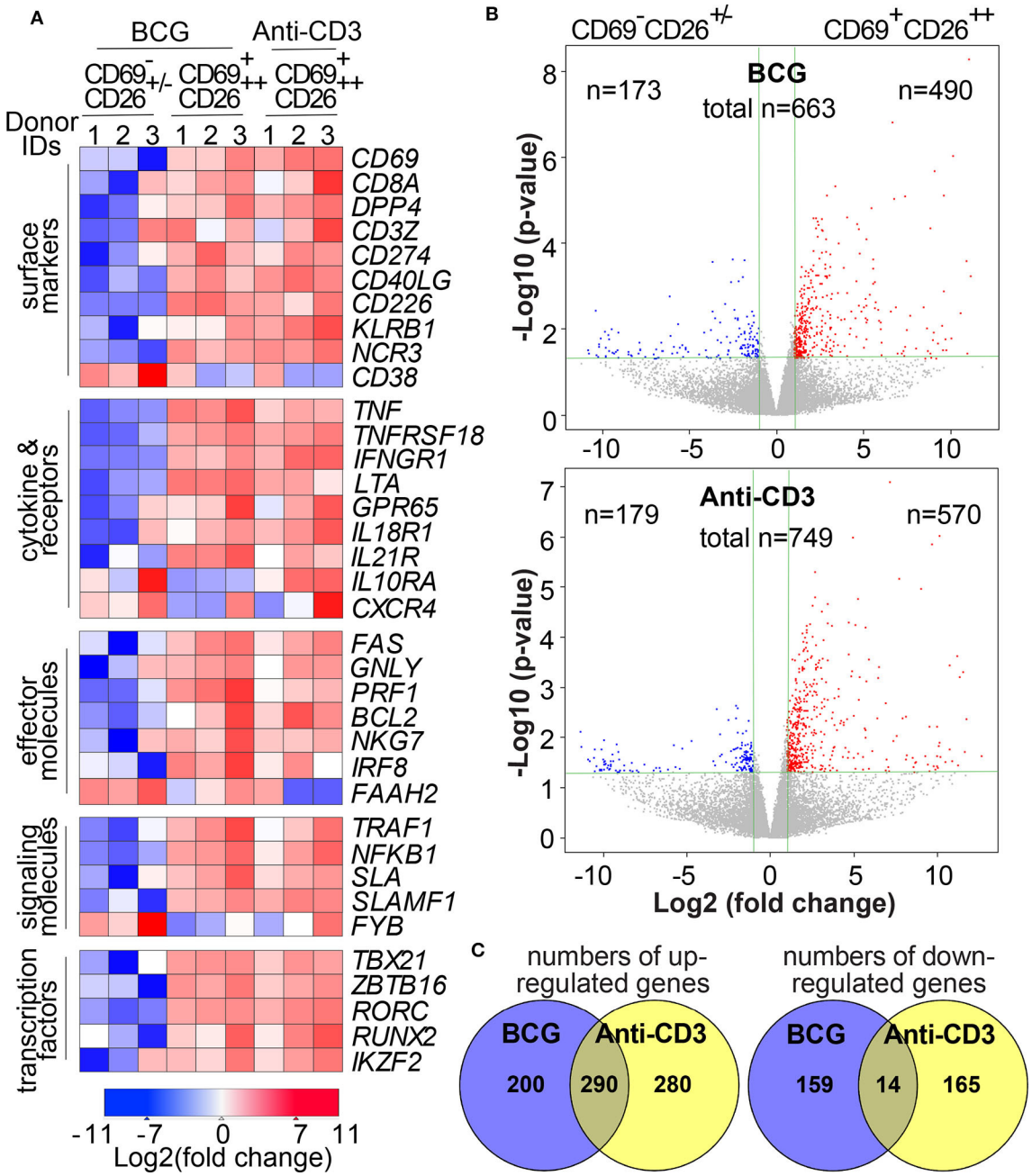

FIGURE 3 | Transcriptomes of Vα7.2^{+}CD161^{+}CD4^{-}CD8^{+}CD69^{+}CD26^{++} MAIT cells depict multiple activation markers. Upon overnight stimulation using BCG-incubated K562.hMR1 cells or anti-CD3 antibody with anti-CD28 co-stimulation in both conditions, anti-Vα7.2-enriched cells were gated on Vα7.2^{+}CD161^{+}CD4^{-}CD8^{+} cells and further sorted into two subsets, CD69^{+}CD26^{++} activated MAITs and CD69$^{+/-}$CD26$^{+/-}$ inactivated MAIT-enriched cells as in **Figure 2** and **Figure S1**. Representative gene expression relevant to MAIT cell activation was compared between BCG-activated MAIT cells and inactivated cells from three healthy donors using anti-CD3 as an activation control. Relative expression levels of these genes are shown in a heatmap with the color scheme representing Log2(fold change) for each gene **(A)**. Volcano plots show up- and down-regulated differentially expressed genes (DEGs) with fold changes (vertical green lines for 2 folds) and *p*-values (horizontal green lines for $p = 0.05$) **(B)**. Venn diagrams show overlapped numbers of DEGs between BCG and anti-CD3 stimulation conditions **(C)**.

stimulated an activated transcriptomic program in MAIT cells. Specifically, we determined whether genes differentially expressed between activated MAIT cells vs. inactivated cells. Further, we characterized whether these DEGs in MAIT cells were similar to other adaptive or innate immune cells to support an innate-like activation program associated with anti-mycobacterial functions. To perform these analyses, we started with volcano plots that revealed hundreds of DEGs between

activated $CD69^{+}CD26^{++}$ $CD8^{+}$MAIT cells and inactivated $CD69^{+/-}CD26^{+/-}$ $CD8^{+}$MAIT-enriched cells in BCG and anti-CD3 stimulations, respectively (**Figure 3B**). The completed lists of up- and down-regulated genes in BCG ($n = 663$ in **Table S1**) and anti-CD3 ($n = 749$ in **Table S2**) stimulations were further used for downstream Venn diagram, enrichment, and pathway analyses. Venn diagrams showed large numbers of different and shared genes in BCG and anti-CD3/CD28 stimulation (**Figure 3C**). In this comparison, 200 upregulated genes (41%) were unique in BCG stimulation and 280 genes (49%) in anti-CD3/CD28 activation. In parallel, 159 downregulated genes (92%) were unique in BCG stimulation and 165 genes (92%) in anti-CD3/CD28 activation. Results supported that BCG stimulation induces a transcriptomic program partially different from that induced by anti-CD3/CD28 antibodies (58), despite that the activated cells were sorted with the same surface markers. To further statistically determine the gene clusters co-expressed with other cell types, we searched the identified BCG-induced DEGs against the gene expression databases in MSigDB (http://software.broadinstitute.org/gsea/msigdb) using the Toppcluster program (37). We identified more than 3,000 of reported gene expression datasets, including conventional $CD8^{+}$ T cells, NKT, NK cells, macrophages, dendritic cells, and other cell types. These T and innate cells co-expressed a large number of genes with BCG-stimulated MAIT cells at a statistically significant level (with a false discovery rate FDR-corrected $p < 0.01$), as shown with multiple representative gene sets (**Figure 4A**). We also use enrichment analyses to show multiple significantly co-expressed datasets, including NK cells, cytotoxic T cells, NKT cells, and monocytes (**Figure 4B**). To further annotate functional pathways of activated MAIT cells overlapped with other immune cells, we searched BCG-induced DEGs against various cellular pathways using Toppcluster and constructed a pathway network using the Cytoscape program (http://www.cytoscape.org), based on the cutoff of an FDR-corrected p-value at 0.01 (**Figure 4C**). This network shows that MAIT cells share gene expression in numerous pathways with multiple cell types, including T and NK cell activation, NFκB signaling, MAPK signaling, cytokine productions, and effector functions, etc. Hence, our data reveal that MAIT cell activation shares gene clusters and functional pathways with conventional T cells and innate immune cells.

$CD69^{+}CD26^{++}$ Human $CD8^{+}$MAIT Cells Display a Gene Profile Co-expressed in the T Cell Activation Pathway

From the comprehensive gene profiles shared between MAIT and other immune cells (**Figure 4B**), it is critical to annotate these shared genes in T cell activation pathways. Therefore, we used Cytoscape to obtain a map of canonical T cell activation pathways (60) to annotate BCG-altered genes. The upregulated genes included *CD3Z* (CD247), *CD8A*, and *LCP2*, downstream transcription factors *NFKB1A, NFKB1*, and *REL*, which together supported a TCR-mediated NFκB pathway (60) (**Figure 5A**). Other genes include *PTPN6* (SHP1) important for TCR signaling (61), *TNF, BCL2*, *TRAF1, IL15Ra, TNFRSF9* (4-1BB), and *FAS* for regulating effector function. Moreover, MAIT cells stimulated with anti-CD3/CD28 antibodies induced additional signaling molecules, including *ITK*, *LCK*, and *VAV1* (**Figure 5A**). Anti-CD3/CD28 antibodies appear capable of stimulating both NFκB- and MAP3 Kinase-mediated signaling pathways (62, 63), likely promoting more comprehensive singling transduction in comparison to BCG stimulation. These data demonstrate that activated MAIT cells share common signature genes in canonical activation pathways with conventional T cells.

$CD69^{+}CD26^{++}$ Human $CD8^{+}$MAIT Cells Display a Gene Profile Co-expressed in the NK Cell Activation Pathway

It is known that MAIT cells express NK cell receptors CD161 and NCR3, as in our findings (**Figures 2**, **3**). To understand whether activated MAIT cells also share gene signatures in activated NK cells, we similarly obtained canonical NK cell activation pathways through database search and comprehensively annotated BCG-altered MAIT cell genes (**Figure 5B**). Co-expressed genes included those encoding cell surface molecules, an IFNγ receptor (*IFNGR1*) for cytokine stimulation and NK cell chemotaxis (64), and a natural cytotoxicity triggering receptor *NCR3 (NKp30)* for CD3ξ molecule (*CD247*) interaction and NK cell differentiation (65). Co-expressed genes also encoded multiple signaling molecules, such as *SH2D1A* (SAP) and *PTPN6* (SHP1), which are critical in both NK and T cells (61, 66). Ultimately, the upregulation of effector molecules, such as cytokine TNF (67, 68), cytolytic molecule perforin (PRF1) (57), and adhesion molecule ICAM1, enhance the protection against infection, killing of bacterial-infected cells, and cell migration to infected tissues. Therefore, activated $CD8^{+}$MAIT cells also share signature gene expression in the NK cell activation pathway (**Figure 5B**).

CD69 and CD26 Label Pro-inflammatory Responses of Human $CD8^{+}$MAIT Cells in *M. tuberculosis* Infection

Pro-inflammatory responses have been demonstrated as critical effector functions against mycobacterial infection (3, 69, 70). Our transcriptomic analyses suggest an enhanced pro-inflammatory response of MAIT cells upon BCG stimulation, including an enhanced expression of *TNF*, *TBX21* (encoding the transcription factor Tbet), and *IFNGR1*. TNFα bears a prominent early anti-tuberculosis function, which was shown in mouse studies (68) and also supported by severe tuberculosis in human autoimmune diseases with anti-TNFα therapies (67). IFNγ is another critical cytokine in controlling the infection of *M. tuberculosis* (71). To determine whether primary human MAIT cells are quickly activated to produce anti-mycobacterial pro-inflammatory cytokines, we similarly stimulated MAIT cells with bacterial-incubated K562.hMR1 cells (**Figure 2**) and validated activated MAIT cells by gating on $V\alpha7.2^{+}CD161^{+}CD4^{-}CD8^{+}CD69^{+}CD26^{++}$ cells (**Figure S1**). Results showed the upregulated frequency of $CD26^{++}TNF\alpha^{+}$ (**Figures 6A,B**) and $CD69^{+}TNF\alpha^{+}$ (**Figures 6C,D**) MAIT

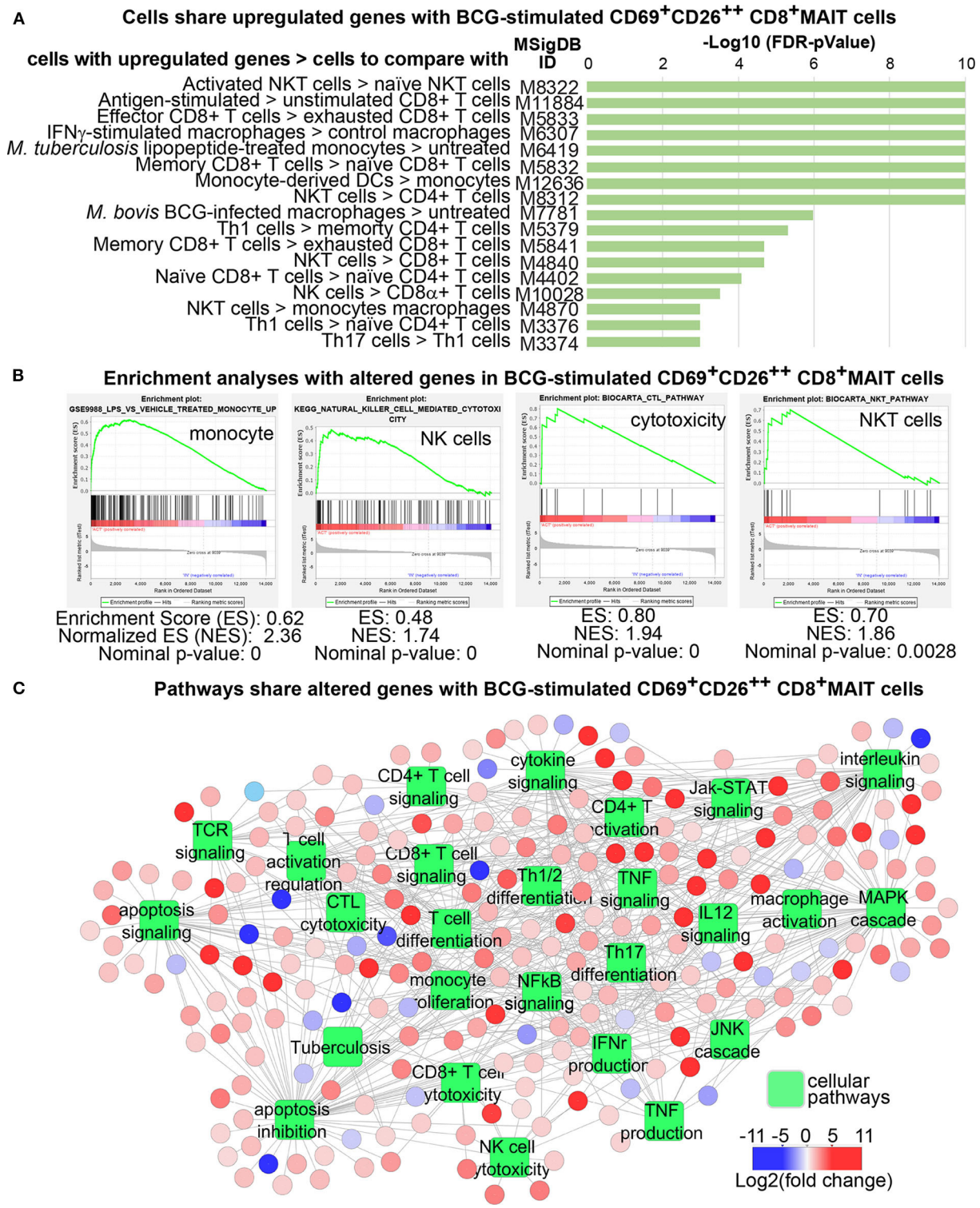

FIGURE 4 | Transcriptomes of Vα7.2^{+}CD161^{+}CD4^{-}CD8^{+}CD69^{+}CD26^{++} MAIT cells display gene clusters and functional pathways shared with T cells and innate immune cells. Upregulated genes from BCG-stimulated MAIT cells were used to search against the Molecular Signatures Database (MSigDB) and identified significant gene co-expression with more than 3,000 gene datasets ($p < 0.01$). Some representative datasets with various p-values are shown **(A)**. Enrichment analyses identified multiple example datasets **(B)**. DEGs from BCG stimulation of MAIT cells significantly overlap with genes in various cellular pathways of activated immune cells ($p < 0.01$) **(C)**.

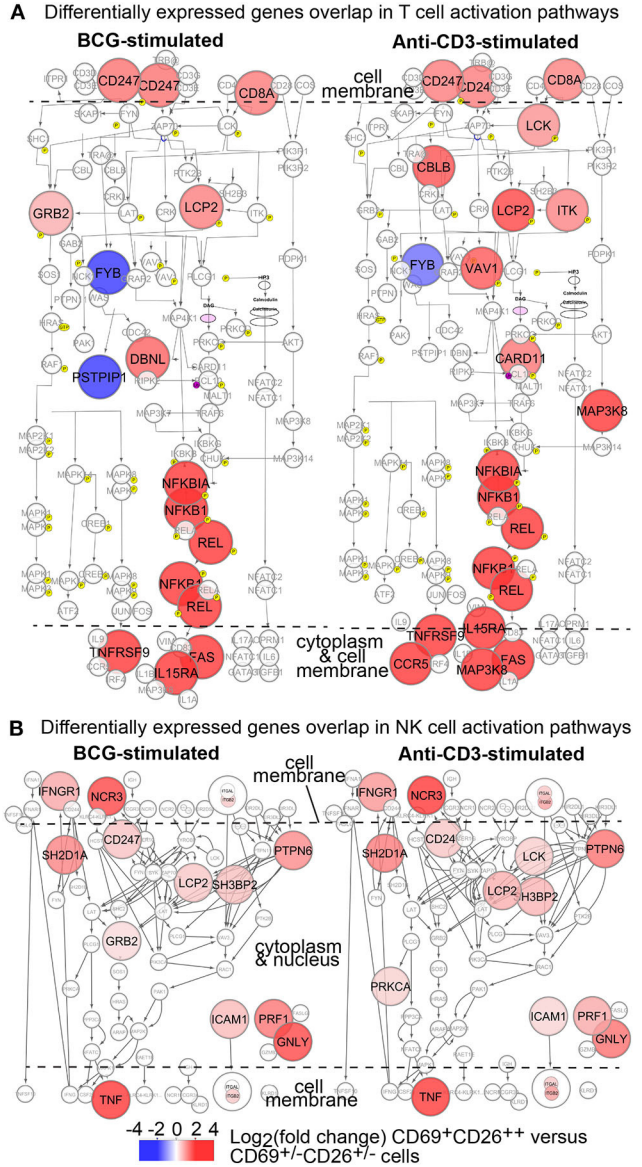

FIGURE 5 | Transcriptomes of Vα7.2^{+}CD161^{+}CD4^{-}CD8^{+}CD69^{+}CD26^{++} MAIT cells depict overlapped genes in conventional T cell and NK cell activation. DEGs from BCG or anti-CD3 stimulation of MAIT cells are annotated in canonical T cell **(A)** and NK cell **(B)** activation pathways as colored.

cells in Vα7.2^{+}CD161^{+}CD4^{-}CD8^{+} upon bacterial and anti-CD3/CD28 stimulation. Further gating on CD69^{+}CD26^{++} from bacterial-stimulated conditions annotated high TNFα expression subset in comparing to un-stimulated CD69$^{+/-}$CD26$^{+/-}$ subset in Listeria-incubated condition (**Figures 6E,F**). Consistently, we demonstrated similar results with IFNγ (**Figure 6**), which display a high degree of enhancement. The expression of transcription factor Tbet is also upregulated (**Figure 6**), similar to the enhanced *TBX21* expression in the transcriptome of BCG-stimulated MAIT cells. Together, anti-mycobacterial pro-inflammatory responses are enhanced in response to *E.coli* and mycobacterial infections.

Stimulated Human MAIT Cells Enhanced Cytolytic Functions and Inhibited Mycobacterial Growth

Human MAIT cells express cytolytic molecules at an un-stimulated condition or upon the activation of fixed *E.coli* (24, 44, 72). However, it is unclear whether mycobacterial infection also stimulates MAIT cells to express cytolytic molecules and display a protective function. Overnight incubation of MAIT cells with BCG- or H37Ra-stimulated K562.hMR1 cells enhanced the % CD26^{++} and CD69^{+} MAIT cells expressing granulysin and Eomesodermin (Eomes) (**Figures 7A–D**). The gated CD69^{+}CD26^{++} MAIT cells also upregulated the fluorescent staining intensity of granulysin and Eomes (**Figures 7E,F**). Results support that bacterial stimulation further upregulates cytolytic molecules over the basal level of expression. To determine whether both pro-inflammatory cytokines and cytolytic function together play a role to inhibit mycobacterial growth, we co-cultured the unstimulated or anti-CD3-stimulated Vα7.2-enriched cells with K562.hMR1 cells or human MoDCs pre-infected with mCherry-labeled BCG (39). Results demonstrated that either rested or pre-stimulated MAIT-enriched cells inhibited the growth of the mCherry-labeled BCG detected with flow cytometry (**Figure 7G**). Since pro-inflammatory cytokines and cytolytic functions of conventional T cells have been shown to fight mycobacterial infections (67, 68), our data support human MAIT cells similarly inhibit mycobacterial growth as in a previous report with mouse MAIT cells (18).

DISCUSSION

The hypothesis of "innate-like T cells" was perhaps raised from the early observation of MAIT cell activation that depends on the interaction of a monomorphic MR1 protein, an antigen, and a semi-invariant TCR (9–14, 73–75). This conserved genetics and physical interaction in the MR1-antigen-TCR complex had likely provided a primordial mechanism that preserved innate features in MAIT cell activation and preceded the peptide antigen presentation (9–14), as similarly exemplified in CD1-restricted T cell activation (53, 75–77). In this study, we defined a combinatorial marker to label rapidly activated MAIT cells for the characterization of transcriptomic programs and effector functions against mycobacterial infections. Results reveal gene expression profiles of mycobacterial-activated MAIT cells share with activated CD8^{+} T cells and NK cells (**Figures 3–5**). Effector responses, including the enhanced production of pro-inflammatory cytokines (TNFα and IFNγ) and the cytolytic molecule (granulysin) (**Figures 3**, **4**, **6**, **7**), and the inhibition of mycobacterial growth (**Figure 7**), are also similar to activated CD8^{+} T cells and NK cells (78). This unique innate-like activation program of MAIT cells is expected to provide a niche of early protection against *M. tuberculosis* infection, by potentially bridging innate (79) and adaptive immune responses (4, 5).

An activation marker is crucial for labeling and defining MAIT cells to further estimate MAIT effector functions in

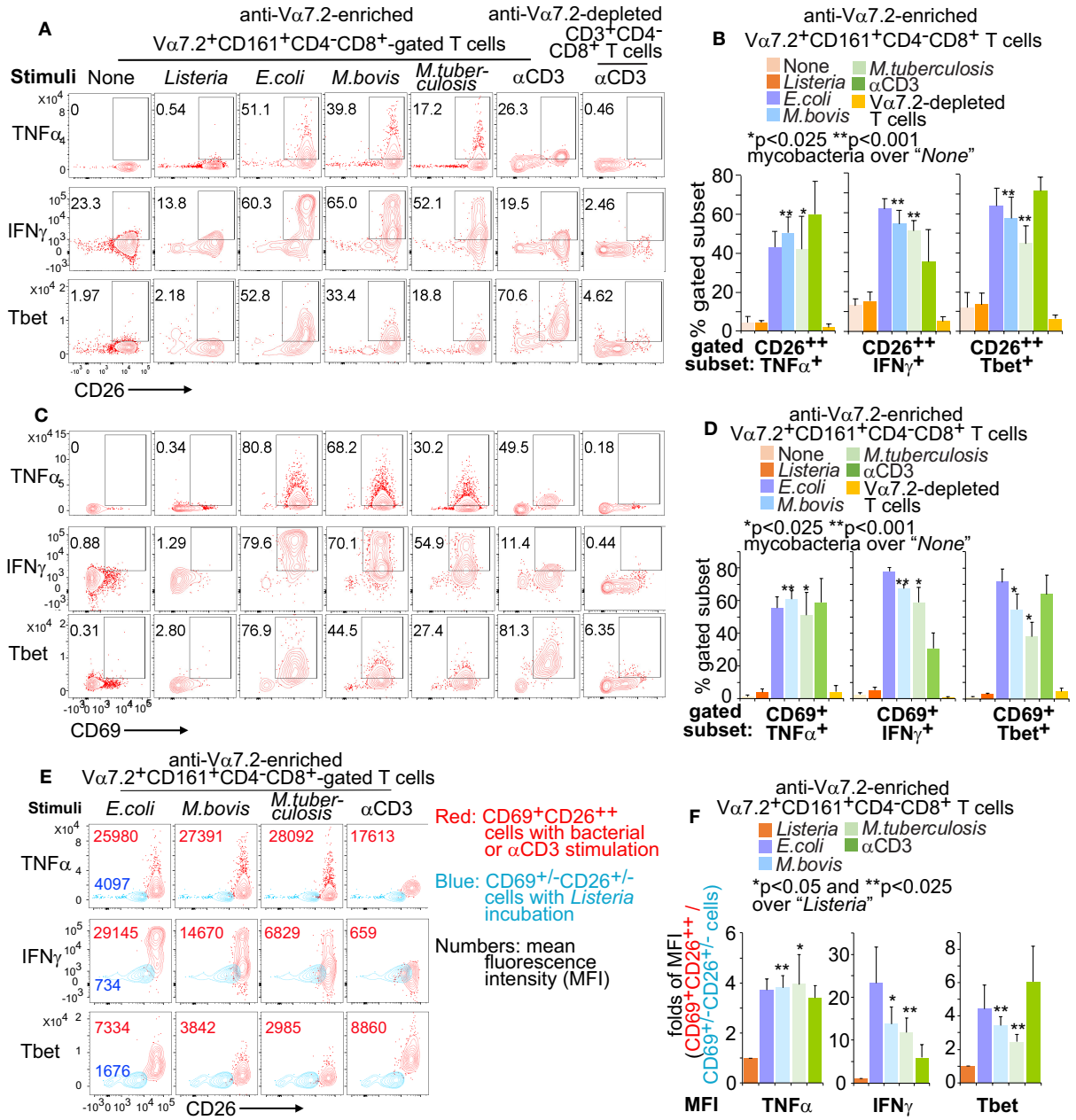

FIGURE 6 | Pro-inflammatory cytokines from activated Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$ cells. Anti-Vα7.2-enriched PBMCs were similarly activated with bacterial-incubated K562.hMR1 cells or anti-CD3 with anti-CD28 presented in all conditions as in **Figure 2**. Flow cytometry gated Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$ cells from anti-Vα7.2-enriched PBMCs and gated CD3$^+$CD4$^-$CD8$^+$ cells from anti-Vα7.2-depleted PBMCs. Contour plots show % positive cells on gated Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$CD26^{++} in one independent assay with PBMCs from one healthy donor **(A)**. Data from four independent assays were plotted with standard errors and analyzed with the paired *t*-test **(B)**. Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$CD69$^+$ MAIT cells are also plotted **(C)** and statistically tested **(D)**. Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$CD69$^+$CD26^{++}-gated activated MAIT cells in bacterial or anti-CD3 stimulation (Red) were further compared with Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$CD69$^{+/-}$CD26$^{+/-}$ inactivated cells in *Listeria* incubation (Blue) by displaying the contour plots and mean fluorescence intensity (MFI) for noted molecules. Data from one independent assay using a blood sample from a healthy donor were shown **(E)**. Fold difference of MFI between gated activated and inactivated cells were plotted with standard errors for data from four independent assays. Paired *t*-tests were performed to test the difference between mycobacterial stimulation and listeria incubation **(F)**.

mycobacterial infections and other diseases. Previous reports mainly focused on detecting MAIT cells from blood samples using Vα7.2, CD161, or MR1 tetramer in humans and mice (7, 8, 24, 45, 47, 48). Nonetheless, it is challenging to isolate activated MAIT cells from the inactivated in bacterially infected conditions, due to the lack of activation-related surface markers for a complete separation and the potential downregulation of TCR expression that minimizing the signals

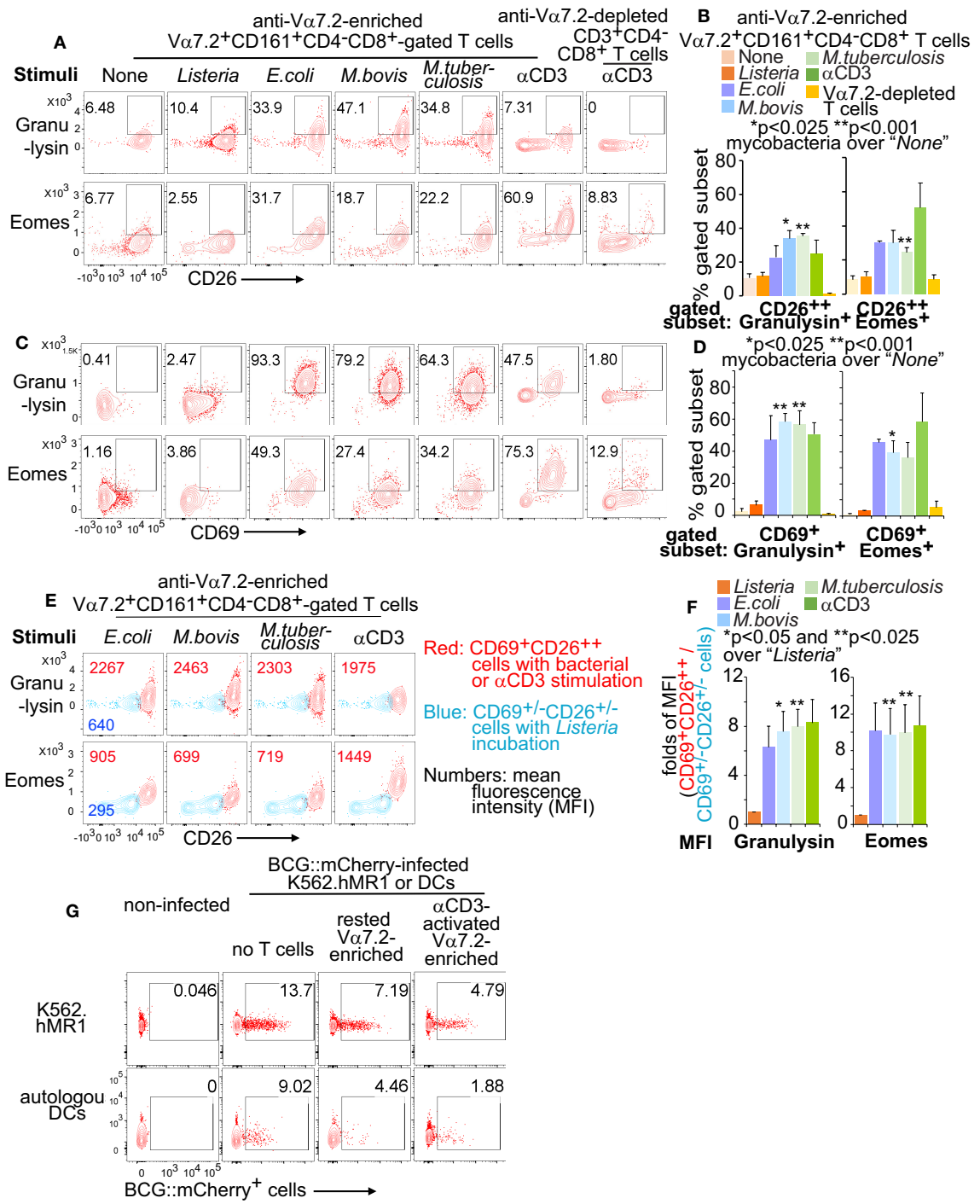

FIGURE 7 | Cytolytic molecules from activated Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$ cells. Anti-Vα7.2-enriched PBMCs were activated and gated as described in **Figure 6**. Contour plots show % positive cells of cytolytic molecules on gated Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$CD26^{++} in one independent assay with PBMCs from one healthy donor **(A)**. Data from three independent assays were plotted with standard errors and analyzed with paired *t*-tests **(B)**. Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$CD69$^+$ MAIT cells are also plotted **(C)** and statistically tested **(D)**. Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$CD69$^+$CD26^{++} activated MAIT cells (Red) were compared with Vα7.2$^+$CD161$^+$CD4$^-$CD8$^+$CD69$^{+/-}$CD26$^{+/-}$ inactivated cells (Blue) with contour plots and mean fluorescence intensity (MFI) for noted molecules as in **Figure 6**. Data from one independent assay were shown **(E)**. Fold difference of MFI between activated and inactivated cells from four independent assays were plotted and statistically tested with paired *t*-tests **(F)**. The BCG.mCherry-incubated MoDCs and K562.hMR1 cells were washed for 4 times and co-cultured with rested or anti-CD3/CD28-activated MAIT-enriched cells for 18 h. Data show remained % mCherry$^+$ cells from an assay using a blood sample of a healthy donor and three independent assays show similar results **(G)**.

of anti-Vα7.2 and tetramer staining on activated MAIT cells (**Figure S3**). Our results showed the TCR downregulation on mycobacterial-stimulated MAIT cells similar to that on activated conventional T cells (80), NKT cells (81), and MAIT cells (51), potentially contributing to a negative feedback regulation of TCR-mediated T cell responses (82). To overcome the difficulty of detecting activated MAIT cells, we used the combinatorial marker $CD69^{+}CD26^{++}$ to label a high percentage of $V\alpha7.2^{+}CD161^{++}CD4^{-}CD8^{+}$ cells at an MR1-dependent activation condition (**Figure 2**) as blocked by the anti-MR1 antibody (**Figure S2**). This combinatorial marker is highly translational to specifically detect activated vs. inactivated MAIT subsets from individual tuberculosis patients without using controls of other bacterial-infected patients. Likewise, $CD69^{+}CD26^{++}$ can be determined whether it is a candidate marker to label activated MAIT cells in *M. tuberculosis*-infected animal models. Particularly, the animal models are highly demanded to replicate abundant MAIT cell frequency, $CD8^{+}$ MAIT cell responses, and *M. tuberculosis*-infected lung pathology in humans. As the major MAIT cell subset in humans (7, 8, 55), $CD8^{+}$ MAIT cells can be stimulated to generate a higher percentage of $CD69^{+}CD26^{++}$ cells than $CD4^{-}CD8^{-}$ MAIT cells in mycobacterial stimulation ($p < 0.01$). Interestingly, CD26 expression on $CD8^{+}$ T cells has been associated with pro-inflammatory responses as a potential co-stimulatory molecule (83) and cytolytic responses as CD26 co-localizes with granulysin, perforin, and granzymes intracellularly (84). Thus, the high expression of CD26 and the differentiation of a $CD69^{+}CD26^{++}$ subset can perhaps label the activated $CD8^{+}$ MAIT cells that produce pro-inflammatory and cytolytic molecules upon *M. tuberculosis* stimulation as in this study. Indeed, rapidly activated MAIT cells in *M. tuberculosis* stimulation consist of a higher percentage of $CD8^{+}$ MAIT cells but a lower percentage of the $CD4^{-}CD8^{-}$ subset than inactivated MAIT cells (**Figure 2**), consistent to the responses of $CD8^{+}$ and $CD4^{-}CD8^{-}$ MAIT cells in *E.coli* stimulation (49). Moreover, the differentiation kinetics and effector functions of $CD8^{+}$ and $CD4^{-}CD8^{-}$ MAIT cells in chronic *M. tuberculosis* infection require comprehensive investigations, as extended *E.coli* stimulation can decrease $CD8^{+}$ MAIT cell frequency (49). To understand the early activation program of $CD8^{+}$MAIT cells in mycobacterial stimulation, we used a heatmap to show the expression of multiple signature genes from these $CD69^{+}CD26^{++}$ $CD8^{+}$MAIT cells, including surface markers, cytokines, cytolytic molecules, and transcription factors. These molecules have been mostly reported in activated MAIT, other T cells, and NK cells (8, 24, 42–46, 57, 58), validating the activation of $CD69^{+}CD26^{++}$ MAIT cells in mycobacterial infection (**Figure 3A**). Our results are also complementary to the findings that MAIT cells can be activated in a manner independent of MR1-mediated antigen presentation. For example, the stimulation with cytokine IL-18 also upregulates the expression of CD69 and IFNγ, independent on anti-MR1 blockage at viral-infected conditions (85, 86). The cellular context and molecular stimulants for MR1-dependent and independent MAIT cell activation are expected to be complementary and similarly important for inducing anti-microbial responses.

Recently, MAIT cell transcriptomes were compared with other T cells (59), associated with tissue repair function (21, 22), and coordination of immune responses (23). However, it remains unclear whether MAIT cells rapidly activated in pathogenic bacterial infections show an innate-like activation program to mediate protective responses. To understand the innateness of MAIT cell activation in mycobacterial infection beyond the conserved molecular interaction of MR1-antigen-TCR complex (12–14, 73, 74), we determined MAIT cell transcriptomes between activated MAIT cells $V\alpha7.2^{+}CD161^{+}CD4^{-}CD8^{+}CD69^{+}CD26^{++}$ and inactivated MAIT-enriched cells $V\alpha7.2^{+}CD161^{+}CD4^{-}CD8^{+}CD69^{+/-}CD26^{+/-}$ in BCG stimulation. Our findings provide multiple lines of evidence to support the innate-like activation program of MAIT cells in mycobacterial stimulation. First, we compared DEGs ($n = 663$) from BCG-stimulated MAIT cells with various innate or adaptive immune cells and revealed thousands of datasets with co-expressed genes at high statistical significance (**Figures 4A,B**). Second, DEGs of $CD69^{+}CD26^{++}$ MAIT cells also significantly overlap with various pathways typically shown in activated $CD8^{+}$ T cells, NK cells, and NKT cells (**Figure 4C**). These overlapped pathways broadly involve activation, differentiation, proliferation, cytotoxicity, cytokine production (e.g., IFNγ, TNF), apoptosis, and intracellular signaling, such as NFκB-, MAPK-, Jak-STAT-mediated signaling, in conventional T cells and innate immune cells (**Figure 4C**). Third, DEGs of activated MAIT cells overlap with both T cells and NK cells in their activation pathways (**Figure 5**). For example, CD3ζ (CD247) is important in coupling the antigen recognition of T cells and the CD16 surface ligation of NK cells to intracellular signaling pathways (87, 88). CD8α is critical in cytotoxic T cell and NK cell responses (89). NFκB contributes to the pro-inflammatory response and cell survival of T cells (90, 91), and receptor-mediated signaling transduction in innate immune cells (92). Upregulated genes also include multiple TNF superfamily factors, which usually interact with the NFκB-mediated pathway in conventional T cells and innate immune cells (93). Shared with NK cell activation pathways as well, genes encoding cell surface receptors *IFNGR1* and *NCR3* (NKp30) are crucial in other innate-like T cell populations, such as γδT cells (94, 95). Together, co-expression, enrichment, overlapped functional pathways, and shared genes in activation pathways between activated MAIT cells and other innate or adaptive immune cells strongly support the innateness of MAIT cell activation.

We expect this innate-like activation program and the combinatorial activation marker can be translated to understand early anti-mycobacterial MAIT cell immune responses in humans and animal models. First, our observation supports that rapid MAIT cell responses are potentially important in early protection against *M. tuberculosis* infection, relevant to previous findings of the conserved tri-molecular interaction in humans (73, 74) and the early inhibition of mycobacterial growth in mice (8, 18). BCG vaccination and *M. tuberculosis* infections are suggested to transiently enhance MAIT cell frequency in rhesus macaques (56), similar to transient MAIT cell responses to BCG vaccination in humans (55). This early MAIT cell response

in vivo is consistent to the innate-like activation program of MAIT cells in this study, although the kinetics, trends, and distributions of MAIT cells or their activation in human *M. tuberculosis* infections requires further investigations (7, 96–98). Second, we observed that both BCG and avirulent H37Ra similarly activated primary human MAIT cells. However, virulent *M. tuberculosis* is usually less efficient in stimulating conventional T cells (99, 100), due to its virulence in causing necrosis and reducing antigen presentation capability of infected cells (101, 102), except for T cell responses specific to the unique antigens of virulent *M. tuberculosis* (103). For MAIT cells, BCG and *M. tuberculosis* have been suggested to comparably enhance peripheral MAIT cell frequency in the early stage of vaccination or infection of rhesus macaques (56), supporting a rapid MAIT cell activation. However, different from BCG vaccination, chronic lung pathology in *M. tuberculosis* infection may contribute to the redistribution of MAIT cells from blood to lung tissues and pleural space (42, 96, 97). Third, we showed that $CD69^{+}CD26^{++}$ $CD8^{+}$MAIT cells upregulated the gene and protein expression of multiple pro-inflammatory and cytolytic molecules, such as TNFα, IFNγ, granulysin, perforin, and corresponding master transcription factors Tbet and Eomes, upon overnight mycobacterial stimulation. Anti-mycobacterial responses have been usually reflected by the production of pro-inflammatory cytokines from $CD4^{+}$ and $CD8^{+}$ T cells, and innate cells (3, 69, 70), together with the cytolytic function of $CD8^{+}$ T cells (4). Relevant to the enhanced production of various pro-inflammatory cytokines and cytolytic molecules of early activated MAIT cells, co-culture of MAIT cells with mycobacterial-incubated K562.hMR1 cells and MoDCs inhibits the growth of mCherry-labeled BCG. These data support the potential protection of human MAIT cells against early mycobacterial infection, similar to the inhibition of BCG growth by mouse MAIT cells (18). Moreover, rapidly stimulated effector functions of activated $CD8^{+}$MAIT cells likely facilitate the kicking in of adaptive immune responses to provide a full set of immune protection, representing a unique niche in anti-mycobacterial immune responses.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/ with an accession number GSE124381.

ETHICS STATEMENT

Blood samples free of detectable infectious or non-infectious diseases were obtained from healthy donors with written informed consent at the Hoxworth Blood Center in the University of Cincinnati. De-identified blood samples were processed according to the protocols approved by the Institutional Review Board of the University of Cincinnati.

AUTHOR CONTRIBUTIONS

All authors: reviewed the manuscript. MS and SZ: perform assays, initial data analyses, and interpretation. LN: initial transcriptomic data analyses. DL: generation of MAIT cell lines. XZ: RNA sample preparation and sequencing. SH: study design, data analyses, and manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01136/full#supplementary-material

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CXCL16 Stimulates Antigen-Induced MAIT Cell Accumulation but Trafficking During Lung Infection Is CXCR6-Independent

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Mucosa-associated invariant T (MAIT) cells are a unique T cell subset that contributes to protective immunity against microbial pathogens, but little is known about the role of chemokines in recruiting MAIT cells to the site of infection. Pulmonary infection with *Francisella tularensis* live vaccine strain (LVS) stimulates the accrual of large numbers of MAIT cells in the lungs of mice. Using this infection model, we find that MAIT cells are predominantly CXCR6^{+} but do not require CXCR6 for accumulation in the lungs. However, CXCR6 does contribute to long-term retention of MAIT cells in the airway lumen after clearance of the infection. We also find that MAIT cells are not recruited from secondary lymphoid organs and largely proliferate *in situ* in the lungs after infection. Nevertheless, the only known ligand for CXCR6, CXCL16, is sufficient to drive MAIT cell accumulation in the lungs in the absence of infection when administered in combination with the MAIT cell antigen 5-OP-RU. Overall, this new data advances the understanding of mechanisms that facilitate MAIT cell accumulation and retention in the lungs.

Keywords: MAIT cells, pulmonary infection, *F. tularensis*, CXCR6, CXCL16

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INTRODUCTION

Mucosa-associated invariant T (MAIT) cells are innate-like T cells that play important roles in protective immunity against microbial infections (1–5). MAIT cells are activated by riboflavin-related metabolites presented by the major histocompatibility complex (MHC) class I related protein (MR1). Riboflavin-related metabolites such as 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) are present in many bacteria, yeast, and plants, but not in mammals and other animals (6–8). Several pathogens such as *Francisella tularensis* live vaccine strain (LVS), *Legionella longbeachae*, and *Salmonella typhimurium* BRD509 have been reported to activate MAIT cells *in vivo* (9–11). MAIT cells have been implicated in anti-tumor responses and the exacerbation/amelioration of autoimmune diseases, including diabetes I, multiple sclerosis and gut-associated diseases like colitis (12–14).

Chemokines are differentially expressed in different tissues and inflammatory microenvironments and guide the homing of specific leukocyte subsets via interactions with differential cell surface chemokine receptors (15–17). Chemokines play critical roles in immunity

and inflammation, including immune system development, leukocyte positioning, lymphocyte migration, and phagocyte activation (15, 18, 19). These important proteins and their receptors have been targeted as clinical therapies and investigated as biomarkers for certain human diseases (19).

In humans, MAIT cells have been observed in numerous tissues, including the lungs, intestinal lamina propria, liver, and peripheral blood (20–23). Human peripheral blood MAIT cells exhibited high levels of CCR6 and CXCR6, heterogenous levels of CXCR4, and intermediate expression of CCR9 under steady-state conditions, which may reflect the potential for circulating MAIT cells traffic to different tissues (21). Indeed, numerous reports have noted a significant decrease in the number of MAIT cells present in the peripheral blood of patients during infections, suggesting their recruitment from the blood to the site of infection. In contrast, MAIT cells in naïve pathogen-free wild type mice were present in low numbers in most tissues (24, 25), including the blood (0.09% of total αβT cells). Nevertheless, MAIT cells in the lungs of naïve mice were largely positive for CXCR6 and low for CCR9 (25).

Little is known about the role of chemokines and their receptors in regulating MAIT cell localization in tissues. It is unclear whether MAIT cells are recruited from peripheral tissues and local lymph nodes during inflammation, or whether they largely proliferate *in situ* at the site of infection. Since our previous work showed that MAIT cells robustly accumulated in the lungs of mice during pulmonary *F. tularensis* LVS infection, we used this model to investigate the role of proliferation and chemokine recruitment in MAIT cell expansion in the lungs after infection (1, 9). Here we find that, despite being predominantly $CXCR6^{+}$, MAIT cells do not require CXCR6 for accumulation at the site of infection. We further find that MAIT cell accumulation is not driven by recruitment from secondary lymphoid organs, and that the majority of MAIT cells proliferate *in situ* in the lungs after pulmonary *F. tularensis* LVS infection. Surprisingly, however, the only known ligand for CXCR6, CXCL16, was sufficient to drive MAIT cell accumulation in the lungs of naïve mice when administered intranasally with the MAIT cell antigen 5-OP-RU (26).

MATERIALS AND METHODS

Mice and Infection

F. tularensis LVS (ATCC) was grown and frozen as previously described (27). MR1 KO mice (28) and $V\alpha19iTgC\alpha^{-/-}MR1^{+/+}$ transgenic mice exclusively expressing the canonical TCR Vα19-Jα33 of mouse MAIT cells (29) were obtained from Ted Hansen (Washington University in St. Louis, St. Louis, MO) and bred at CBER/FDA. Wild type mice (C57BL6J #000664) and $CXCR6^{-/-}$mice (#005693) were purchased from The Jackson Laboratory. Animals were housed in a barrier environment at CBER/FDA, and procedures were performed according to approved protocols under the FDA Animal Care and Use Committee guidelines. Bacteria were diluted in PBS (Gibco, Life Technologies), and intranasal (IN) infections were performed by delivering 1 or 2×10^2 LVS colony-forming units (CFU) in a volume of 25 μl to anesthetized mice.

For *in vivo* MAIT cell induction therapy, the first dose consisted of a combination of 30 μg Pam_2CSK_4 (Invivogen) and MAIT cell ligands (5-OP-RU or Ac-6-FP, 37.5 μl of an 8 μM solution) administrated IN per mouse. The second and third doses consisted of 5-OP-RU or Ac-6-FP (37.5 μl of a 4 μM solution) administrated IN per mouse. For MAIT cell induction therapies using chemokines, the relevant recombinant chemokines (CXCL16, heat-denatured CXCL16, and CCL24) were administered in the following regimen: the first dose consisted of a combination of 3 μg chemokine and MAIT cell ligands (5-OP-RU or Ac-6-FP, 37.5 μl of an 8 μM solution) administrated IN per mouse. The second and third doses consisted of 3 μg chemokine and 5-OP-RU or Ac-6-FP (37.5 μl of a 4 μM solution) administrated IN per mouse. CXCL16 and CCL24 were purchased from R&D systems. CXCL16 was heat-denatured by incubation at 100°C for 10 min. In all cases, lungs were harvested and processed for flow cytometry analyses 6 days after the first dose.

For FTY720 (Sigma) treatment, mice were injected daily i.p. with 0.5 mg/kg/day of FTY720 dissolved in sterile PBS after day 2 of LVS IN infection. Untreated control mice received an equivalent volume of sterile PBS. For BrdU treatment, mice were administered BrdU (62.5 μl of 10 mg/ml of BrdU) IN daily on days 6–9 after infection. BrdU staining was performed according to the manufacturer's instructions (BD).

Preparation of Synthetic Ligands

Synthetic 5-OP-RU (as a DMSO solution) and Ac-6-FP were prepared according to previously published procedures (26, 30).

Preparation of Single-Cell Suspensions

To prepare lung cells for flow cytometry, the lungs were excised and transferred to a Petri dish and chopped with a sharp scissors for ∼30 s, or until no large pieces were visible. The pieces should be small enough to pass through 10 ml serological pipettes. The lung pieces were incubated with 3 ml of DMEM containing 10% FBS, collagenase D (Sigma, 0.5 mg/ml) for 1 h at 37°C in 5% CO_2. Released cells were filtered through a 40-μm filter, subjected to ACK lysis, washed in PBS + 2% FBS, and passed through a 40-μm filter again. Live cells were enumerated using a hemocytometer after dilution in trypan blue. For the experiments shown in **Figures 3A,B**, the airway lumen cells and lung parenchyma cells were processed separately for each lung. For each mouse, the bronchoalveolar lavage (BAL) fluid was harvested by inserting a 18G catheter into an incision in the trachea and the airway lumen was washed four times with PBS. The resulting cells were processed for flow cytometry staining (airway lumen cells). After removal of the airway lumen cells, the lungs were then excised, and the tissue processed for flow cytometry as described above (lung parenchyma cells). For cells from draining LNs, thymus, and spleen, the organs were minced and the cell suspensions were passed through a 40-μm filter. Cells from the lamina propria were isolated according to a previous publication (31).

In vitro Bone Marrow-Derived Macrophage Co-cultures

BMMØ were cultured as previously described. Briefly, bone marrow was flushed from femurs of healthy wild type mice with Dulbecco minimal essential medium (DMEM; Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone), 10% L-929 conditioned medium, 0.2 mM L-glutamine (Life Technologies), 1 mM HEPES buffer (Life Technologies), and 0.1 mM non-essential amino acids (Life Technologies) [complete DMEM (cDMEM)]. Cells were washed, a single cell suspension prepared, and cells plated at 1×10^6 in 48-well plates, or 2×10^6 in 24-well plates, in cDMEM supplemented with 50 μg/ml gentamycin (Life Technologies) and incubated at 37°C in 5% CO_2. After 1 day of incubation, the medium was replaced with antibiotic-free cDMEM, and the cells were incubated for an additional 6 days at 37°C in 5% CO_2. The medium was replaced with fresh, gentamycin-free cDMEM every 2 days during the 7-day incubation. Vα19iTgCα$^{-/-}$MR1$^{+/+}$ mice were used as source of MAIT cells for co-culture with BMMØs. Total Thy1.2$^+$ T cells were purified from Vα19iTgCα$^{-/-}$MR1$^{+/+}$ mouse spleens using a Thy1.2$^+$ cell enrichment column (Life Technologies), according to the manufacturer's recommendations. MAIT cells were added to the wells at a ratio of 1 T cell to 2 macrophages. Recombinant CXCL16 (200 ng/ml) and 5-OP-RU (9.3 μM) were added to the cultures at the same time as the MAIT cells, and supernatants were collected for cytokine analyses (ThermoFisher Luminex assay for Cytokine & chemokine 26-Plex Mouse ProcartaPlex™ Panel 1) 16 h later.

Flow Cytometry Analyses and Intracellular Cytokine Staining

Cells were stained for a panel of murine cell surface markers and analyzed by using a BD LSR Fortessa flow cytometer and FlowJo software. Ab clones used included GK1.5 (anti-CD4), 53-6.7 (anti-CD8α), H57-597 (anti-TCR β-chain), MP6-XT22 (anti-TNFα), TC1118H10.1 (anti-IL17A), XMG-1.2 (anti-IFN-γ), 2E7 (anti-CD103), M1/70 (anti-CD11b), 2G12 (anti-CCR4), HM-CCR5 (anti-CCR5), 29-2L17 (anti-CCR6), CXCR3-173 (anti-CXCR3), DATK32 (anti-α4β7), 2E7 (anti-CCR9), SA051D1 (anti-CXCR4), SA203G11 (anti-CCR2), J073E5 (anti-CCR3), CXCR3-173 (anti-CXCR3), 4B12 (anti-CCR7), SA051D1 (anti-CXCR6) were obtained from BioLegend. MR1 tetramers were obtained from the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Live/Dead Near IR stain (Molecular Probes) was included in all staining protocols. Optimal antibody concentrations were determined in separate experiments, and appropriate fluorochrome-labeled isotype control antibodies or "fluorescence minus one" (FMO) controls were used throughout. In all cases, cells were first gated on singlets (forward scatter-width or forward scatter-area vs. -height) and live cells (Live/Dead Near IR negative) before further analyses.

To monitor the expression of TNF, IFN-γ, and IL-17A, lung cells were incubated in complete Dulbecco's modified eagle medium (cDMEM) containing 5 μg/mL Brefeldin A at 37°C in 5% CO_2 for 4 h in the presence or absence of PMA and ionomycin. Cells were stained for cell surface markers and followed by intracellular staining. Intracellular staining was performed by using the BD Biosciences buffer system according to the manufacturer's instructions.

Quantitation of CXCL16 in Lung Homogenates

The left side of the lung from each mouse was homogenized in 500 μl of T-PER™ tissue protein extraction reagent (ThermoFisher Scientific) containing proteinase inhibitor using a FastPrep-24™ 5G Instrument (MP Biomedicals). The lung homogenates were assayed using the mouse CXCL16 ELISA kit (ThermoFisher Scientific) according to the manufacturer's instructions.

Statistical Analyses

All experiments were performed and repeated at least two to three time to assess reproducibility using three to five mice per experimental group unless otherwise stated in the figure legend. Data are represented as mean ± SEM and data were analyzed via one-way ANOVA followed by the Student-Newman-Keuls multiple stepwise comparison (for experiments with >2 experimental groups). A P <0.05 was considered a significant difference ($^*P < 0.05$, $^{**}P < 0.01$, $^{**}P < 0.001$).

RESULTS

Lung MAIT Cells Are Predominantly CXCR6$^+$ During Pulmonary *F. tularensis* LVS Infection

To identify important chemokine receptors that may be used by MAIT cells for homing during infection, we first sought to characterize MAIT cell surface expression of different chemokine receptors during steady state and infectious conditions. Previous studies have shown that MAIT cell numbers in naïve pathogen-free wild type (WT) C57BL/6 mice are exceptionally low, constituting <0.2% of cells in the lungs, spleen, and thymus (24). In order to reliably assess the expression of chemokine receptors by MAIT cells under steady state conditions, we utilized transgenic mice that exclusively express the canonical MAIT cell Vα19-Jα33 TCRα chain (Vα19iTg mice). The gating strategy used to identify MAIT cells is shown in **Figure S1**. As expected, naïve Vα19iTg mice had high levels of MAIT cells in their lungs (7.8%), spleen (5.7%), thymus (3.3%), and lamina propria (LP; 1.1%), as shown in **Figure 1A**. Fourteen days following primary sublethal *F. tularensis* LVS intranasal (IN) infection, Vα19iTg mice exhibited significant accumulation of MAIT cells as compared to their naïve counterparts in the lungs (24.1 ± 2.3% vs. 9.1 ± 0.7%, $P < 0.01$; $2.1 \times 10^6 \pm 7.2 \times 10^4$ vs. 3.1 x $10^5 \pm 5.9 \times 10^3$ total MAIT cells/lung, $P < 0.01$), but no significant changes were observed in the spleen (4.8 ± 0.3% vs. 5.2 ± 0.3%), thymus (2.0 ± 0.5% vs. 3.0 ± 0.6%), and LP (3.2 ± 0.4% vs. 1.9 ± 0.5%).

We next examined the surface expression of a panel of chemokine receptors and integrins by MAIT cells found in the lungs of naïve and LVS-infected Vα19iTg mice (**Figures 1B–F**, controls are shown in **Figure S2**). Of the ten chemokine receptors

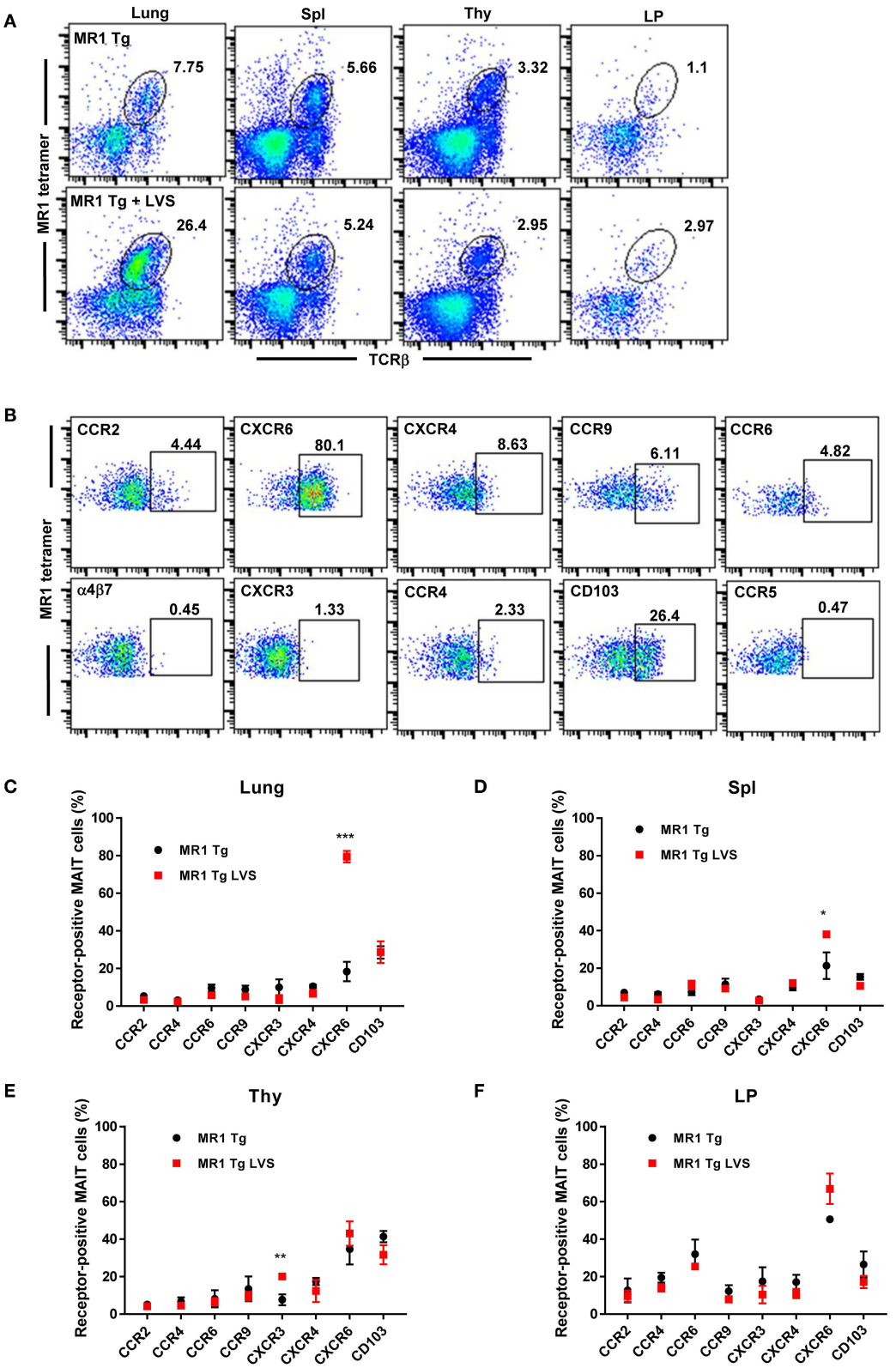

FIGURE 1 | MAIT cells in the lungs of Vα19iTg mice predominantly express the chemokine receptor CXCR6 during *F. tularensis* LVS intranasal infection. **(A)** Representative flow cytometry dot plots of MAIT cells in the lungs, spleen (Spl), thymus (Thy), and intestinal lamina propria (LP) of naïve Vα19iTg mice (upper panel)

(Continued)

FIGURE 1 | and LVS-infected Vα19iTg mice on day 14 after infection (lower panel). MAIT cells are gated on live 5-OP-RU MR1 tetramer$^+$ TCRβ$^+$ cells in the total cell population for each organ. **(B)** Representative flow cytometry dot plots depicting expression of different chemokine receptors on MAIT cells in LVS-infected Vα19iTg mouse lungs on day 14 after infection. Plots show live 5-OP-RU MR1 tetramer$^+$ TCRβ$^+$ cells. A graphical representation of the percentage of chemokine receptor-positive MAIT cells in the lungs **(C)**, spleen **(D)**, thymus **(E)**, and intestinal lamina propria **(F)** in naive and LVS-infected Vα19iTg mice on day 14 after infection (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ as compared to naïve mice). Data are presented as the mean ± SEM ($n = 4$–5) and are representative of two independent experiments.

examined, only CXCR6 exhibited a significant change in the level of cell surface expression on MAIT cells in the lungs 14 days after LVS IN infection. As shown in **Figure 1C**, the percentage of CXCR6-expressing MAIT cells in the lungs increased almost five-fold in infected Vα19iTg mice (79.4% ± 1.7) as compared to naïve Vα19iTg mice (18.4% ± 3.0). A small but significant increase in the levels of CXCR6-expressing MAIT cells was also observed in the spleen, but not the thymus and LP after infection (**Figures 1D,F**). We found that relatively few MAIT cells expressed CCR2, CCR4, CCR6, CXCR3, CXCR4, and CCR9 in the lungs (**Figure 1C**), spleen (**Figure 1D**), thymus (**Figure 1E**), and LP (**Figure 1F**) both before and after infection. Of those chemokine receptors, only a slight increase in the percentage of CXCR3-expressing MAIT cells was observed after infection, and this only occurred in the thymus ($P < 0.05$). In addition, the mucosal integrin CD103 was expressed by about 20% of MAIT cells in the lung, spleen, thymus, and LP, but these levels did not significantly change following LVS IN infection.

We next sought to determine whether MAIT cells exhibit a similar pattern of chemokine receptor expression in WT C57BL/6 mice during *F. tularensis* LVS IN infection. Fourteen days after LVS IN infection, large numbers of MAIT cells were observed in the lungs (9.1% ± 2.0), while relatively few were found in the spleen (0.9% ± 0.1), LP (0.5% ± 0.1), and thymus (0.2% ± 0.05; **Figure 2A**). Because such low numbers of MAIT cells were present in the LP and thymus, we did not examine chemokine receptor expression in these tissues. Consistent with our observations in LVS-infected Vα19iTg mice, MAIT cells in the lungs of infected WT mice were predominantly CXCR6$^+$ (∼80%; **Figures 2B,C**; controls are shown in **Figure S2**). In the spleen, ∼60% of MAIT cells expressed CXCR6 and 20% expressed CCR6 (**Figure 2C**). Thus, a large proportion of MAIT cells in the lungs express the chemokine receptor CXCR6 during pulmonary LVS infection, suggesting that this receptor may participate in MAIT cell trafficking to the lungs.

The CXCR6 Ligand CXCL16 Is Up-Regulated in the Lungs During Pulmonary *F. tularensis* LVS Infection

The only known ligand for CXCR6 is CXCL16, which was previously found to be expressed at high levels in the liver and lungs of naïve mice (32–34). Since the vast majority of MAIT cells responding to LVS pulmonary infection in the lungs were CXCR6$^+$, we next investigated whether CXCL16 is elevated in the lungs during pulmonary LVS infection. To this end, we gave WT mice a sublethal LVS IN infection and CXCL16 levels were assessed in the lungs over time. As shown in **Figure 2D**, approximately 2 ng/ml of CXCL16 was detected in in the lungs of naïve WT mice. The levels of CXCL16 significantly increased as early as 4 days after LVS IN infection and peaked 7–10 days post-infection (an approximately five-fold increase as compared to naïve mice on day 10; $P < 0.001$). By 21 days after LVS infection, at a time when bacterial CFUs were fully cleared, the CXCL16 levels had diminished but not fully returned to that of naïve mice ($P<0.05$). Thus, CXCL16 is significantly up-regulated in the lungs during LVS pulmonary infection and may serve as a mechanism to recruit CXCR6-expressing MAIT cells.

CXCR6 Is Not Required for MAIT Cell Accumulation in the Airway Lumen and Lung Parenchyma During Pulmonary *F. tularensis* LVS Infection

To further elucidate the role of CXCR6 in MAIT cell localization and function during infection, we compared MAIT cell accumulation in the lungs of CXCR6 knock out mice (CXCR6$^{-/-}$) and WT mice after LVS IN infection. We previously found that MAIT cell numbers increased substantially after the first week of LVS pulmonary infection (days 8–9), reached a peak during the clearance phase of infection (days 10–14), and declined slowly thereafter but remained a notable presence in the lungs for a long time (>56 days) (9).Thus we examined MAIT cell accumulation in the lungs of CXCR6$^{-/-}$and WT mice on days 10 and 54 after LVS IN infection. In addition, since recent research reported that the CXCR6/CXCL16 signaling axis regulated localization of tissue resident memory CD103$^+$ CD69$^+$ CD8$^+$ T cells to the airway lumen (35), we examined MAIT cell partitioning in the lungs by assessing MAIT cell numbers in the lung parenchyma and airway lumen (BAL fluid). There were no differences in the total numbers of MAIT cells in the lung parenchyma (**Figure 3A**) and airway lumen (**Figure 3B**) on day 10. However, MAIT cell numbers in the airway lumen, but not the lung parenchyma, were significantly reduced in CXCR6$^{-/-}$mice as compared to WT mice on day 54, long after clearance of the LVS infection (at approximately day 18). Of note, we observed no significant differences in LVS growth in the lungs between WT and CXCR6$^{-/-}$ mice (**Figure S2**). MAIT cell numbers in the spleen were not significantly different between LVS-infected WT and CXCR6$^{-/-}$ mice at a time when MAIT cell numbers peaked in that tissue (day 14) (**Figure 3C**). Overall, these data show that CXCR6 is not essential for MAIT cell accumulation in the lungs and spleen during LVS IN infection but has a significant role in maintaining MAIT cells in the airway lumen long after clearance of the infection.

Previous studies have shown that the loss of CXCR3 expression by MAIT cells in patients with end stage renal disease was coupled to increased expression of CCR6 and CXCR6 (36).

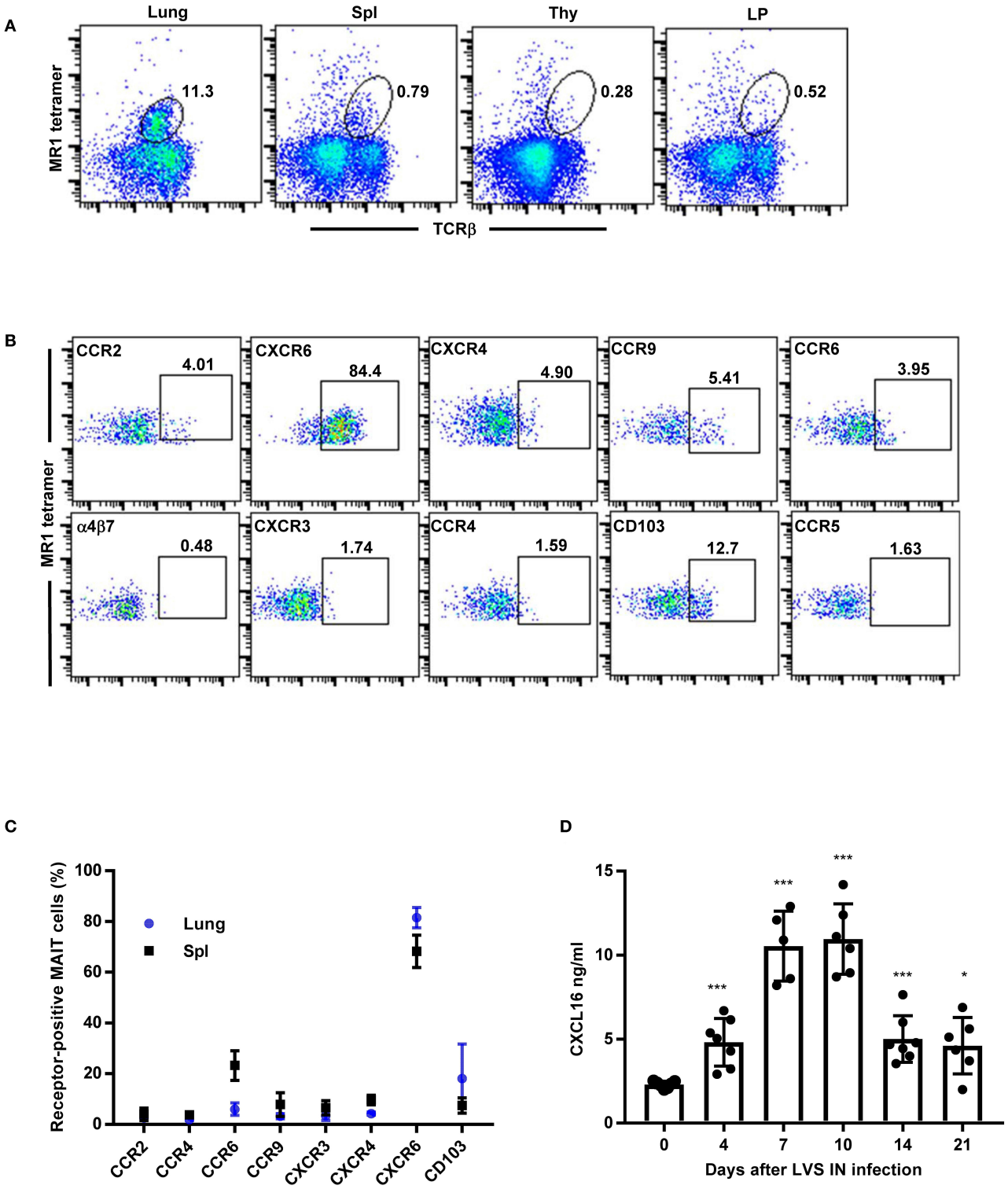

FIGURE 2 | MAIT cells in the lungs of WT mice predominantly express the chemokine receptor CXCR6 during *F. tularensis* LVS intranasal infection. **(A)** Flow cytometry analysis of MAIT cells in the lungs, Spl (spleen), Thy (thymus), and LP (lamina propria) of LVS-infected WT mice on day 14 after LVS infection, showing reactivity to MR1-5-OP-RU tetramer in total lung cells. **(B)** Representative flow cytometry dot plots depicting expression of different chemokine receptors on MAIT cells in LVS-infected WT mice lungs (day 14 after infection). Plots show live 5-OP-RU MR1 tetramer^{+} TCRβ^{+} cells. **(C)** A graphical representation of the percentage of MAIT cells positive for the indicated chemokine receptors in the lungs and spleens (Spl) of LVS-infected WT mice on day 14 after infection. **(D)** The levels of CXCL16 in lung homogenates of naïve and LVS-infected WT mice at the indicated time points (*$P < 0.05$; ***$P < 0.001$ as compared to naïve Day 0 mice). Data are presented as the mean ± SEM ($n = 5$–6) and are representative of two independent experiments.

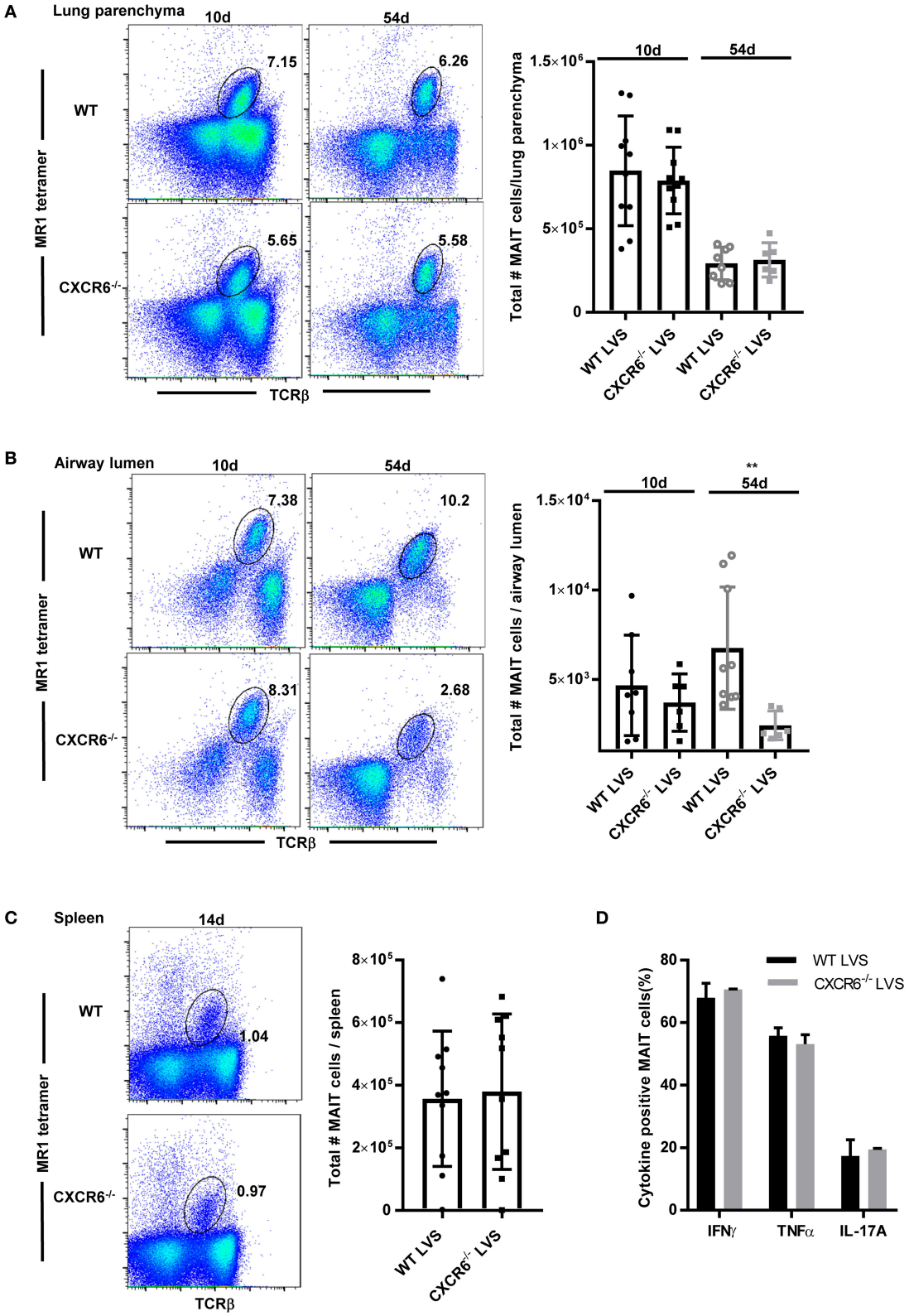

FIGURE 3 | CXCR6 is not required for MAIT cell accumulation in the lung parenchyma or spleen during *F. tularensis* LVS intranasal infection but contributes to long-term retention in the airway lumen (BAL). **(A)** Representative flow cytometry dot plots showing the percentage of MAIT cells in the lung parenchyma of WT and

(Continued)

FIGURE 3 | CXCR6$^{-/-}$ mice on days 10 and 54 after LVS IN infection. MAIT cells are gated on live MR1-5-OP-RU tetramer^{+} TCRβ^{+} cells in the lungs. A graphical representation of the number of MAIT cells in the lung parenchyma is shown. **(B)** Representative flow cytometry dot plots of the percentage of MAIT cells in the airway lumen (BAL) of WT and CXCR6$^{-/-}$ mice on days 10 and 54 after LVS IN infection. MAIT cells are gated on live MR1-5-OP-RU tetramer^{+} TCRβ^{+} cells in the lungs. A graphical representation of the number of MAIT cells in the airway lumen is shown (**$P < 0.01$ as compared to WT mice). **(C)** Representative flow cytometry dot plots of the percentage of MAIT cells in the spleen of WT and CXCR6$^{-/-}$ mice on day 14 after LVS IN infection. MAIT cells are gated on live MR1-5-OP-RU tetramer^{+} TCRβ^{+} cells in the spleen. A graphical representation of the number of MAIT cells in the spleen is shown. **(D)** Lungs harvested from LVS-infected WT and CXCR6$^{-/-}$ mice on day 14 after infection were stimulated *in vitro* in the presence of PMA and ionomycin. A graphical representation of the percentage of MAIT cells positive for IFN-γ, TNF, and IL-17A is shown. Data are presented as the mean ± SEM ($n = 5$–7) and is representative of at least two independent experiments.

To assess the possibility that other chemokine receptors may have compensated for the loss of CXCR6 in the CXCR6$^{-/-}$ mice, we examined the cell surface expression of a panel of chemokine receptors on MAIT cells present in the lungs of infected WT mice and CXCR6$^{-/-}$ mice. No significant changes were observed in the proportion of MAIT cells expressing the chemokine receptors CCR2, CCR4, CCR5, CCR6, CCR9, CXCR3, CXCR4, and α4β7 in LVS-infected CXCR6$^{-/-}$ mice as compared to WT mice (data not shown). Thus, our evidence suggests that MAIT cell accumulation in the lungs of CXCR6$^{-/-}$ mice is not the result of compensation via any of the aforementioned chemokine receptors.

Although we were unable to detect a difference in the number of MAIT cells present in the lungs of WT and CXCR6$^{-/-}$ mice after LVS IN infection, it was possible that the MAIT cells accumulating in the lungs of the CXCR6$^{-/-}$ mice had functional deficits. To this end, we compared cytokine production by MAIT cells in the lungs of LVS-infected WT and CXCR6$^{-/-}$ mice. No significant differences were observed in the production of IL-17, IFN-γ, or TNF by MAIT cells obtained from the lungs of LVS-infected WT and CXCR6$^{-/-}$ mice (data not shown). Similarly, no differences in cytokine production were detected by MAIT cells obtained from the same mice and further stimulated *ex vivo* with PMA/ionomycin (**Figure 3D**). Thus, MAIT cells from CXCR6$^{-/-}$ mice do not have obvious deficits in IL-17, IFN-γ or TNF production.

The Majority of MAIT Cells Proliferate in the Lungs During Pulmonary *F. tularensis* LVS Infection

Since we were unable to identify any chemokine receptors that were likely to promote MAIT cell recruitment to the lungs during LVS infection, we next determined whether MAIT cells proliferated in the lungs during pulmonary LVS infection. As shown in **Figure 4A**, MAIT cell numbers in the lungs increased significantly starting on day 6 after LVS infection as compared to naïve mice. To determine whether this increase was the result of MAIT cell recruitment to the site of infection or *in situ* proliferation in the lungs, MAIT cells present in the lungs were given the opportunity to incorporate bromodeoxyuridine (BrdU) delivered via the IN route. BrdU is a synthetic nucleoside analog of thymidine that is only incorporated into actively replicating cells. BrdU was administered daily days 6–9 after infection, and BrdU^{+} MAIT cells were assessed in the lungs on day 10. Approximately 60% of MAIT cells were BrdU^{+} on day 10 after infection (**Figure 4B**), indicating that the majority of these cells had proliferated in the preceding 4 days.

Since previous studies showed that some of the BrdU delivered IN to mice travels to the lung-draining lymph nodes (37), it remained possible that some of the BrdU^{+} MAIT cells observed in the lungs had migrated from local lymph nodes. To address this possibility, we first examined the MAIT cell population in the mediastinal and cervical lymph nodes on day 10 after LVS IN infection. Although MAIT cells were a very small population in the lung-draining lymph nodes (**Figure 4C**), a significant proportion of these cells were BrdU^{+} at day 10 (**Figure 4D**). Therefore, to determine whether MAIT cells located in the lymph nodes traffic to the lungs during LVS IN infection, mice were administered the sphingosine-1-phosphate receptor agonist FTY720. FTY720 (fingolimod) is an FDA-approved drug for multiple sclerosis that inhibits lymphocyte egress from the thymus and secondary lymphoid organs (38). Mice were treated with FTY720 starting on day 2 after LVS IN infection, before MAIT cells increased significantly in the lungs, and daily administration was continued until we investigated the numbers of MAIT cells, CD4^{+} T cells, and CD8^{+} T cells in the lungs on day 10. As shown in **Figure 4E**, the numbers of CD4^{+} and CD8^{+} T cells were significantly reduced in the lungs of mice administered FTY720 as compared to control mice, indicating that a large proportion of these T cells trafficked to the lungs from secondary lymphoid organs. In contrast, the number of MAIT cells in the lungs of mice administered FTY720 was not significantly different from control mice, demonstrating that emigration from the lymph nodes and other secondary lymphoid organs does not substantially contribute to the MAIT cell population found in the lungs during infection. This evidence, coupled with the high levels of BrdU incorporated by lung MAIT cells after IN administration, suggest that the majority of MAIT cells in the lungs during pulmonary infection arise from *in situ* proliferation as opposed to trafficking from other sites.

Intranasal Administration of CXCL16 and 5-OP-RU Induces MAIT Cell Accumulation in the Lungs Under Non-inflammatory Conditions

Previous studies have shown that intranasal administration of a TLR agonist (e.g., CpG, Pam_2CSK_4) and a MAIT cell activating antigen (e.g., 5-OP-RU) induced robust accumulation of MAIT cells in the lungs of naïve mice (10). In contrast, intranasal inoculation of a TLR agonist in combination with the MAIT cell inhibitory antigen Ac-6-FP failed to stimulate

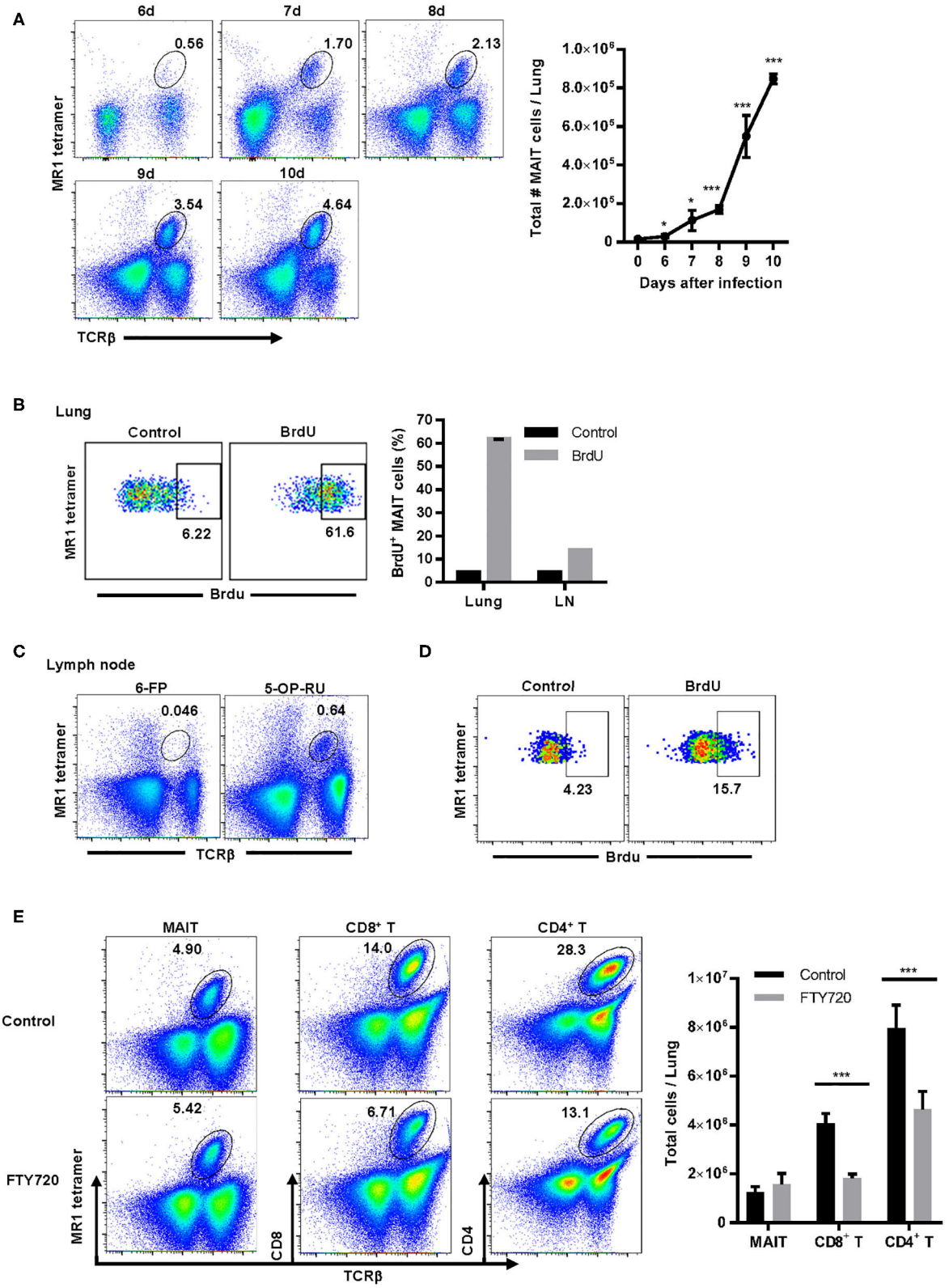

FIGURE 4 | MAIT cells are not recruited from secondary lymphoid organs and largely proliferate *in situ* in the lungs during *F. tularensis* LVS pulmonary infection. **(A)** Representative flow cytometry dot plots of MAIT cells (gated on live 5-OP-RU MR1 tetramer$^+$ TCRβ$^+$ cells) in the lungs of WT mice on days 6–10 after LVS IN

(Continued)

FIGURE 4 | infection. A graphical representation of the total number of MAIT cells in the lungs is shown (*$P < 0.05$; ***$P < 0.001$ as compared to naïve mice). **(B)** Representative flow cytometry dot plots of MAIT cell incorporation of BrdU in the lungs of WT mice on day 10 after LVS IN infection. Mice were administered BrdU IN daily on days 6–9 after infection. Control staining was performed using an isotype control Ab on mice that received BrdU. MAIT cells were gated on live 5-OP-RU MR1 tetramer$^+$ TCRβ$^+$ cells. This is accompanied by a plot depicting the percentage of MAIT cells in the lungs and draining lymph nodes (LN) that were BrdU$^+$ following staining with anti-BrdU Ab (BrdU) or isotype control Ab (Control). **(C)** Representative flow cytometry dot plots of MAIT cells in the cervical and mediastinal lymph nodes of WT mice on day 10 after LVS IN infection. Plots show staining with the negative control MR1 tetramer (6-FP) and the MAIT cell-reactive MR1 tetramer (5-OP-RU) in total lymph node cells. **(D)** Representative flow cytometry dot plots of MAIT cell incorporation of BrdU in the lymph nodes on day 10 after LVS IN infection. These dot plots show BrdU uptake by MAIT cells found in the lymph nodes of mice shown in **(B)** above. **(E)** Representative flow cytometry dot plots of MAIT cells, CD4$^+$ T cells, and CD8$^+$ T cells in the lungs of WT control mice and mice treated with the sphingosine-1-phosphate agonist FTY720 on days 2–9 after LVS IN infection. The lungs were harvested on day 10 after infection for flow cytometry analysis. The accompanying panel is a graphical representation of this data (***$P < 0.001$ as compared to control). Data are presented as the mean ± SEM ($n = 3$) and are representative of two independent experiments.

MAIT cell accumulation. Further, the TLR agonist and the MAIT cell antigen were both essential, as administration of either component individually was not sufficient to increase MAIT cell numbers in the lungs. TLR agonists are highly immunostimulatory and thus far it remains unclear which of the many cytokines and chemokines produced in response to these agents are sufficient to induce MAIT cell accumulation in combination with 5-OP-RU.

Although our data show that CXCR6 is not required to increase MAIT cell numbers in the lungs during the peak of infection, this does not rule out a redundant role for the CXCR6/CXCL16 axis in MAIT cell accumulation. Since the majority of MAIT cells expressed CXCR6, we next sought to determine whether administration of CXCL16 might be sufficient to replace the aforementioned TLR agonists in the promotion of MAIT cell accumulation when combined with 5-OP-RU. To this end, recombinant CXCL16 and 5-OP-RU were administrated intranasally to naïve WT mice on day 1, and 5-OP-RU alone was administrated intranasally on days 2 and 3. MAIT cell numbers in the lungs on day 7 were then compared to several different control immunostimulatory combinations (**Figures 5A,B**). Consistent with previous research (10), the TLR2/6 agonist Pam_2CSK_4 + 5-OP-RU dramatically induced MAIT cell accumulation in the lungs (7.5 ± 0.1% of total lung cells). Significant accumulation of MAIT cells was also observed in mice that received CXCL16 + 5-OP-RU (3.7 ± 0.7%) on days 1, 2, and 3, but not in those that received CXCL16 + Ac-6-FP (0.4 ± 0.1%) or PBS (0.2 ± 0.02%) (**Figures 5A,B**). Recombinant CCL24 and heat denatured CXCL16 were used as controls to confirm the role of CXCL16 in the enrichment of MAIT cells. CCL24 binds the chemokine receptor CCR3, which is crucial for directing migration and priming of eosinophils and is unlikely to contribute to MAIT cell trafficking (39). When CCL24 + 5-OP-RU (0.6 ± 0.2%) or heat-denatured CXCL16 + 5-OP-RU (0.6 ± 0.1%) were administrated intranasally to naïve WT mice (**Figures 5A,B**), no significant MAIT cell accumulation was observed. Of note, mice treated with CXCL16 + 5-OP-RU did not exhibit significant differences in the numbers of conventional CD4$^+$ and CD8$^+$ T cells or inflammatory monocytes (Ly6C^{hi} CD11b$^+$) as compared to mice given PBS (**Figure S4**). In contrast, mice administered CXCL16 + 5-OP-RU exhibited significantly more antigen presenting cells (CD11c$^+$ MHCII$^+$) as compared to PBS-treated mice, but not CXCL16 + Ac-6-FP-treated mice, indicating that this effect was not related to MAIT cell accumulation (**Figure S4**). Overall, these data demonstrate that CXCL16 in combination with 5-OP-RU is sufficient to induce MAIT cell accumulation *in vivo*.

To assess the ability of CXCL16 and CXCL16 + 5-OP-RU to stimulate MAIT cell cytokine production, purified transgenic murine MAIT cells were co-cultured with uninfected macrophages (for antigen presentation), recombinant CXCL16, and 5-OP-RU. As shown in **Figure 5C**, 5-OP-RU alone stimulated MAIT cells to produce multiple cytokines, including TNF, IL-17A, IL-22, GM-CSF, and IFN-γ. In contrast, CXCL16 alone failed to stimulate MAIT cell cytokine production. MAIT cells stimulated with the combination of CXCL16 + 5-OP-RU did not exhibit a significant increase in cytokine production as compared to those treated with 5-OP-RU alone. These data show that MAIT cells respond to CXCL16 + 5-OP-RU by producing critical cytokines, but that CXCL16 does not significantly augment the levels of MAIT cell effector cytokines produced in response to 5-OP-RU alone.

DISCUSSION

The chemokine receptor CXCR6 was originally described as the HIV and SIV co-receptor (40–42) and is expressed on NKT cells, a subset of activated T cells, and some NK cells (43–45). CXCR6 and its ligand CXCL16 have been shown to mediate homing of lymphocytes to non-lymphoid tissues as well as NK T cell homeostasis and activation (43, 45–48). Since several studies have shown that MAIT cells also express CXCR6 (21, 25), we were interested in determining its role in MAIT cell trafficking, cytokine production, and tissue localization.

Using the *F. tularensis* LVS pulmonary infection model, we found that the vast majority of MAIT cells in the lungs expressed CXCR6 during infection. Despite this finding, CXCR6 was not required for accumulation of MAIT cells in either the lung parenchyma or the airway lumen during LVS infection. Similarly, MAIT cell production of IFN-γ, TNF, and IL-17A was not significantly affected in CXCR6$^{-/-}$ mice. Interestingly, however, CXCR6 had a significant role in maintaining MAIT cells in the airway lumen long after clearance of LVS infection. Importantly, CXCL16 is expressed in both soluble and membrane-bound forms (49), with the latter inducing adhesion of cells expressing CXCR6 (50). This is consistent with findings that suggest CXCL16 is weakly

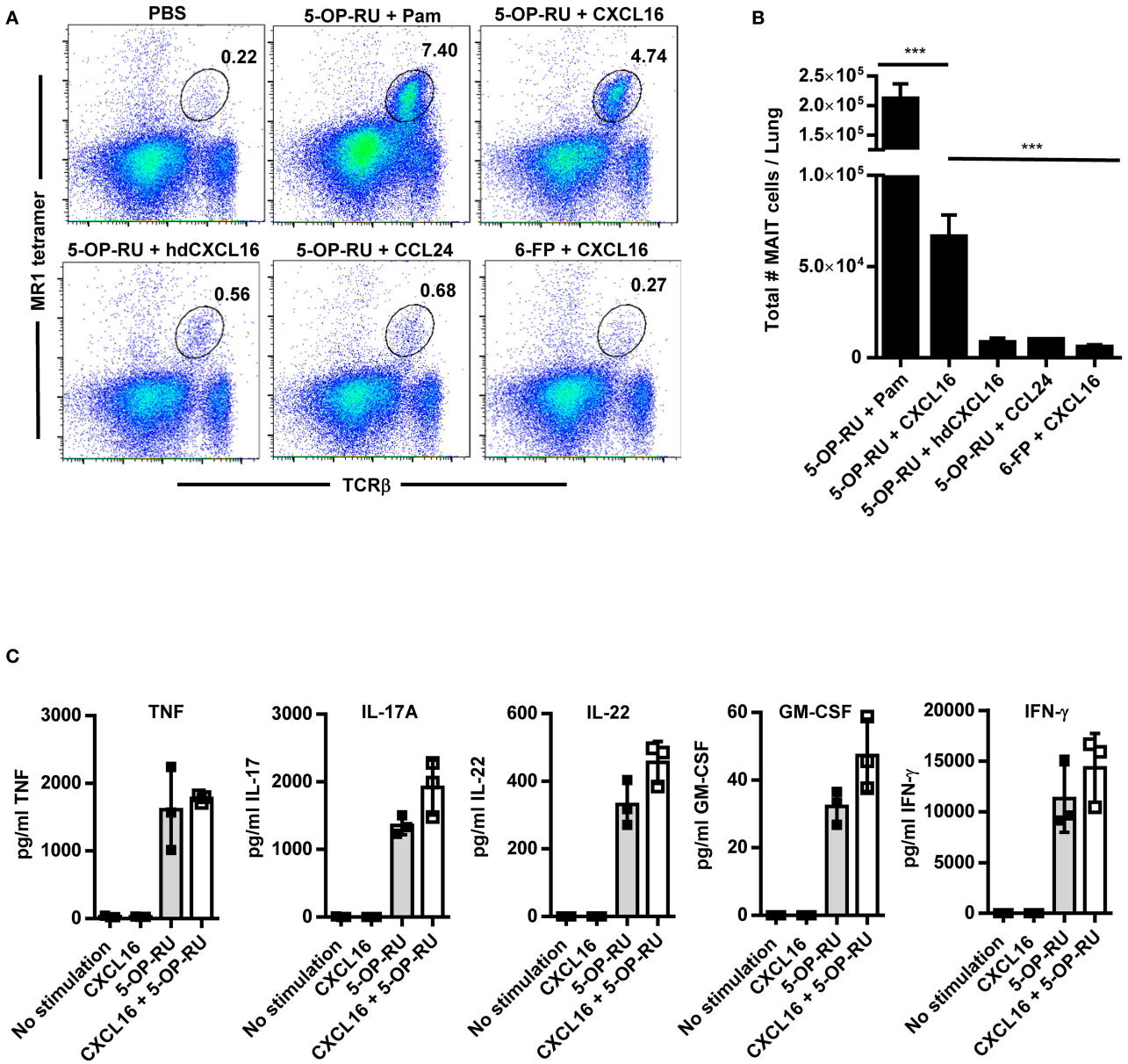

FIGURE 5 | Intranasal instillation of CXCL16 and 5-OP-RU induces MAIT cell accumulation in the lungs in the absence of infection. Naïve WT mice were intranasally administered 5-OP-RU or control Ac-6-FP (6-FP) with one of the chemokines shown on days 1, 2, and 3. Mice given 5-OP-RU + Pam were intranasally administered 5-OP-RU and Pam on day 1, and 5-OP-RU alone on days 2 and 3. On day 7, the lungs were harvested for flow cytometry analysis. "PBS" mice received only PBS at the indicated time points. **(A)** Representative flow cytometry dot plots showing 5-OP-RU MR1 tetramer$^+$ TCRβ$^+$ MAIT cells in naïve WT mice treated as indicated. Total live singlet lung cells for individual mice are shown. **(B)** A graphical representation of the number of MAIT cells in the lungs of mice on day 7 (***$P < 0.001$). **(C)** Vα19iTgMR1$^{+/+}$ transgenic murine MAIT cells were co-cultured with uninfected macrophages, recombinant CXCL16, and 5-OP-RU, and cytokine production was measured after 16 h. "No stimulation" = uninfected macrophages and MAIT cells. hd CXCL16 = heat-denatured CXCL16. Pam = Pam_2CSK_4. Data are presented as the mean ± SEM ($n = 3$) and is representative of three independent experiments.

chemotactic (51) and instead has a role in controlling the localization of CXCR6$^+$ cells within different compartments of the lung rather than directly recruiting them from the circulation (35). Indeed, CXCL16 is constitutively expressed by bronchial epithelial cells, which could facilitate retention of T lymphocytes in the lungs (35, 40, 52). Our own data identified CXCL16 as being up-regulated in the lungs during LVS infection and remained significantly elevated even after the bacterial CFUs had fully cleared. Overall, our data show that CXCR6 is not required for MAIT cell recruitment during infection but instead supports long-term retention of MAIT cells in the airway lumen.

Although our data does not rule out a role for chemokine receptors in MAIT cell recruitment, it raised the question of whether MAIT cells proliferate *in situ* in the lungs during pulmonary LVS infection. Of particular interest, Wang et al. found that MAIT cells in both the lungs and mediastinal lymph nodes proliferated during pulmonary *Legionella longbeacheae* infection (11). This study delivered BrdU via the intraperitoneal route and used a short labeling period (2 h) to avoid detecting cells that had proliferated elsewhere and migrated to the lungs. In our study, we delivered BrdU directly to the lungs by intranasal instillation and investigated the possibility that MAIT cells migrate from secondary lymphoid organs during infection. We found that the majority of MAIT cells in the lungs (∼60%) and a smaller proportion in the lung-draining lymph nodes (∼15%) incorporated BrdU during LVS pulmonary infection. Further, MAIT cell accumulation in the lungs was not impacted by treatment with the sphingosine-1-phosphate agonist FTY720, which inhibits lymphocyte trafficking from the thymus and secondary lymphoid organs (38). This was in sharp contrast to conventional $CD4^+$ and $CD8^+$ T cells, which exhibited impaired recruitment to the lungs after FTY720 treatment. Although FTY720 does not inhibit the migration of T cells found in non-lymphoid tissues and the blood, our combined data suggest that the majority of MAIT cells in the lungs arise from *in situ* proliferation during infection.

Several studies have demonstrated that MAIT cell accumulation in the lungs can be induced by intranasal instillation of a TLR agonist and a MAIT cell antigen in naïve mice (10, 53). Since TLR agonists stimulate production of multiple cytokines and chemokines, the precise agents that facilitate MAIT cell accumulation remain unknown. Further, TLR agonists are broadly inflammatory and may be detrimental in some situations where MAIT cells could be targeted as an immunotherapy. Here we found that intranasal administration of CXCL16 with 5-OP-RU was sufficient to increase MAIT cell numbers in the lungs of naïve mice, although it was not as effective as the TLR agonist Pam_2CSK_4. This is similar to the finding that intranasal delivery of the *Mycobacterium tuberculosis* antigen rec85A with CXCL16 induced enrichment of antigen-specific $CXCR6^+$ T cells in the airways of previously immunized mice and provided significant protection against pulmonary *M. tuberculosis* infection (54). Although our data showed that CXCR6 was not essential for MAIT cell recruitment during LVS infection, this does not rule out a non-redundant role for the CCR6/CXCL16 axis in MAIT cell accumulation. However, it is particularly interesting to note that CXCL16 stimulated proliferation of Jurkat T cells and primary human $CD4^+$ T cells *in vitro* (55). In this light, it is possible that CXCL16 in combination with 5-OP-RU stimulated MAIT cell proliferation rather than recruitment to the lungs. Overall, our data demonstrate that CXCL16 can partially replace TLR agonists in regimens used to induce MAIT cell accumulation, offering a more targeted method to achieve this goal.

In summary, here we found that the vast majority of MAIT cells were $CXCR6^+$ during pulmonary *F. tularensis* LVS infection, but that CXCR6 was not required for MAIT cell accumulation in the lung parenchyma. However, CXCR6 contributed to long-term retention of MAIT cells in the airway lumen. Our data show that MAIT cells are not recruited from secondary lymphoid tissues and largely proliferate *in situ* in the lungs during infection. Importantly, we found that CXCL16 can partially substitute for TLRs in therapeutic regimens that induce MAIT cell accumulation via administration of 5-OP-RU. Overall, our data advance the understanding of the mechanisms that facilitate MAIT cell accumulation and retention in the tissues.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by FDA White Oak Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

SC and HY designed the study and analyzed and interpreted data. HY and AY performed experiments. LL, JM, and DF provided reagents. SC, HY, LL, JM, and DF wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01773/full#supplementary-material

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REVIEW
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The Immune Modulating Properties of Mucosal-Associated Invariant T Cells

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Mucosal-associated invariant T (MAIT) cells are unconventional T lymphocytes that express a semi-invariant T cell receptor (TCR) recognizing microbial vitamin B metabolites presented by the highly conserved major histocompatibility complex (MHC) class I like molecule, MR1. The vitamin B metabolites are produced by several commensal and pathogenic bacteria and yeast, but not viruses. Nevertheless, viral infections can trigger MAIT cell activation in a TCR-independent manner, through the release of pro-inflammatory cytokines by antigen-presenting cells (APCs). MAIT cells belong to the innate like T family of cells with a memory phenotype, which allows them to rapidly release Interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and in some circumstances Interleukin (IL)-17 and IL-10, exerting an immunomodulatory role on the ensuing immune response, akin to iNKT cells and γδ T cells. Recent studies implicate MAIT cells in a variety of inflammatory, autoimmune diseases, and in cancer. In addition, through the analysis of the transcriptome of MAIT cells activated in different experimental conditions, an important function in tissue repair and control of immune homeostasis has emerged, shared with other innate-like T cells. In this review, we discuss these recent findings, focussing on the understanding of the molecular mechanisms underpinning MAIT cell activation and effector function in health and disease, which ultimately will aid in clinically harnessing this unique, not donor-restricted cell subtype.

Keywords: MAIT cells, TCR-dependent, TCR-independent, dendritic cell maturation, inflammation, tissue repair

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INTRODUCTION

MAIT cells are unconventional T lymphocytes that were first described by Porcelli et al. as one of two cell populations enriched in the $CD4^-$ $CD8^-$ T cell fraction (the other being $V\alpha24^+$ iNKT cells) (1). It is now established that canonical MAIT cells express a semi-invariant TCRα-chain (in humans mostly Vα7.2-Jα33/Jα20, in mice Vα19-Jα33) paired with a number of TCR β-chains, contributing to a limited TCR repertoire (2, 3). Because of the limited TCR repertoire, and their similarities with iNKT cells, it was initially proposed by Tilloy and colleagues that these cells could be restricted by a non-classical MHC like molecule presenting either an endogenous ligand or a ubiquitous pathogen (4). Subsequently, it was demonstrated that MAIT cells are restricted to the highly conserved MHC-class I-related protein 1 (MR1) (5, 6). MR1 is β2m-associated, nonpolymorphic, and conserved across various mammalian species, with 90% of sequence similarity between mice and humans (7). The high inter-species conservation of MR1 and TCRα chain results in cross-reactivity between human and non-human species such as bovine,

mouse, and rat (8, 9). Interestingly, a murine autoreactive MAIT hybridoma was shown to strongly recognize cells expressing bovine or rat MR1, but not human MR1, and this was pinpointed to residue Q151, present in the human but not the murine sequence (9). In the same study, polyclonal human MAIT cells were activated by rat, murine, and bovine MR1, but were not autoreactive (9).

MAIT cells preferentially locate in mucosal-associated tissue such as gut, lamina propria, and lung, in both humans and mice (6). Recent research has demonstrated that MAIT cells are also present in liver and human blood, where they can represent up to 50 and 10% of circulating $CD8^+$ T cells, respectively (10, 11). Through the combined use of MR1 tetramers and $J\alpha33^{-/-}$ mice, other populations of MR1-restricted T cells have been described, which express TCRs distinct from the canonical Vα7.2-Jα33, and may play antimicrobial as well as immunoregulatory functions (3, 11–13).

For many years after the discovery of MAIT cells restriction to MR1, the nature of the antigen MAIT cells detect in association with MR1 was unclear. In 2012, Kjer-Nielsen et al. demonstrated that MAIT cells TCRs recognize intermediates of the vitamin B2 (riboflavin) biosynthetic pathway (14). Several bacteria and fungi previously associated with MAIT cell activation (15, 16) produce agonist vitamin B2 metabolites that stimulate MAIT cell activation in a TCR-dependent manner. Viruses are unable to synthesize vitamin B2 metabolites and cannot elicit TCR-dependent MAIT cell activation. Nevertheless, viral infections can elicit MAIT cell activation in a TCR-independent manner, through the release of different cytokines such as IL-12 and IL-18 (17, 18). MAIT cell activation results in the production of a variety of chemokines and pro-inflammatory cytokines, associated with both Th1 (IFN-γ and TNF-α) (16, 19) and Th17 immunity (IL-17 and IL-22) (20), but in certain tissues or upon prolonged stimulation MAIT cells can also release IL-10 and IL-13 (21, 22). Like conventional T cells, cytokine secretion is controlled by key transcription factors, mainly T-bet and RORγt (23). In addition, MAIT cells efficiently lyse bacterially-infected epithelial cells through granzyme and perforin molecules (24, 25) and MAIT-derived granulysin and granzyme B may be effective against antibiotic resistant bacterial species (26).

Like iNKT cells, MAIT have a unique developmental pathway and their effector-memory phenotype is controlled by the master transcription factor PLZF (27). Several recent reviews have extensively discussed MAIT cell development, their antimicrobial role, and their contribution to cancer and inflammatory diseases (23, 28–30). Herein, we will discuss their role at the interface between innate and adaptive immunity and recent results describing an important contribution of MAIT cells to tissue homeostasis, with a view to potentially harnessing their immunomodulatory properties.

TIGHT REGULATION OF MAIT CELL ACTIVATION

Like other populations of lymphocytes straddling across innate and adaptive immunity, such as iNKT and γδ T cells (31, 32), MAIT cells are emerging as important modulators of immune responses. As MAIT cells are particularly abundant at mucosal surfaces, where antigen might also be available at higher concentrations, tight regulation of their activity is required to avoid immunopathology: for example, accumulation of activated MAIT cells has been reported in the inflamed mucosa in ulcerative colitis (33) and in the gastric mucosa during *Helicobacter pilori* infection (34). Tight regulation of MAIT cell activity is likely to occur through several mechanisms, from regulation of MR1 expression, to antigen availability, stability, and modulation of MAIT cell activation through cognate interactions.

MR1 Ligands and Their Importance in Modulating MAIT Cell Function

MR1 is ubiquitously expressed at the transcript level (7), although the protein is retained in the ER and surface expression is tightly regulated by antigen availability (35). The most potent natural MAIT cell agonists known to date are intermediates of the vitamin B2 biosynthetic pathway (14, 36), present in a number of bacteria, commensals and pathogenic (37, 38). Despite stabilizing MR1 molecules at the cell surface, folate derivatives are not recognized by the MAIT TCR (14, 36), although more in depth analysis with folate-loaded MR1 tetramers has identified small subsets of circulating $TRAV1.2^+$ and $TRAV1.2^-$ reactive T cells (3).

The structure-activity relationship of MR1 ligands has been well characterized and while MR1 surface upregulation correlates with the ability of the compounds to form a Schiff base with Lys43 of MR1, the agonist activity correlates with binding of the compound ribityl moiety to the TCR, via its Tyr95α residue (2, 39–41). Recently, a very elegant study with 20 altered metabolite ligands and 11 crystal structures of TCR-MR1-ligand ternary complexes has refined the molecular basis underpinning the potency and specificity of MAIT cell antigens, with the identification of an "interaction triad" between Tyr95α (in the MAIT TCR), Tyr152 (in the MR1 groove), and 5' and 2' OH groups in 5-OP-RU, which needs to be preserved for maximal agonist activity (42). These findings will be invaluable in future investigations exploring how to design ligands to better harness MAIT cell activity.

The full spectrum of MAIT cell ligands is still under appreciated, although two studies have reported agonist activity of drugs and drug like molecules (43) and of synthetic compounds identified *in silico* (44). The weak agonist activity of drugs like diclofenac and the antagonist activity of salicylates potentially underscores a much broader involvement of MAIT cells in several physio-pathological processes. Inhibitory ligands have the potential to be used to downregulate MAIT cell activation. Indeed, a synthetic derivative of the vitamin B9 metabolite 6-FP (i-6FP) has been used to inhibit MAIT cell activation and improve the course of the autoimmune disease lupus in $FcγRIIb^{-/-}$ mice, a spontaneous model of systemic lupus erythematosus in which MAIT cells have been shown to enhance autoantibody production and tissue inflammation (45).

While the majority of antagonists stabilize MR1 through a Schiff base but lack a moiety capable of interacting with the MAIT TCR, a novel mechanism of inhibition has recently been identified (44). Two synthetic non-microbial compounds, DB28 and its derivative NV-18, retain MR1 in the endoplasmic reticulum in an immature ligand-receptive form and compete with stimulatory ligands for MR1 binding. Neither DB28 nor NV18 form a Schiff base with MR1, but they are both sequestered in the A' MR1 pocket by a network of hydrophobic and polar contacts (44).

Antigen Stability

The potent MAIT cell antigens 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) derive from enzymatic and non-enzymatic condensation of 5-amino-6-(1-D-ribitylamino)uracil (5-A-RU) with glyoxals and methylglyoxals (host or bacteria derived). However, 5-OP-RU and 5-OE-RU are unstable and unless bound to MR1 via a Schiff base with Lys43 of the antigen presenting groove, they rapidly cyclize to less potent lumazines (36, 46). Furthermore, the biological activity of 5-A-RU is affected by long-term storage and spontaneous oxidation, unless prepared in dimethylsulfoxide solutions (46). To overcome the intrinsic instability of 5-A-RU, Lange et al. synthesized a pro-drug modifying the 5′ aminogroup with a cleavable valine-citrulline-p-aminobenzyl carbamate (47). The prodrug is stable and is cleaved intracellularly by cathepsin B, leading to preferential loading in the recycling endosomes.

Antigen Availability

Antigen availability influences MAIT cell population expansion throughout life. 5-OP-RU MR1-tetramer binding cells are few at birth, but they rapidly increase within the first year of life, accounting for the majority of $V\alpha7.2^{+}$ $CD161^{++}$ cells in the circulation (48). Germ free mice lack MAIT cells (6, 49–51) and mono-colonization with riboflavin producing bacteria, or exposure to synthetic 5-OP-RU, is sufficient to rescue MAIT cell development (51). These results, although surprising in view of the instability of 5-OP-RU, underscore the high sensitivity of TCRs in detecting cognate antigens bound to the relevant antigen presenting molecule.

At steady state, microbial diversity and density increases from the upper to the lower gastrointestinal tract (52) and mucosal conditions affect the relative abundance of MAIT-stimulatory metabolites, thus influencing MAIT cell activation (37). It was shown that *E. coli* bacteria grown in anaerobic conditions, stationary phase, and in medium supplemented with glucose, xylose, ribose, or glycerol stimulated more potently MAIT cell activation (37). These growth conditions correlated with increased accumulation of stimulatory MAIT cell ligands, detected by mass spectrometry (37). Furthermore, location of bacteria in the luminal space vs. areas adjacent to the epithelium (such as for *bacteroides* spp., *proteobacteria*) is also likely to influence antigen availability (37). Finally, the relative expression of individual enzymes in the vitamin B2 biosynthetic pathway influences the balance of MAIT cell-activating and inhibitory metabolites, as shown for *Salmonella typhimurium* and *Streptococcus pneumoniae* isolates (53–55).

Direct and Indirect MAIT Cell Activation

When the phenotype of MAIT cells from paired mucosal and blood samples has been analyzed, important differences have been highlighted. Colon resident MAIT cells are more activated (higher expression of CD137, CD69, HLA-DR, and CD25), but they also express higher levels of inhibitory receptors, such as TIGIT, CTLA-4, PD1, and LAG3 (37). This phenotype might reflect continuous exposure to metabolites derived from commensals and/or pathogenic bacteria and the expression of inhibitory receptors may balance this exposure. During bacterial infections, in addition to MR1-antigen complexes, MAIT cells are exposed to a variety of inflammatory cytokines that co-stimulate and enhance their activation, potentially overcoming inhibitory signals (**Figure 1**). The relative importance of TCR-driven vs. cytokine driven MAIT cell stimulation also changes during the course of an infection, with the former dominating at earlier stages of the response (56). Unlike conventional memory T cells, *in vitro*, MAIT cells are poorly responsive to anti CD3/anti CD28 stimulation, but their responsiveness is greatly enhanced by IL-12 and IL-18 (**Figure 1A**) (57). Freshly isolated blood MAIT cells and MAIT cell lines are potently activated by synthetic 5-OP-RU presented by myeloid cells, and in this setting their activation is mostly MR1-dependent (58). Yet, a high fraction of cells undergoes activation induced cell death, and perhaps cytokine dependent signals, including IL-12, IL-18, and IL-15, increase MAIT cell viability through changes in expression of pro and anti-apoptotic proteins, such as Bcl2 and Bax1 (59, 60).

The high expression of IL-12 and IL-18 receptors by MAIT cells [as well as by iNKT cells (61, 62)] facilitates their activation in a TCR-independent manner, during viral infections [reviewed in (63)]. In addition, cytokine stimulation is important during infections with *Group A streptococcus* bacteria, lacking the vitamin B2 biosynthetic pathway (64). In this paper the authors showed that MAIT cell activation occurs in response to streptococcal exotoxins of the superantigen family, recognized by the Vβ2 TCR chain, but independently of MR1 (64). Similarly, MAIT cell responsiveness to *Staphylococcus enterotoxin B* (SEB) superantigen occurs in a TCR Vβ13.2 dependent manner but is MR1-independent and is largely contributed by IL-12 and IL-18 (65).

The importance of cytokines signaling for achieving full MAIT effector function is also underscored by the observation that chronic stimulation by type I IFN (such as in HIV infected patients) results in impaired MAIT cell responses to bacteria, via IL-10-dependent suppression of IL-12 secretion by APCs (66). However, in other settings, type IFNs synergize with other signals to enhance MAIT cell activation, consistent with the notion that type I IFNs are an important third signal that shapes the differentiation of memory conventional T cells (67). Accordingly, in HCV infected patients treated with type I IFN and antivirals it was reported that MAIT cells express higher CD69, indicative of activation (17), and a synergistic activity of type I IFN and IL-15 was also demonstrated. Furthermore, type I IFN has also been shown to co-stimulate TCR dependent MAIT cell activation

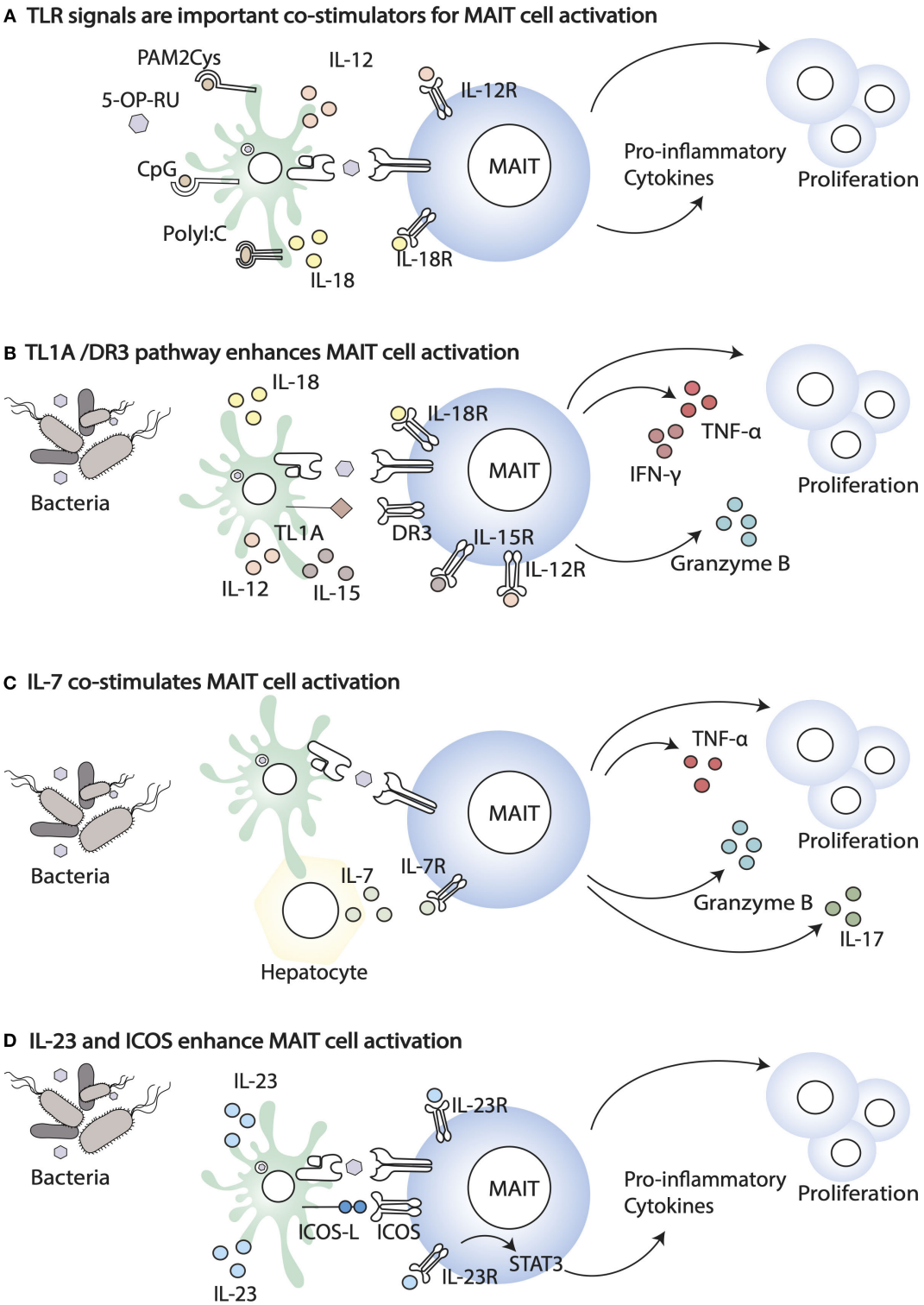

FIGURE 1 | Co-stimulatory signals that enhance MAIT cell activation. **(A)** TLR agonists, including PAM2Cys, CpG, and Poly I:C, enhance MAIT cell response in the presence of the ligand. **(B)** Co-stimulation through the TL1A-DR3 pathway results in enhanced MAIT cell proliferation and the release of Granzyme B, IFN- γ, and TNF- α. **(C)** Infections trigger IL-7 release by different cells, including hepatocytes. IL-7 co-stimulates MAIT cells enabling cell proliferation and the release of IL-17, Granzyme B, and TNF- α. **(D)** Bacterial infection induces the expression of ICOS ligand (ICOSL) and ICOS (ICOS) on APC and MAIT, respectively. The increased expression of ICOS and IL23R signalling in MAIT cells enhance secretion of pro-inflammatory cytokines.

(68) in addition to the TCR independent activation discussed above (17).

Other inflammatory cytokines that have been shown to enhance MAIT cell responsiveness to antigen include the gut-associated TNF superfamily member TL1A (via death receptor 3, DR3 (**Figure 1B**) (69, 70) and IL-7 (**Figure 1C**). IL-7 is essential for proliferation of liver-derived MAIT cells, which like the colonic MAIT have a semi-activated state yet they have higher expression of negative regulators (SOCS1 and SOCS3), in addition to lower expression of TCR signaling components, which may be an adaptation to constant antigen exposure (71). Furthermore, in HIV patients, IL-7 has been shown to rescue the defective MAIT cell cytolytic capacity and cytokine secretion, both *in vitro* and *in vivo*, in patients on anti-retroviral therapy (72, 73).

In vivo, population expansion of MAIT cells following infection with *S. typhimurium* depends on riboflavin metabolites and microbial signals (74). Indeed, synthetic 5-OP-RU injection intranasally or intravenously is not sufficient to increase MAIT cell frequency and numbers in the lung and draining lymph nodes, unless it is accompanied by TLR-derived signals (Pam2Cys, polyI:C, or CpG) (**Figure 1D**). Likewise, infection with riboflavin-deficient *S. typhimurium* mutants does not lead to MAIT cells population expansion, unless complemented with synthetic 5-OP-RU (74). However, more recently, intraperitoneal injection (51) or skin application of 5-OP-RU (50) was shown to be sufficient to activate and expand MAIT cells, locally and systemically. Whether these discrepancies are due to differences in the microbial flora of the animal colonies of the different investigators, to different sensitivity of lung, skin vs. thymic MAIT cells, or to different antigen preparations and doses of antigens reaching the sites, it remains to be determined.

Nevertheless, for sustained expansion and cytokine secretion, some form of co-stimulation of MAIT cells seems to be required. Following cutaneous application of *S. epidermidis*, population expansion of MAIT cells is reduced in the absence of IL-18, but not IL-23, while IL-1 signaling is required for licensing of IL-17A production by MAIT cells (50). Using a mouse model of conditional MR1 targeting, the authors also demonstrated that MAIT cell population expansion following *S. epidermidis* application is MR1 dependent, although homeostatic maintenance of MAIT cells is not, as their frequency is unchanged 3 weeks post deletion of MR1.

Specific costimulatory requirements have been identified for population expansion of RORγt$^+$ MAIT cells in response to intranasal *Salmonella typhimurium* or *Legionella longbeachae* (75). In this paper, through mixed bone marrow chimeras the authors showed that APCs-bone marrow derived and epithelial-are important in MR1-dependent antigen presentation. In addition, they demonstrated a role for ICOS-mediated co-stimulation and IL-23/IL-23R signaling in MAIT cell expansion and activation (**Figure 1D**). Both ICOS-deficiency and IL-23 deficiency impaired expansion mainly of the RORγt MAIT subset, suggesting they are required for maintenance of RORγt expression and IL-17 secretion. IL-23 was also shown to be sufficient on its own to co-stimulate MAIT cell proliferation, in the presence of 5-OP-RU and to up-regulate ICOS expression, although IL-23 and ICOS double deficient mice were not tested, nor was IL-23 measured in ICOS deficient mice (75). These results are consistent with the high expression of both ICOS and IL-23R in MAIT cells, compared to conventional T cells in naïve mice. In this context IL-23 signaling is important, presumably via STAT3, to maintain the Th17 signature of tissue resident MAIT cells. As homeostatic IL-23 contributes to MAIT cell development/accumulation in the presence of a normal microbial flora (50), these results also are consistent with the observation that patients with STAT3 loss of function mutations have, amongst others, a deficiency of MAIT cells (76).

A TCR-DEPENDENT TISSUE REPAIR FUNCTION OF MAIT CELLS

Overall, the above results are consistent with the hypothesis that due to ubiquitous MR1 expression and abundance of MAIT cells in tissues, their function needs to be tightly regulated, and for maximal effector function (i.e., during an infection) a combination of TCR, cytokine, and co-stimulation signals is required. In the absence of inflammatory signals, only minimal cell activation might be elicited. To understand the extent of MAIT cell functional plasticity and changes in their functional program in the course of an infection, several groups have analyzed their transcriptional signatures, in bulk and at the single cell level. At steady state, in both humans and mice, distinct transcriptional patterns have been identified, according to tissue specificity: despite different frequencies in different organs, MAIT/iNKT1 subsets, and MAIT/iNKT17 subsets share transcriptional signatures that are developmentally imprinted, including their tissue residency (77). Transcriptional changes occur at each developmental stage in the thymus and are underpinned by expression of the key transcription factors PLZF, T-bet and RORγt (27, 78), imprinting specific tissue residence signatures and Th1 or Th17 bias.

Recently, three groups compared the transcriptional signature of human and murine MAIT cells activated by bacterial infection (*Legionella longbeachae*), cytokines, and/or TCR stimulation (60, 69, 79). Despite differences in experimental models, common signatures were observed by the three groups. As expected from previous analysis at the protein level, upon activation, both human and murine MAIT cells expressed genes encoding for proinflammatory cytokines (such as GM-CSF, IL-17, INF-γ) and chemokines (such as XCL1, CCL3, CCL4, CXCL16) (60, 69). Human MAIT activated by anti-CD3/CD28 displayed a signature intermediate between unstimulated MAIT cells and those activated by cytokines and anti-CD3/28 (69). In turn, the transcriptional signature of human MAIT activated by cytokines and anti-CD3/CD28 (69) resembled that of 5-OP-RU activated human MAIT cells (60). Some species-specific differences were observed, such as higher expression of LIGHT and IL-2 in human MAIT cells, and higher TRANCE and RANKL in murine cells, but these could also be explained by different activation modes (60). Furthermore, and in line with their innate-like function, it was reported that the transcriptome of TCR-activated murine MAIT cells resembled that of iNKT cells, while upon

resolution of infection it was more similar to γδ T cells (60). Interestingly, γδ T cells are a population of unconventional T cells that crucially contribute to tissue integrity and repair (80). Accordingly, an interesting finding common across these studies was the identification of a tissue repair signature upon TCR-dependent activation of MAIT cells. This signature was previously identified in H2M3 restricted CD8 Tc17 cells upon exposure to the commensal *S. epidermidis* (81) and was also seen in skin MAIT cells upon *S. epidermidis* topical application (50). Key genes in this signature are involved in tissue repair and remodeling (*MMP25, FURIN, PDGFB, TGFB1*) and angiogenesis (*CSF2, VEGFB, PDGFB*). Other MAIT cell-derived factors with potential role of tissue homeostasis were IL-26, OSM, and HBEGF (69, 79). Hence an important function of tissue resident MAIT cells could be maintenance of tissue homeostasis in the presence of commensals, limiting inflammation, and associated tissue injury. In agreement with these findings, MAIT cells in the female genital tract, in the oral mucosa, and in fetal mucosal tissues show a bias toward IL-22 and IL-17 secretion, and a potential role in barrier immunity and tissue homeostasis (20, 82, 83). This hypothesis is also consistent with *in vitro* results demonstrating that supernatants of activated MAIT cells promote closure of scratches of monolayers of Caco2 cells (69) and *in vivo* results showing that MR1 deficient mice have reduced re-epithelization of skin wounds (50). In addition, the MAIT tissue repair function could account for increased gut permeability in $MR1^{-/-}$ NOD mice, compared with MR1 sufficient littermates (84). Finally, alteration of gut permeability and MAIT cell numbers following conditioning regimens and allogeneic bone marrow transplantation could contribute to intestinal symptoms of Graft versus host disease (GvHD) (85, 86). In the future it will be interesting to investigate whether there is an alteration in the homeostatic tissue repair function of MAIT cells in chronic fibrotic diseases, in which a pathogenic MAIT cell role has been suggested (87–89).

MAIT CELLS: THE NEW iNKT/γδ T CELLS?

Common features across the three major populations of innate-like lymphocytes—iNKT, γδ, and MAIT cells—are their peculiar thymic differentiation program, after which the cells emerge as pre-set memory/effectors, poised to a rapid response upon antigen encounter (31, 32, 90, 91). Through secretion of a variety of cytokines and chemokines, iNKT cells and γδ T cells regulate the function of several immune cell subsets and have emerged as central players in immunobiology and immunopathology, bridging innate and adaptive responses (**Figure 2**). We will discuss the existing evidence in favor of the immunomodulatory activity of MAIT cells.

Interactions With Myeloid Cells

MAIT cells can modulate myeloid cell function directly, following MR1-cognate interactions, or indirectly, through soluble factors. During *in vivo* pulmonary infection with *Francisella tularensis* live vaccine stain (LVS), it has been shown that MAIT cells produce critical antimicrobial cytokines (IFN-γ, IL-17A, TNF-α) and in $MR1^{-/-}$ mice there is a higher bacterial burden and delayed bacterial clearance (92). In addition, through early GM-CSF production, pulmonary MAIT cells promote the differentiation of $CCR2^{+}$ inflammatory monocytes into dendritic cells (DC) (93). As DC are critical for priming adaptive immunity, recruitment of activated $TCR\beta^{+}$ $CD4^{+}$ and $CD8^{+}$ cells is significantly delayed in MR1 deficient mice (92), but it can be rescued by adoptive transfer of *in vivo* differentiated DC (93). In this experimental system, MAIT cells influence monocyte differentiation into DC in a GM-CSF dependent but MR1 independent way (93). In other models, however, MAIT cells can modulate DC function in an MR1-dependent manner. Indeed, it has been shown that primary human MAIT cells and MAIT cell lines induce maturation and activation of monocyte-derived DC and primary DC upon MR1-dependent recognition of 5-OP-RU complexes (58). DC maturation is dependent on CD40-CD40L signaling and results in secretion of bioactive IL-12, which can further modulate NK cell activation (58). Interestingly, murine MAIT cells can also upregulate CD40L upon activation (75), although it remains to be determined whether they are able to induce antigen specific adaptive T cell responses through CD40L dependent DC maturation, as is the case for iNKT cells (94, 95).

Additionally, MAIT cells have been shown to exert a protective function in non-alcoholic fatty liver disease, inducing M2-macrophages polarization through IL-4 secretion (96).

Interactions With B Cells

MAIT cells final differentiation and peripheral expansion depends on cognate interactions with B cells in mice, although this does not seem to be critical in humans (6, 97). MAIT can be directly activated by bacterial infected B cells with up-regulation of CD69, and secretion of IFN-γ, TNF-α, and IL-17 (98). The observation that in *Vibrio cholera* infection (99) and *Shigella dysenteriae* vaccination (24) the circulating frequency of MAIT cells correlates with the pathogen-specific antibody response suggests some form of helper activity from MAIT cells. In a murine model of lupus, MAIT cell activity correlates with autoantibodies, germinal center reaction, and severity of disease (45). In this paper, the authors also reported that, *in vitro*, MAIT cells enhanced IgG production by LPS-stimulated B cells through CD40-CD40L cognate interactions (45).

In vitro, MAIT cell supernatants have been shown to induce plasmablast differentiation and antibody secretion from memory B cells (100). In this system, help for B cells is provided in an MR1-dependent, but CD40L independent manner, likely via cytokines like IL-6, IL-10, and IL-21 (100). However, the authors only blocked soluble CD40L and did not address whether during cognate interaction between B cells and MAIT cells the CD40/CD40L axis has a role for B cell differentiation. Furthermore, as the CD161 ligand LLT1 has been shown to play a role in the germinal center reaction (101), there remains the possibility that $CD161^{++}$ MAIT cells, in addition to the more abundant $CD161^{+}$ follicular DC subset, might contribute to B cell differentiation through cognate interactions. Lastly, it is not yet established whether a T-follicular helper subset of MAIT cells exists, akin to $Bcl\text{-}6^{+}$ iNKT cells providing cognate B cell help in the lymph node (102–104).

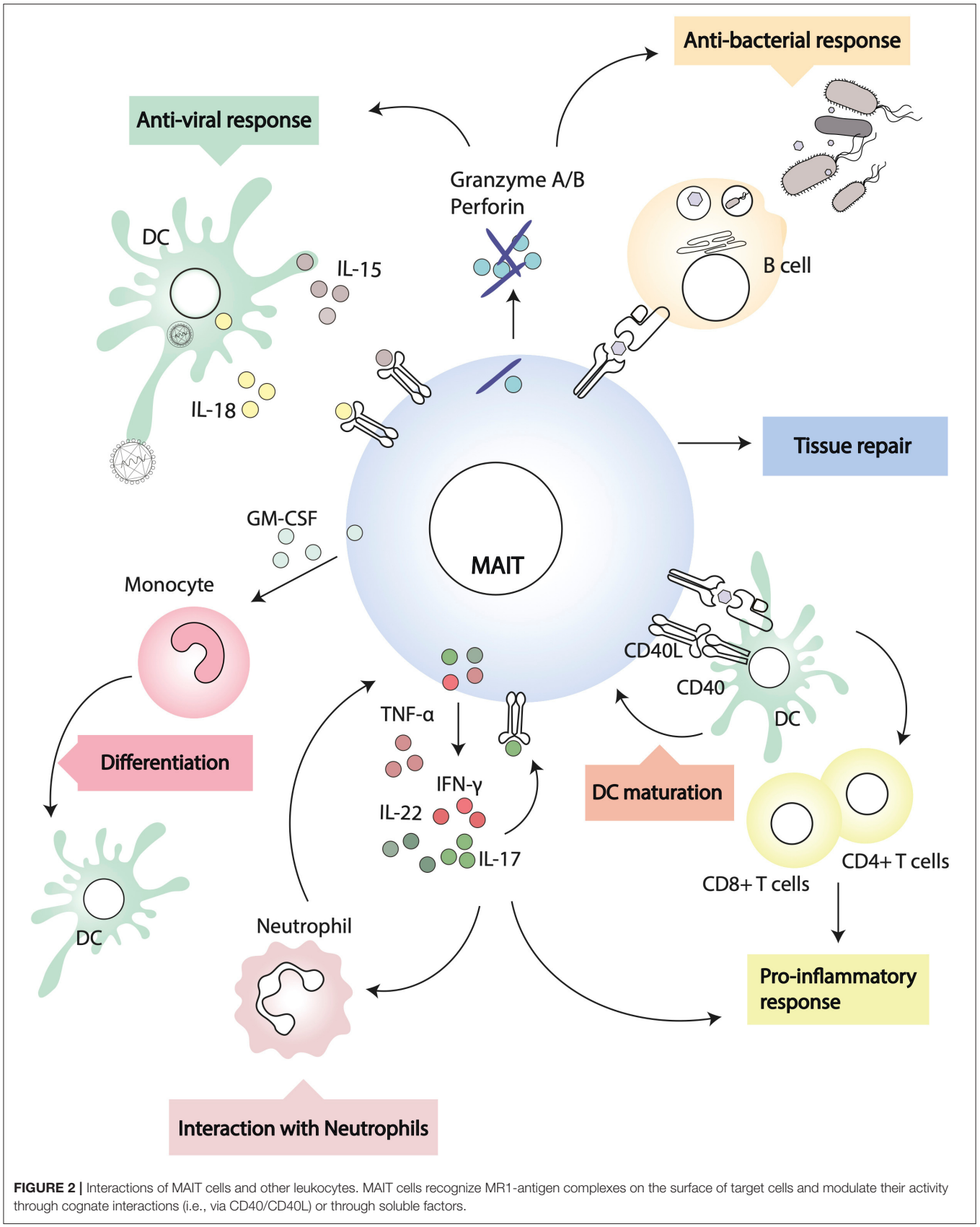

FIGURE 2 | Interactions of MAIT cells and other leukocytes. MAIT cells recognize MR1-antigen complexes on the surface of target cells and modulate their activity through cognate interactions (i.e., via CD40/CD40L) or through soluble factors.

Interactions With Neutrophils

In the presence of monocytes, MAIT (and γδ) T cells rapidly respond to bacterial infected neutrophils, and through secretion of GM-CSF, IFN-γ, and TNF-α they increase neutrophil survival and promote their differentiation into antigen presenting cells, expressing CD64, CD83, HLADR, CD54, CD40, and HLA A, B, C (105). In addition, in this manuscript, MAIT and γδ T cells activated neutrophils acquired the ability to uptake exogenous antigens, and cross-present antigenic peptides to $CD8^+$ T cell clones (105). The ability of MAIT cells to modulate neutrophils function could play an important role in sepsis patients (106), where the number of circulating MAIT cells might affect the outcome of infection, particularly in the aging population with declining numbers of MAIT cells (107). Furthermore, as MAIT cells can become anergic upon recognition of microbial superantigens that often are associated with sepsis (65), and are then impaired in their ability to respond to bacterial infected cells, licensing of activated neutrophils to present antigens to $CD4^+$ and $CD8^+$ T cells may become crucial for the establishment of protective immunity (105).

Contradicting the above results, a recent study found that neutrophils inhibit MAIT cell activation through cell contact and hydrogen peroxide and that MAIT cells-derived TNF-α induces neutrophil death (108). The discrepancies might be related to different experimental settings, for example, one study isolated neutrophils by dextran sedimentation followed by Ficoll-Plaque centrifugation and hypotonic lysis of remaining red blood cells, while the other study purified them from whole blood or Lymphoprep separated granulocytes by HetaSep sedimentation and negative selection with the EasySep neutrophil enrichment kit; one study activated MAIT cells with 5-OP-RU, while the other with anti-CD3/28 beads, hence the amount of cytokines in the supernatants would be different. Therefore, further research is required to understand the exact outcome of MAIT-neutrophils interactions.

It also remains to be determined whether, like iNKT cells, MAIT cells are able to modulate the suppressive function of granulocytic myeloid derived suppressor cells, which could be of relevance to relive cancer immunosuppression and amenable to clinical harnessing (109, 110).

CONCLUDING REMARKS

Because of their high numbers in humans and of the restriction by a monomorphic molecule, MAIT cells represent an interesting population to target to enhance antigen specific immunity, through their multiple interactions with cells of the innate immune system. Despite great progress since the first discovery of T cell populations bearing semi-invariant TCRs, several aspects of MAIT cell biology remain to be fully unraveled before harnessing MAIT cells can be taken forward into the clinic. We need to better understand the role of MAIT cells in several diseases in which their numbers or functionality is altered, such as cancers and autoimmune diseases. The molecular mechanisms of ligand antigen presentation are not completely defined and a characterization of endogenous antigens, if they exist, is still lacking. Known ligands occupy the MR1 A' pocket and it remains to be determined if ligands or chaperones will bind the F' pocket. Pathogen evasion mechanisms from MAIT immune-surveillance have been identified (55, 111), but further research in this field is also needed. Lastly, the recently described MAIT tissue-repair function needs to be molecularly defined as well as the interplay with the host microbiota, which is key in homeostasis.

AUTHOR CONTRIBUTIONS

MI wrote the first draft. MS edited and extended it. The paper is dedicated to the memory of VC. All authors contributed to the article and approved the submitted version.

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REVIEW
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Understanding the Role of Mucosal-Associated Invariant T-Cells in Non-human Primate Models of HIV Infection

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Chronic HIV infection causes systemic immune activation and dysregulation, resulting in the impairment of most T-cell subsets including MAIT cells. Multiple human cohort studies demonstrate MAIT cells are selectively depleted in the peripheral blood and lymphoid tissues during HIV infection, with incomplete restoration during suppressive antiretroviral therapy. Because MAIT cells play an important role in mucosal defense against a wide array of pathogens, fully reconstituting the MAIT cell compartment in ART-treated populations could improve immunity against co-infections. Non-human primates (NHPs) are a valuable, well-described animal model for HIV infection in humans. NHPs also maintain MAIT cell frequencies more comparable to humans, compared to other common animal models, and provide a unique opportunity to study MAIT cells in the circulation and mucosal tissues in a longitudinal manner. Only recently, however, have NHP MAIT cells been thoroughly characterized using macaque-specific MR1 tetramer reagents. Here we review the similarities and differences between MAIT cells in humans and NHPs as well as the impact of SIV/SHIV infection on MAIT cells and the potential implications for future research.

Keywords: MAIT cells, MR1, non-human primate, comparative immunology, HIV/SIV infection, HIV/TB co-infection

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INTRODUCTION

Unconventional T-cells, including mucosal-associated invariant T-cells (MAIT cells), have emerged as important immune mediators in both infectious and inflammatory diseases [Reviewed in Godfrey et al. (1)]. While their role in cancer development and progression has not been fully elucidated, MAIT cells and specific populations of major histocompatibility complex class I-related (MR1)-restricted T-cells have been shown to infiltrate the tumor microenvironment and are able to lyse cancer cells *in vitro* and *in vivo*, indicating a potential role in cancer immunotherapy (2); Reviewed in Lukasik et al. (3). In contrast to conventional T-cells which recognize antigens through MHC-mediated antigen presentation, MAIT cells recognize antigens by MR1-mediated presentation of vitamin B metabolites predominately through a Vα7.2 semi-invariant T-cell receptor (TCR) (4, 5). MAIT cells are also capable of being activated via a cytokine-mediated,

antigen independent pathway, indicating they have properties of both innate and adaptive immune cells (6). These features of MAIT cells highlight their importance in rapid immune responses to a wide array of pathogens.

While the ability of MAIT cells to mount an immune response against bacterial pathogens is well established, their role in viral infection is not as clear. Viral replication cycles do not involve vitamin B synthesis pathways, which are necessary for TCR-dependent MAIT cell activation. Multiple studies have shown increased MAIT cell activation in patients with active viral infections, including dengue (7, 8), influenza (7), chronic hepatitis B (9), hepatitis C (7), and Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) (10). Similar findings have also been made in mice following influenza infection (11), as well as *in vitro* with hepatitis C (7), influenza (12), and zika virus (8). Virus-induced MAIT cell activation is mediated through TCR-independent pathways, as shown for influenza (7, 12), dengue (7), hepatitis C (7), or zika virus (8) exposure *in vitro*. This stands in contrast to MAIT cell stimulation with paraformaldehyde fixed *E. coli*, which is blocked by treatment with an anti-MR1 blocking antibody. Beyond MAIT cell activation, data on MAIT cell contributions to viral immunity is limited. Supernatant from activated MAIT cells suppresses hepatitis C virus replication in a dose-dependent manner (7). $MR1^{-/-}$ mice, which are deficient in MAIT cells, have increased morbidity and mortality following influenza challenge; compared to wild type controls (11). Increased morbidity and mortality is ameliorated with adoptive transfer of murine pulmonary MAIT cells prior to viral challenge. Adoptive transfer of pulmonary MAIT cells also reduced morbidity and mortality in $Rag^{-/-}$ IL-$2R\gamma^{-/-}$ mice following influenza challenge (11). Taken together, these studies indicate that MAIT cells are capable of suppressing viral replication and providing clinical improvements during viral infections against certain viruses. Additional studies are needed to better characterize the impact MAIT cells have, both beneficial and deleterious, on viral infections, as well as the breadth of viral infections that MAIT cells are able to mount an immune response against.

A better understanding of MAIT cell function will require continued evaluation of human MAIT cell dynamics during various disease states, along with *in vivo* models that best represent the relevant pathologic processes. The MR1 gene, the primary receptor for MAIT activation through antigen presentation, is highly conserved among mammalian species, but is absent in non-mammalian species (13). Additionally, there have been 3 separate losses of functional MR1 among mammals, including in Lagamorpha (rabbits), and in Carnivora (dogs, cats, and ferrets) (14). Mice carry a functional MR1 gene but have a relatively low abundance of MAIT cells in the peripheral blood (median: 0.1%) necessitating the generation of transgenic mice expressing an invariant mVα19-Jα33 TCRα to increase MAIT cell frequencies (15, 16), or the boosting of tissue MAIT cell frequencies by administration of antigen and TLR agonists (17). In contrast, non-human primates (NHPs) express a functional MR1 gene and maintain MAIT cells at frequencies more comparable to humans, providing a superior *in vivo* model to study MAIT cell immunological dynamics. Herein, we discuss the current state of MAIT cell characterization in NHPs [which has focused on rhesus macaques (RM), pigtail macaques (PTM), and Mauritian cynomolgus macaques (MCM)] and the changes in MAIT cell populations that occur during simian immunodeficiency virus (SIV) and simian-human immunodeficiency virus (SHIV) infection, which are the critical animal models for HIV infection.

PHENOTYPE OF NON-HUMAN PRIMATE MAIT CELLS

Human MAIT cells were originally identified as $V\alpha7.2^+$ $CD161^+$ cells among the bulk T-cell population [Reviewed in Garner et al. (18)]. Recently, the development of antigen loaded MR1 tetramers has allowed for a more refined identification of MAIT cells by flow cytometry (19, 20). Similar approaches have been utilized to phenotype macaque MAIT cells, via identification of $V\alpha7.2^+$ and/or MR1-5-OP-RU^+ T-cells (21–29). One important consideration for determining tetramer reactivity in macaque MAIT cells is the utilization of species specific MR1 tetramers. Two studies have identified incomplete cross reactivity of human MR1 tetramers with macaque MAIT cells (23, 25). Identification of these cells is improved with the use of macaque specific MR1 tetramers. Furthermore, the inclusion or exclusion of Vα7.2 expression in the definition of a MAIT cell should be carefully considered. There is growing evidence of a unique $V\alpha7.2^-$MR1 $tetramer^+$ T-cell population in humans (30, 31), which has also been identified in the peripheral blood of PTMs, RMs, and MCMs (23, 25, 28). Additional work is needed to characterize these cells and to compare their phenotypic and functional properties to their human counterparts.

Human MAIT cells are predominately $CD8\alpha\alpha^+$ or $CD4^-CD8^-$, with a minor population of CD4-expressing cells (19). In contrast, NHP MAIT cells are almost uniformly $CD8\alpha^+$, with 3 studies noting an absence of $CD4^-CD8^-$ MAIT cells in NHP (23, 25, 28). One additional study identified peripheral blood MAIT cells as predominately $CD8\alpha^+$ (36.3%) or $CD8^-CD4^-$ (44.9%) in RMs, with minor populations of $CD8^+CD4^+$ (2.9%) and $CD4^+$ (15.8%) MAIT cells (29). MAIT cells were identified based on reactivity to NHP-specific MR1 tetramers without concurrent expression of Vα7.2, which may partially explain the presence of $CD8^-CD4^-$, $CD8^+CD4^+$, $CD4^+$ MAIT cells that were not observed in other studies. It is presently unknown if NHP $CD8\alpha^+$ MAIT cells express a homodimeric ($CD8\alpha\alpha^+$) or heterodimeric ($CD8\alpha\beta^+$) receptor. The cause for this absence of $CD4^-CD8^-$ MAIT cells in the majority of NHP studies is unknown, and additional studies are needed to characterize this variation from human peripheral MAIT cells. Human $CD8^+$ and $CD4^-CD8^-$ MAIT cells have been shown to have distinct phenotypic and functional profiles (32). $CD8^+$ MAIT cells express higher levels of cytotoxic and coactivating markers compared to $CD4^-CD8^-$ MAIT cells, and produce higher levels of IFNγ and TNFα following stimulation. $CD4^-CD8^-$ MAIT cell can be derived *in vitro* from $CD8^+$ MAIT cells following TCR-dependent activation. Potential causes for the relative paucity of $CD8^+$ MAIT cells in captive NHPs

include species-specific variation in MAIT cell development or differentiation between humans and NHPs, or environmental factors related to husbandry practices which drives the altered frequencies in NHP peripheral $CD8^+$ and $CD4^-CD8^-$ MAIT cells. A lack of $CD4^-CD8^-$ MAIT cells in NHPs may also impact the immune response to certain disease states, and should be considered when utilizing NHPs as a model for humans. While NHPs predominately lack $CD4^-CD8^-$ MAIT cells, we caution against pre-gating on $CD8^+$ T cells prior to identifying MR1 $tetramer^+$ cells in NHPs, as this could hinder observing shifts in MAIT cell CD4 and CD8 co-receptor usage during disease states or experimental interventions.

CD161, a C-type lectin like-receptor, is almost ubiquitously expressed by human MAIT cells in the peripheral blood and has classically been used as a co-staining marker to gate on $V\alpha7.2^+$ or MR1 $tetramer^+$ cells (15, 33, 34)This marker is associated with innate-like, TCR-independent responses to IL-12 + IL-18 stimulation (35, 36). Three studies have utilized CD161 expression to identify MAIT cells in NHPs (22, 26, 27). However, two studies have noted poor cross-reactivity of anti-human CD161 antibodies against RM and PTM CD161 antigen (23, 25). Additionally, CD161 downregulation has been identified in MAIT cells from chronic HIV infected individuals (33, 37, 38). As a result, we strongly caution against the use of CD161 to define the total MAIT cell population in NHPs; rather, MR1 tetramer in combination with TCR Vα7.2 can distinguish major MR1-restricted T-cell populations, which can then be phenotyped for additional surface markers. PLZF (ZBTB16) is most consistently expressed on MAIT cells in NHPs (21, 23–25, 27, 28), as well as in humans (34, 39), and is a marker to consider when identifying the bulk MAIT cell population. Additional markers that are expressed on a relatively high frequency of MAIT cells and may be considered for MAIT cell identification are CCR6 and IL-18Rα, which have been shown to be expressed on MAIT cells from multiple NHP species (21, 25, 27, 28).

DISTRIBUTION OF MUCOSAL-ASSOCIATED INVARIANT T-CELLS IN NON-HUMAN PRIMATES

MAIT cells constitute approximately 2.6% (range: 0.1–9.2%) of the total T-cell population in human peripheral blood (**Table 1**) (40). By comparison, MAIT cell frequencies in the peripheral blood range from 0.3 to 4.8% in RMs (21, 27), 0.026–1.28% in PTM (25), and 0.6–17% of $CD3^+CD8^+$ T-cells in MCMs (28). In general, NHPs maintain peripheral MAIT cell frequencies at 1–2% of the bulk CD3 T-cell population. While MAIT cell frequencies are lower than what is observed in humans, it is substantially higher than baseline MAIT cell frequencies in mice (16), and is sufficient to evaluate changes in peripheral MAIT cell frequencies and phenotype with different experimental interventions.

Similar to MAIT cell frequencies in the blood, NHPs MAIT cell frequencies in tissues tend to be lower compared to the frequencies in humans, but higher compared to murine MAIT cell frequencies. Both humans and NHPs maintain relatively higher MAIT cells frequencies in the liver and mucosal tissue sites (e.g., Gastrointestinal tract, lung), compared to their respective peripheral blood frequencies. MAIT cell frequencies in the human liver vary from 20 to 50% of the bulk $CD3^+$ T-cell population [reviewed in Kurioka et al. (41)], and are significantly enriched in the liver of PTM (approximately 5% of $CD3^+$ T-cell population) compared to paired PBMCs (approximately 1% of $CD3^+$ T-cell population) (25). MAIT cells comprise a range of ∼0.5–9.8% of the bulk $CD3^+$ T-cell population in the liver of RMs (21, 23, 26, 27). Within the gastrointestinal tract, MAIT cell frequencies in humans vary from 1.5% of the bulk T-cell population to approximately 60% of the $CD3^+CD8^+$ population, with the highest concentration of MAIT cells occurring in the jejunum [(19), reviewed in Kurioka et al. (41)]. MAIT cell frequencies range from 0.02 to 0.03% of $CD3^+$ T-cells in the rectal mucosa of PTM (25). However, there was no significant MAIT cell enrichment in the rectal mucosa compared to paired PBMC's (0.15–1.31% of $CD3^+$ T-cell population). This was attributed to a relatively low expression of the mucosal homing marker α4β7 in circulating MAIT cells in PTM. MAIT cells comprised ∼1–3% of the bulk $CD3^+$T-cell population in the gastrointestinal tract of RMs (23, 26, 27) with one study noting the highest frequency occurring in the colon compared to the ileum and jejunum (23).

MAIT cells are relatively abundant in the human lung, accounting for approximately 2–4% of the bulk T-cell population, with the most dramatic enrichment occurring in the trachea, and the proximal and distal bronchi [(42), reviewed in Kurioka et al. (41)]. By comparison, MAIT cells account for approximately 1.1% of the bulk T-cell population, or 16% (range: 9–28%) of the bulk $CD3^+CD8^+$ T-cell population, in the lungs of RMs (22, 27). Additionally, MAIT cell frequencies are highly variable in RM bronchoalveolar lavage fluid (BAL), comprising 0.5–14.7% of the bulk $CD3^+$ T-cell population (21, 23, 24, 26), or approximately 1–17% of the bulk $CD3^+CD8^+$ T-cell population (29). Similar findings have also been made in MCMs, with MAIT cell frequencies ranging from approximately 12–40% of the bulk $CD3^+CD8^+$ T-cell population in the BAL (28).

TABLE 1 | Comparison of MAIT cell tissue frequencies between humans, non-human primates, and mice.

	Human (% of $CD3^+$); Mean (range)	Non-human primate (% of $CD3^+$); Mean (range)	Mouse (% of $CD3^+$); Mean (range)
Blood	2.6 (0.1–9.2)	1–2 (0.026–6.5)	0.1
	(40)	(21, 25, 27–29)	(16)
Liver	20–50	4–7 (0.5–17)	0.60
	(41)	(21, 23, 25–27)	(16)
Gastrointestinal tract	1.5–10	0.02–3	0.70
	(41)	(21, 23, 25–27)	(16)
Lung	2–4	1.10	3.30
	(41)	(27)	(16)
Lymph node	1	0.03–0.2	0.20
	(38, 45)	(21, 25)	(16)

MAIT cells are consistently maintained at low frequencies in secondary lymphoid organs (lymph nodes and spleen) compared to the peripheral blood, in both humans and NHPs. This is attributed to the relative lack of CCR7 and CD62L expression, both required for lymphoid tissue homing, on peripheral MAIT cells [reviewed in Kurioka et al. (41)]. Additionally, it has been postulated that MAIT cells in the lymph represent a distinct population that recirculate from the tissues, separate from circulating MAIT cells in the blood (20). While similar patterns of tissue recirculation in NHPs still need to be investigated, differential distribution of MAIT cell phenotypes between lymphoid and extralymphoid tissues has been described in RMs. MAIT cells within secondary lymphoid tissues are predominately $CCR7^{+}CD28^{+}$ (central memory), while MAIT cells in extralymphoid tissues are predominately $CCR7^{-}CD28^{+}$ (transitional memory) or $CCR7^{-}CD28^{-}$ (effector memory) (23).

FUNCTIONAL ACTIVITY OF NON-HUMAN PRIMATES MUCOSAL-ASSOCIATED INVARIANT T-CELLS

Species specific variations in T-cell functional activity are important to consider in the context of different animal models. Stimulation of peripheral blood mononuclear cells (PBMCs) from PTM with the riboflavin metabolite–based antigen 5-OP-RU results in a dose-dependent upregulation of CD69 and selective proliferation of MR1 $tetramer^{+}V\alpha7.2^{+}$ MAIT cells (**Table 2**) (25), demonstrating that human and macaque MAIT cells are similarly activated by antigen (**Figure 1**). Surprisingly, 5-OP-RU stimulated PTM MAIT cells produced relatively low amounts of TNFα and IFNγ (although IFNγ secretion could be augmented with IL-12 and IL-18 supplementation to the cultures). In contrast to the PTM data, stimulation of human peripheral MAIT cells with 5-A-RU resulted in significant production of TNFα and IFNγ (43). In contrast to humans and NHPs, murine MAIT cells predominately produce IL-17 following stimulation with 5-OP-RU (16), highlighting the potential for species specific differences in MAIT cells functional activity, and the value of the NHPs in being able to better model the human peripheral MAIT cell cytokine profile.

The use of fixed *E. coli* to stimulate human MAIT cells is now a standard assay, and activates MAIT cells through both MR1-dependent and -independent mechanisms (6, 44). Similarly, LPS or *E. coli* stimulation resulted in significant IFNγ and TNFα production in RM and MCM MAIT cells (23, 28), as well as CD107a upregulation in MCM MAIT cells (28). In contrast, MCM MAIT cell stimulation with *Mycobacterium smegmatis* resulted in no significant increase in IFNγ and TNFα production, or CD107a upregulation.

Following mitogenic stimulation with PMA/ionomycin, both PTM and RM MAIT cells readily produce IFNγ, TNFα, and IL-17 (21, 25, 27). Variations in the amount of IL-17 production have been reported, with PTM MAIT cells producing relatively moderate levels of IL-17, and RM MAIT cells producing higher levels of IL-17. These differences may reflect species specific variability in MAIT cell IL-17 production capacity. These findings are similar to PMA/ionomycin stimulated human MAIT cells, which results in IFNγ, TNFα, and IL-17 production (45). Compared to humans, NHP MAIT cells have fairly conserved functional capacities, further highlighting the utility of NHP model to study immune dynamics.

TABLE 2 | Comparison of MAIT stimulation responses between humans, non-human primates, and mice.

	Human (% of $CD3^{+}$); Mean (range)	Non-human primate (% of $CD3^{+}$); Mean (range)
5-OP-RU/5-A-RU	CD69: ∼95 TNFα: 14–18 IFNγ: 20–60 (43, 59)	CD69: 20–80 TNFα: 5–13 IFNγ: 0.1–4 (25)
PMA/Ionomycin	TNFα: ∼80–95 IFNγ: ∼0.5–5 IL-17: ∼4–10 (45, 60)	TNFα: ∼20–80 IFNγ: ∼5–80 IL-17: ∼7–20 (25, 27)
Fixed E. coli	TNFα: 10–50 IFNγ: 10–75 (43, 61)	TNFα: ∼25–35 IFNγ: ∼20–35 (28)

MUCOSAL-ASSOCIATED INVARIANT T-CELLS IN ACUTE SIV/SHIV INFECTION

HIV infection in humans is marked by significant immune dysregulation, with significant MAIT cell depletion occurring in the peripheral circulation within the first 4 months of infection (33). However, within the first weeks of infection, longitudinal data suggests that there is a transient increase in MAIT cell frequencies in the peripheral circulation (46). Consistent with this observed MAIT cell expansion during acute HIV infection, NHP MAIT cells also appear to be activated and/or proliferating during the first month post-SIV/SHIV infection. Two studies have evaluated longitudinal MAIT cell dynamics in macaques following SIV/SHIV infection (25), and both noted increased expression of the proliferation marker, Ki-67, in circulating MAIT cells during acute SIV/SHIV infection. Increases in peripheral MAIT cell frequency were only observed in one of the two studies (25), but large fluctuations in pre-infection MAIT cell frequencies, high inter-animal variability in MAIT cell frequency, and small sample size, may have contributed to the lack of observed trends in the second study (28). MAIT cell frequencies also increase in the rectal mucosa and lymph nodes following SHIV challenge, with a peak frequency at ∼3 weeks post-infection (25). These results highlight the conservation of blood and tissue MAIT cell frequencies between humans during acute HIV infection, and NHPs following SIV/SHIV infection.

Evaluation of activation and tissue trafficking markers in circulating MAIT cells have shown variable changes following acute SIV/SHIV infection. There is no change in CCR5, CCR6, CXCR3, CD69, and HLA-DR expression on PTM peripheral

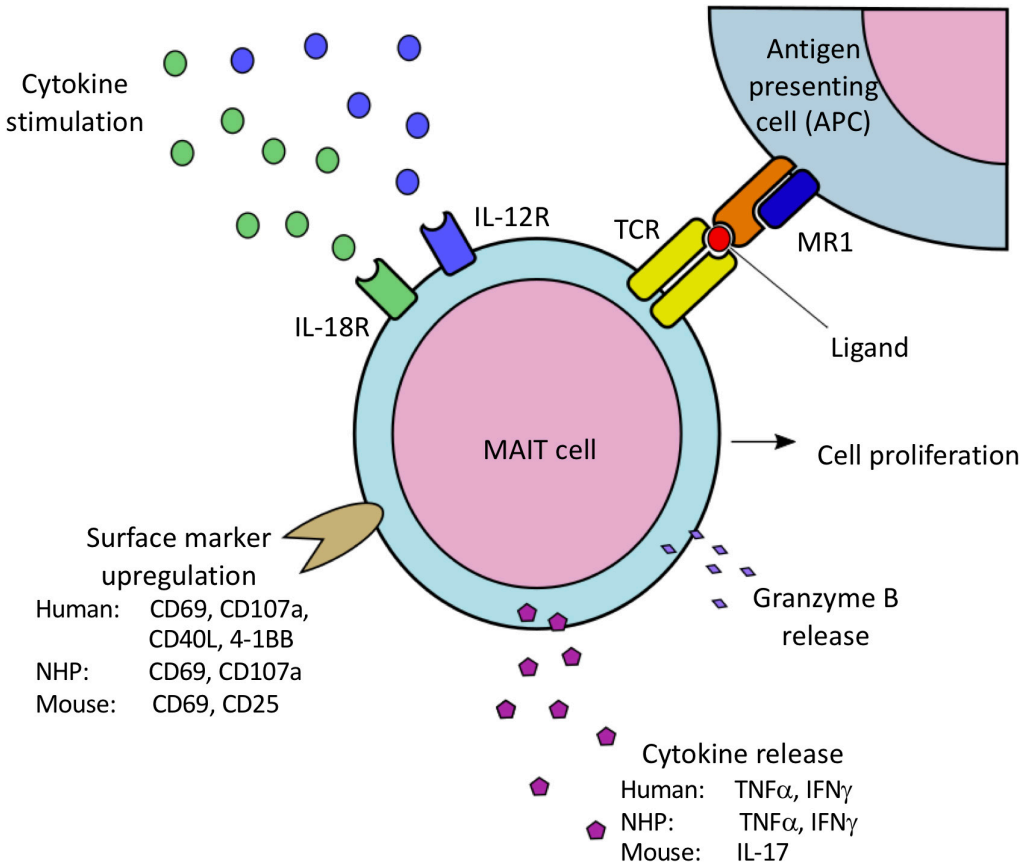

FIGURE 1 | Species-specific variations in MAIT cell activation via MR1-mediated presentation of bacterial-derived vitamin B metabolites to the MAIT cell TCR, as well as IL-12/IL-18 mediated cytokine stimulation. Activation results in MAIT cell proliferation, cell surface marker upregulation associated with cell activation, and release of proinflammatory cytokines and granzyme B. Both human and NHP MAIT cells upregulate CD69 and CD107a, and produce IFNγ and TNFα. Human MAIT cells have also been shown to upregulate CD40L and 4-1BB. In contrast, murine MAIT cells have been shown to upregulate CD69 and CD25, and predominately produce IL-17 when activated.

blood MAIT cells during acute SHIV infection (25). Similarly, there was no change in CD69 expression on the effector memory ($CD28^{-}CD95^{+}$) MAIT cell population of MCMs following SIV challenge (28). However, a transient but significant increase in CD69, CD39, T-bet, and RORγT was observed in the central memory ($CD28^{+}CD95^{+}$) MAIT cell population of MCMs at 2–3 weeks post-SIV challenge (28).

SIV/SHIV infection is associated with a transient increase in peripheral blood $\alpha4\beta7^{+}$ MAIT cells in PTMs at 3–4 weeks post infection (25). This increase correlated with peak viral load, as well as increased MAIT cell frequencies with rectal mucosa. One hypothesis is that early viral replication and increased antigen availability in the lymph node may drive α4β7 expression on MAIT cells. Peripheral blood MAIT cells upregulated α4β7 when cocultured with mesenteric lymph node cells and 5-OP-RU, demonstrating that circulating MAIT cells can upregulate α4β7 when stimulated with antigen in the context of mesenteric lymph node cells (25). These findings are similar to what has been observed with conventional T-cells in humans and mice, where α4β7 induction is mediated by retinoic acid-producing dendritic cells from gut-associated lymphoid tissues and mesenteric lymph nodes (46–50). Similarly, α4β7 expression is significantly increased on peripheral MAIT cells from chronic SHIV-infected RMs, compared to naïve controls (27). In humans, β7 integrin expression is increased in HIV^{+} individuals, suggesting this relationship may be conserved between humans and NHP models [reviewed in Saeidi et al. (51)]. This may also partially explain the mechanism for the transient increase in MAIT cell frequencies observed in the rectal mucosa of HIV^{+} individuals during initial infection (46). Alternatively, increased $\alpha4\beta7^{+}$ MAIT cells in the peripheral blood following SIV/SHIV infection may be due to expansion and subsequent trafficking of MAIT cells into the blood from mucosal tissues sites. These changes correlate with peak viremia, suggesting that early viral replication may drive α4β7 induction and increase mucosal tissue trafficking during acute infection. This is potentially driven by direct viral replication, or immune dysregulation with microbial translocation in the gastrointestinal tract. The lack of MAIT cell depletion during acute infection is consistent with what has been observed in humans during initial HIV infection and emphasizes the importance of the NHP-SIV/SHIV model to study MAIT cell dynamics during peracute infection.

MUCOSAL-ASSOCIATED INVARIANT T-CELLS IN CHRONIC SIV/SHIV INFECTION

Chronic HIV-1 infection in humans is associated with persistent MAIT cell depletion in the peripheral circulation [(33, 37), reviewed in Saeidi et al. and Juno et al. (51, 52)]. In contrast, chronic SIV/SHIV infection has inconsistent effects on peripheral MAIT cell frequencies in NHPs. One study reported a reduced frequency and absolute number of peripheral MAIT cells in RMs during chronic SIV infection (21). A reduction in $CCR6^+$ MAIT cells was also noted during chronic SIV infection, which was hypothesized to reflect impaired tissue trafficking capabilities of the remaining MAIT cells in the periphery. In contrast, a second study noted increased peripheral MAIT cell frequencies in RMs during chronic SHIV infection, which correlated with plasma viral load (27). Despite the overall increase in frequency, MAIT cell frequencies in the peripheral blood were inversely correlated with plasma viral load, and directly correlated with peripheral $CD4^+$ T-cell frequencies. Human MAIT cells are not preferentially infected by HIV (53), suggesting that the observed correlation may reflect reduced MAIT cell frequencies due to reduced CD4 T-cell frequencies, which is secondary to virus-mediated CD4 T-cell depletion. Other potential causes for the observed correlation includes increased MAIT cell consumption in response to higher amounts of virus mediated gastrointestinal inflammation and bacterial translocation. A third study identified no significant difference in peripheral MAIT cell frequencies during chronic SHIV infection in PTMs (25). Increased Ki-67 expression in the peripheral MAIT cell populations was identified in 2 studies, despite the variability in MAIT cell frequency (21, 27).

Similar variability has also been observed in MAIT cell frequencies in tissues during chronic SIV/SHIV infection in NHPs. Two separate studies have reported increased and decreased MAIT cell frequencies in the BAL fluid of chronic-SHIV infected PTMs and chronic-SIV infected RMs, respectively (21, 25). Additionally, one study identified decreased MAIT cell frequencies in the inguinal lymph nodes, with concurrent maintenance of MAIT cells in the mesenteric lymph nodes of chronic SHIV-infected PTMs (25). In contrast, a separate study identified a reduction in MAIT cell frequencies in the mesenteric lymph nodes of chronic SIV-infected RMs (21). However, MAIT cell frequencies are consistently maintained in the liver, spleen, and gastrointestinal tract of chronic SIV/SHIV-infected macaques (21, 25).

Chronic HIV infection is associated with altered expression of a variety of cytokine and chemokine receptors on peripheral MAIT cells [reviewed in Saeidi et al. (51)]. Similar alterations have also been observed in chronic SIV/SHIV infected NHPs. Chronic SHIV-infection is associated with reduced Tbet and Eomes expression of peripheral MAIT cells in PTMs, while CCR5 and CXCR3 expression levels are unchanged (25). In contrast to humans where CCR6 and HLA-DR expression is decreased and increased, respectively in MAIT cells from chronic HIV-infected individuals, CCR6 and HLA-DR expression on peripheral MAIT cells are unchanged in chronic SHIV-infected PTMs. Chronic SIV infection in RMs results in reduced CD28 and PLZF expression on peripheral MAIT cells, while caspase 3 expression is unchanged (21). Reduced Tbet and Eomes expression in both humans and NHPs during chronic viral infections indicates some conservation of the immune dysregulation within the model. However, the NHP SIV/SHIV model likely does not capture every aspect of the immune dysregulation that is observed in HIV patients. Some examples where immune dysregulation varies includes MAIT cell activation (HLA-DR), antigen presentation (HLA-DR), and tissue trafficking (CCR6). Further studies are needed to better characterize MAIT cell functional and phenotypic changes that occur during SIV/SHIV infection.

Immune effector functions of peripheral MAIT cells are significantly impaired during chronic HIV infection. This is marked by reduced cytokine production and CD69 upregulation following *E. coli* stimulation, which is only partially restored with combination antiretroviral therapy [(37), reviewed in Saeidi et al. (51)]. Similar findings were also reported in one study evaluating chronic SHIV-infected macaques, with reduced TNFα and IL-17 production capacity following mitogenic stimulation (27). However, two studies looking at chronic SIV and SHIV infected MCMs and PTMs, respectively, report no alterations in cytokine stimulation capacity (25, 28). Additionally, chronic SHIV-infection in PTMs is associated with increased CD69 expression on peripheral MAIT cells (25). SIV/SHIV infection does not consistently alter MAIT cells' ability to produce granzyme, with 2 studies noting no significant changes in the frequency of granzyme B^+ MAIT cells in the peripheral blood during SIV or SHIV infection (27, 29). These findings are in contrast to what is observed in HIV^+ individuals, where peripheral granzyme B^+ MAIT cells are increased in frequency compared to uninfected control samples at resting state (54) and have reduced perforin and granzyme expression following *E. coli* stimulation (54, 55). In one of the NHP studies, $perforin^+$ and $perforin^+$granzyme B^+ MAIT cell frequencies were elevated following SHIV challenge (27).

The cause for the variability in MAIT cell frequencies, receptor expression, and effector functions observed in these studies is unknown. The authors hypothesize these may reflect species differences between RMs and PTMs, and/or differences in viral pathogenesis between different SIV/SHIV strains. Additional sources of variation may be due to inter-study differences in gating strategies to identify MAIT cells, including the usage of CD161 expression and $CD8^+$ pre-gating. There is also evidence of significant variability in MAIT cell frequencies within the peripheral circulation and different tissue compartments, both between different species and individual animals. This emphasizes the need for longitudinal studies, in order to evaluate changes in MAIT cell trends following different experimental manipulations, as opposed to cross sectional studies. It will also be important to consider standards for identifying NHP MAIT cells in future studies, including the usage of the Vα7.2 TCR

and species specific MR1 tetramer reactivity, due to evidence of limited cross-reactivity with human MR1 tetramers.

MUCOSAL-ASSOCIATED INVARIANT T-CELLS IN CHRONIC SIV/*Mtb* CO-INFECTION

One important consideration for HIV^+ individuals is the risk of co-infection with secondary opportunistic bacterial and fungal pathogens, for which HIV-mediated dysregulation of MAIT cells may be a predisposing factor. While data on the impact of MAIT cell responses on fungal infections is currently lacking, MAIT cells have been shown to become activated in response to *Candida* spp. [reviewed in Wong et al. (56)] and *Aspergillus* spp. (57). *Mycobacterium tuberculosis* (*Mtb*) is one of the most common opportunistic bacterial infection among HIV^+ individuals. Individuals with active or latent *Mtb* infections tend to have lower peripheral MAIT cell frequencies, compared to uninfected individuals (58). Additionally, MAIT cells from HIV/*Mtb* co-infected individuals tended to express lower levels of CCR6 compared to HIV infected individuals and healthy controls, though statistically significant differences were only seen between the HIV/*Mtb* co-infected individuals and the healthy controls. This suggests that HIV-mediated MAIT cell dysregulation may predispose individuals to opportunistic infection with *Mtb*.

In RMs, vaccination with Bacillus Calmette–Guérin, a vaccine historically used to prevent tuberculosis, resulted in significantly increased expression of Ki-67 and granzyme B in MAIT cells in the peripheral blood, the vaccine sites, and the draining lymph nodes, compared to pre-vaccination frequencies (23). While there was evidence of significant MAIT cell activation following vaccination, no significant changes in MAIT cell frequencies in the blood or tissue sites were observed. Increased peripheral MAIT cell Ki-67 expression, without an increased in peripheral blood MAIT cell frequencies, was also observed in RMs following intrabronchial *Mtb* infection (23). No significant difference in MAIT cell frequencies in the peripheral blood or tissue has been reported with SIV/*Mtb* co-infection, similar to what has been observed in humans (26, 28, 58). However, a weak negative correlation between MAIT cell frequency and bacterial burden was observed in SIV/*Mtb* co-infected MCMs, which was not maintained in NHPs mono-infected with *Mtb*. There was also a significant reduction in TNFα producing capacity of MAIT cells from the peripheral blood and *Mtb* granulomas of SIV/*Mtb* co-infected MCMs, compared to *Mtb* mono-infected controls (28). The results reflect the potential impact of MAIT cells on the susceptibility of *Mtb* in HIV^+ individuals, in an NHP animal model.

CONCLUSION AND FUTURE DIRECTIONS

There is growing evidence that MAIT cells are capable of being activated in response to viral pathogens, despite the lack of vitamin B metabolites that are necessary for TCR-dependent MAIT cell activation. Additionally, there is evidence of altered MAIT cell frequencies, phenotypes, and functional activity during both acute and chronic HIV infection. However, the underlying pathophysiology for why these changes occur is not well understood. Having a better understanding of this interaction may provide key insights into developing vaccines and/or therapies for HIV infection and related co-morbidities. The NHP animal model provides a valuable platform to study these changes, and understand the underlying mechanisms for why they occur.

While SIV/SHIV infection in NHPs does not reliably recapitulate all aspects of the MAIT cell changes that occur during HIV infection, notably the lack of MAIT cell depletion, it does model many aspects of HIV infection well. This includes an initial increase in MAIT cell frequency with upregulation of α4β7 during acute viral replication, as well as evidence of impaired MAIT cell function in SIV/mTB co-infected NHPs. Future studies should focus on characterizing the perturbations in the MAIT cell populations within different tissue compartments during SIV/SHIV infection; with a special focus on variables that may alter these perturbations, such as macaque species, age, and gender, as well as strain, dose, and route of administration of the virus. By better understanding how these factors impact MAIT cell dynamics during SIV/SHIV infection, we will ideally be able to refine the NHP model to better recapitulate the MAIT cell changes that occur during HIV infection in humans. Additionally, the robust response that NHP MAIT cells generate to both antigen and cytokines, including cytokine independent cell activation, which is comparable to humans, may be advantageous for studying MAIT cell dynamics with other pathogens.

AUTHOR CONTRIBUTIONS

IB-A wrote the initial draft of the manuscript. IB-A, SK, and JJ revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Biased MAIT TCR Usage Poised for Limited Antigen Diversity?

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Mucosal-associated invariant T (MAIT) cells are a subset of unconventional T cells that recognize the evolutionarily conserved major histocompatibility complex (MHC) class I-like antigen-presenting molecule known as MHC class I related protein 1 (MR1). Since their rise from obscurity in the early 1990s, the study of MAIT cells has grown substantially, accelerating our fundamental understanding of these cells and their possible roles in immunity. In the context of recent advances, we review here the relationship between MR1, antigen, and TCR usage among MAIT and other MR1-reactive T cells and provide a speculative discussion.

Keywords: MR1, MAIT cells, antigen diversity, TCR repertoire diversity, T cell subsets

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INTRODUCTION

Conventional T cells recognize peptide antigens presented by the major histocompatibility complex (MHC) class I or class II (MHC-I or MHC-II) molecules and elicit a cellular immune response to provide anti-microbial and anti-tumoral immunity for the host. Conventional T cells utilize a cell surface bound T cell receptor (TCR) to recognize peptide-MHC complexes presented on the surface of antigen-presenting cells (APCs). The TCR is a heterodimer comprised of a TCR α- and β-chain, each consisting of a variable (V) and constant (C) domain. Three sets of finger-like extensions on the TCRα- and β-chain variable domains, known as complementarity determining loops (CDR1, CDR2, and CDR3), are responsible for recognizing the peptide-MHC-complex and are collectively unique to each TCR. CDR1 and CDR2 loops are germline-encoded by the V gene segments, while the CDR3 loops are formed from somatic recombination of the V gene segments with a joining (J) (V-J) and/or diversity (D) gene segment (V-D-J) during T cell development (1). Together with random nucleotide additions and deletions in the CDR3 loop gene regions, this creates an enormous diversity of TCRs, clonally distributed amongst T cells (2).

Mucosal-associated invariant T (MAIT) cells are a subset of unconventional T cells that are restricted by a monomorphic MHC-I-like molecule, known as MHC class I related protein 1 (MR1) (3–6). In contrast to conventional T cells, MAIT cells express a "semi-invariant" αβ TCR, meaning they typically express the same TCRα chain paired to a preferred array of TCRβ chains (3). In humans, the "classical MAIT TCR" comprises a TCRα chain encoded by the V gene segment *TRAV1-2* juxtaposed to the J gene segment *TRAJ33*, *TRAJ20*, or *TRAJ12* (5, 7–9) that pairs preferentially with a TCRβ chain encoded by *TRBV6* or *TRBV20 V* gene segment family members (7). In mice, the MAIT TCR is composed of homologous gene segments, a *TRAV1-TRAJ33* TCRα chain, that pairs with a TCRβ chain composed of *TRBV19* or *TRBV13 V* gene segments, both of which are murine orthologous segments of human *TRBV6* (3, 5, 10). MAIT cells are abundant in healthy adults, representing on average 3% of blood T cells (11) and can be found throughout peripheral tissues (6, 7, 10, 12–23). The frequency of MAIT cells in laboratory mice is distinctly lower than in humans, although murine MAIT cells are also found in many

peripheral organs (24, 25). The prototypical antigen presented by MR1 to MAIT cells is the small molecule 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), an adduct of the riboflavin biosynthetic precursor 5-amino-6-D-ribitylaminouracil (5-A-RU) and methylglyoxal (26) (**Figure 1**). See recent reviews for details on the riboflavin biosynthesis and formation of 5-OP-RU from 5-A-RU (31, 32). Riboflavin biosynthesis is absent in mammals. Thus, by recognizing 5-OP-RU (25, 33, 34), and potentially other riboflavin-based ligands presented by MR1 (35), MAIT cells are able to sense a broad range of riboflavin biosynthesis proficient microbes in a highly conserved, innate-like manner, reviewed in (32). Human MAIT cells stimulated with 5-OP-RU rapidly secrete T helper (Th)1 and Th17 type cytokines (11, 36, 37) as well as cytotoxic granules (38). In mice, lung infection with riboflavin-synthesizing bacteria or co-administration of synthetic 5-OP-RU with adjuvant leads to a significant expansion of MAIT cells with Th1/17 cytokine secreting capacity (25, 34, 39), enabling MAIT cells to contribute to protection against several pathogens, including *Klebsiella pneumoniae* (40), *Mycobacterium bovis* BCG (41), *Francisella tularensis* (39), *E. coli* (42), *Legionella longbeachae* (34), and *Clostridium difficile* (43). Thus, observations to date suggest MAIT cells are poised, but perhaps not limited to, protecting peripheral tissues from microbial pathogen or commensal breach. In particular, MAIT cells have recently been shown to contribute to tissue repair at barrier sites (44–47). MAIT cells may also be involved in the tumoral immune response (48–52), however, elevated MAIT cell numbers at the tumor site in some cancers correlate with a poorer prognosis (49, 52). Notably, MAIT cells appear to be subject to a similar fate as conventional T cells during the anti-tumoral immune response, namely: T cell exhaustion, altered functional response, altered frequency, and drug sensitivity (50, 52–57). A cytokine-modulated (IL-7, IL-12, IL-18) tumor response that occurs independent of, or concurrent with, TCR stimulation should also be considered in the context of tumoral immunity, as MAIT cells are known to respond to inflammatory stimuli in this manner (15, 58, 59). Furthermore, MAIT cells from healthy donors can efficiently lyse MR1 proficient tumor cells presenting microbial agonists such as 5-OP-RU, suggested as a potential strategy to harness the MAIT cell response therapeutically (56). Perhaps similar in mechanism, disruption of barrier tissues (i.e., colorectal cancers) by tumors may allow invasive growth of commensal bacteria, providing a source of microbial ligand in the context of an inflammatory environment which may trigger anti-tumor MAIT cell responses (48–50, 60). Much is still unknown regarding the response by MAIT cells in the tumoral environment, particularly whether tumor associated, MAIT cell specific MR1 ligands exist and

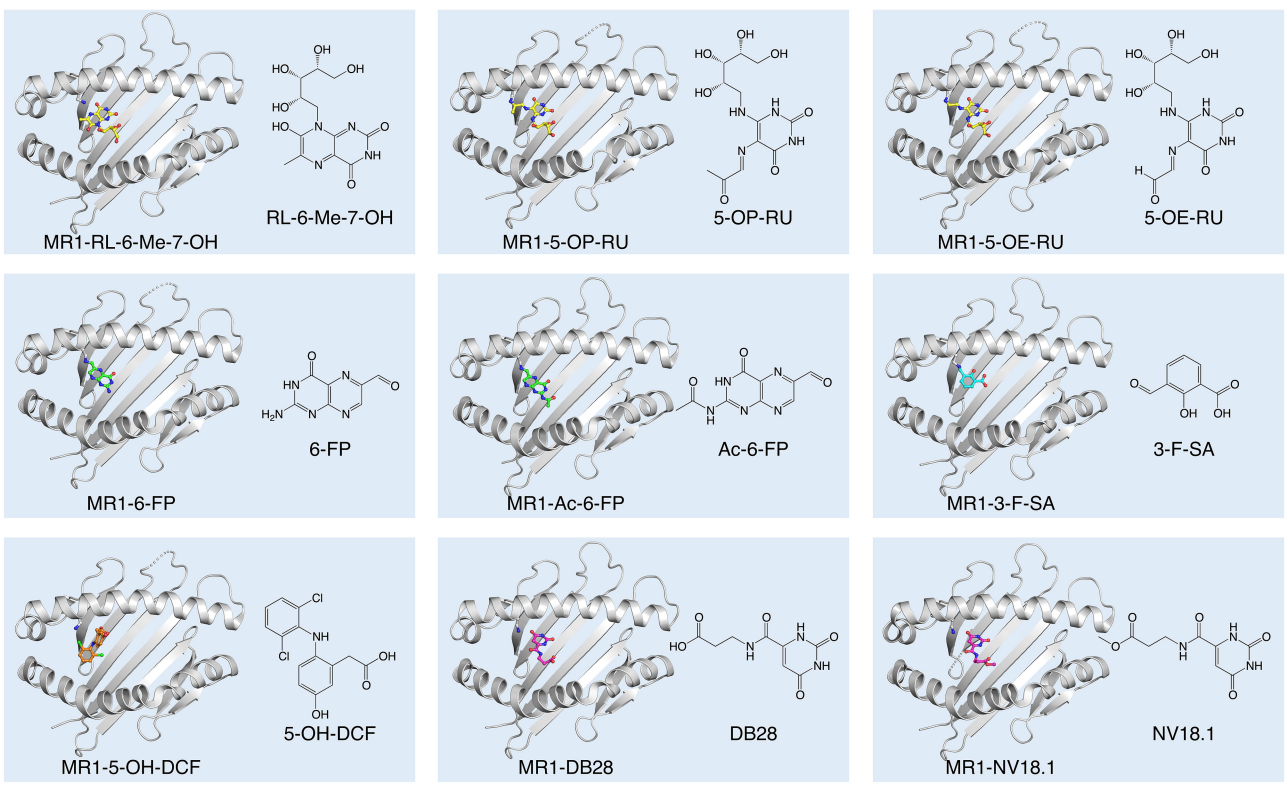

FIGURE 1 | Diversity of small molecule ligands presented by MR1. Cartoon display (light gray) of the MR1 antigen-binding cleft (top-view) and ball-and-stick display of the antigen (colored) based on the protein data bank (PDB) deposited crystal structures, featuring the human A-F7 MAIT TCR in complex with human MR1-RL-6-Me-7-OH [PDB ID: 4L4V (27)], MR1-5-OP-RU and MR1-5-OE-RU [PDB IDs: 4NQC, 4NQE (26)], MR1-6-FP [PDB ID: 4L4T (27)], MR1-Ac-6-FP [PDB ID: 4PJF (28)], MR1-3-F-SA and MR1-5-OH-DCF [PDB IDs: 5U6Q, 5U72 (29)], and MR1-DB28 and MR1-NV18.1 [PDB IDs:6PVC and 6PVD (30)].

the factors that might drive MAIT cell to become pro- or anti-tumoral. MAIT cells have, however, attracted some interest as a potential immunotherapeutic target as they possess a number of favorable attributes such as a high precursor frequency, wide tissue distribution, potent cytokine response and cytotoxicity and a donor unrestricted nature (61).

THE RIBOFLAVIN-BASED MR1 LIGANDS

Independent observations from Gold et al. and Bourhis et al. demonstrated that a wide range of bacteria and yeasts, and their supernatants, are capable of stimulating MAIT cells in an MR1-dependent manner (36, 62). On the assumption that MR1 would likely adopt a "MHC-I-fold" (63) in the presence of ligand, Kjer-Nielsen et al. folded soluble recombinant MR1 proteins in the presence of bacterial supernatant to capture ligands in the form of stable MR1-ligand-complexes (35). This approach of ligand-capture, combined with mass-spectrometry, and subsequent genetic manipulation of the riboflavin biosynthetic pathway in bacteria, led to the discovery of the pyrimidines; 5-OP-RU and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), and the substantially less potent, cyclised ribityllumazines; 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH); and 7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe) as riboflavin-based, MR1-presented, MAIT cell stimulating antigens (26, 27, 32, 35) (**Figure 1**). While both 5-OP-RU and 5-OE-RU were detected in the supernatant of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, 5-OP-RU is the dominant MAIT cell antigen found in these bacterial supernatants (26). Nevertheless, MR1 tetramers loaded with 5-OE-RU stain a similar proportion of T cells compared to those loaded with 5-OP-RU (26) and synthetic versions of both antigens are similarly potent (64, 65). 5-A-RU co-incubated with methylglyoxal is, however, more potent in activating MAIT cells than 5-A-RU co-incubated with glyoxal (26, 66), presumably due to differences in the catabolic and metabolic kinetics of 5-OP-RU and 5-OE-RU (64). 5-OP-RU has emerged as the preferred antigen for research, both as synthetic antigen and loaded in MR1 tetramers. Accordingly, MR1-5-OP-RU tetramers have become the gold standard for identifying MAIT cells (67), enabling more accurate MAIT cell detection than using antibodies specific for surrogate markers (TRAV1-2, CD161, and CD8) (11). MR1 presentation of 5-OP-RU and 5-OE-RU involves the formation of a Schiff base between the Lys43 of MR1 and a residual carbonyl group on the ligand. This Schiff base, unique to MR1 ligand presentation amongst MHC molecules, stabilizes, and guides 5-OP-RU and 5-OE-RU into their position on MR1 in the A'-pocket amongst a cradle of aromatic residues (26). Notably, even in the absence of the Schiff base, by mutating Lys43 in MR1 to Ala (68), 5-OP-RU is presented by MR1 in the identical position (26), although the stability of this complex is markedly reduced (28). Unlike the pyrimidine ligands, RL-6-Me-7-OH does not form a Schiff base with MR1, however residues within the A'-pocket of MR1 help orient the ligand into a similar position (27). In a physiological context, neutralization of MR1 Lys43 by covalently bound ligand is essential for complete MR1 folding and trafficking from the endoplasmic reticulum (ER) to the cell surface (69).

All classical MAIT TCRs studied to date recognize MR1 presenting riboflavin-based antigens using a surprisingly consistent mode of docking, orthogonally over the cleft of MR1 and forming multiple contacts with aromatic residues that surround the ligand-binding site, notably residues Tyr62 and Tyr152 (26–28, 68, 70). The TCR also directly contacts the antigen, which occupies only 0.6% of the exposed surface in the MR1 cleft (70). Crucial to antigen recognition by classical MAIT TCRs is Tyr95α, a highly conserved residue in the CDR3α loop which directly contacts the 2′-OH group on the antigen ribityl moiety (**Figure 2A**) (26–28, 68). Structure-function studies with synthetic derivatives of 5-OP-RU and other riboflavin-based antigens have provided valuable insights on which chemical group modifications affect ligand antigenicity within the MAIT-MR1 axis (64, 65). While improving ligand stability, modification of the pyrimidine backbone of 5-OP-RU (C-5 and C-6) (**Figure 2A**), had only small effects on altering antigen potency (64), perhaps unsurprising as these atoms make no direct contact with the MAIT TCR (26, 28, 64, 65). In contrast, modifications to the ribityl moiety of 5-OP-RU and ribityllumazine ligands had profoundly negative effects on MAIT TCR recognition and antigen potency (65, 71). Particularly, removal of the ribityl 2′- and 3′-OH groups or the entire ribityl tail largely abolished MAIT TCR recognition of relevant 5-OP-RU analogs (65, 71). In contrast, shortening of the ribityl tail, while preserving the 2′- and 3′-OH groups (ethyl- or propyl-5-OP-RU analogs) did not appreciably affect MAIT TCR recognition (65, 71). Notably however, 2′-OH was not required for MAIT TCR recognition of a modified analog of the ribityllumazine RL-6-Me-7-OH (72). Structural modeling suggested the modified ribityl tail of the 2′-deoxy-RL-6-Me-7-OH is flexible enough to be oriented for recognition by the MAIT TCR Tyr95α via a hydrogen bond with the 5′-OH in place of the 2′-OH group (**Figure 2A**) (72). Although MAIT cells could recognize MR1 presenting the 2-deoxyribityllumazine, this interaction was not sufficient to lead to MAIT cell activation (72), stressing that distinct requirements exist for MAIT cell activation that are not satisfied by TCR engagement alone. Thus, the series of altered metabolite ligands helped to define the boundaries of riboflavin-based antigen diversity tolerated by the MAIT TCR and emphasized the importance of the ribityl tail and Tyr95α in MAIT TCR recognition. In light of this, new microbial ligands have been identified that share the ribityl moiety and some structural features with the riboflavin-based antigens (73). The lumazines 6-(2-carboxyethyl)-7-hydroxy-8-ribityllumazine (photolumazine I; PLI) and 6-(1H-indol-3-yl)-7-hydroxy-8-ribityllumazine (photolumazine III; PLIII) and the riboflavin analog 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) were isolated from soluble recombinant MR1 expressed in the context of live bacterial infection (**Figure 2B**) (73). Although all three ligands had ribityl moieties, only PLI and PLIII, possible adducts of 5-A-RU, were antigenic to MAIT cells, while FO was antagonistic (73). Similarly, three pyrimidines, mercaptopurine, floxuridine, and doxofylline (**Figure 2C**) were identified as putative MR1 ligands in an *in silico* screen of a range of small

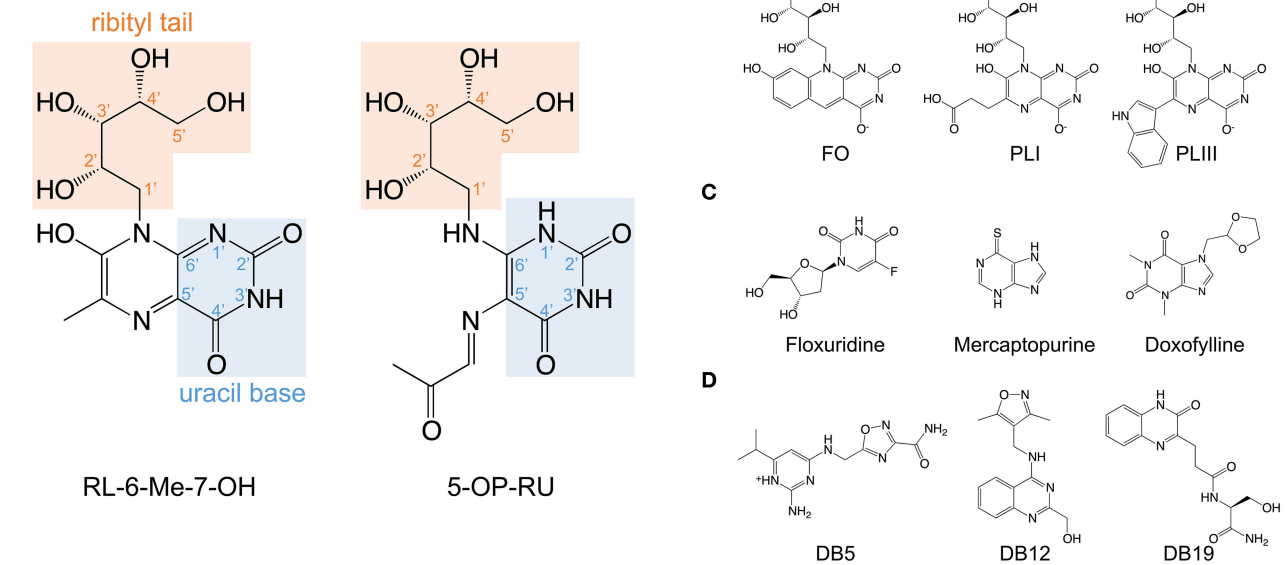

FIGURE 2 | Structures of the riboflavin-based and related MR1 ligands. **(A)** Chemical structure of 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH) and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) annotated with the position numbers for relevant functional groups. **(B)** MR1 ligands isolated from soluble recombinant MR1 expressed with *E. coli* or *M. smegmatis*; lumazines 6-(2-carboxyethyl)-7-hydroxy-8-ribityllumazine (photolumazine I; PLI) and 6-(1H-indol-3-yl)-7-hydroxy-8-ribityllumazine (photolumazine III; PLIII) and the riboflavin analog 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO). **(C,D)**. A selection of MAIT cell agonist and non-agonist MR1 ligands identified from *in silico* screens of multiple chemical libraries; mercaptopurine, floxuridine, doxofylline, and 2-amino-4-(((3-carbamoyl-1,2,4-oxadiazol-5-l)methyl)amino)-6-isopropylpyrimidin-1-ium (DB5), (4-(((3,5-dimethylisoxazol-4-yl)methyl)amino)quinazolin-2-yl)methanol (DB12) and (S)-N-(1-amino-3-hydroxy-1-oxopropan-2-yl)-3-(3-oxo-3,4-dihydroquinoxalin-2-yl)propenamide (DB19).

organic molecules, including drugs, drug metabolites, and drug-like molecules (29). In cellular assays, floxuridine and mercaptopurine were weakly agonistic but did not upregulate MR1 to detectable levels, while doxofylline weakly upregulated MR1 but was not agonistic (29). Further *in silico* screening of commercial compound libraries recently identified seven weakly agonistic MR1 ligands (**Figure 2D**) (30). None of the novel agonist ligands were as potent as 5-OP-RU in cellular assays or were able to upregulate MR1 surface expression, likely due to the inability to form a Schiff base with MR1 Lys43 (30). In contrast, in the same study, two pyrimidines were identified; 3-[(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)formamido] propanoic acid (DB28) and its derivative methyl 3-[(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)formamido] propanoate (NV18.1) that significantly modulated MR1 surface expression (30). These ligands bound to MR1 non-covalently (**Figure 1**), causing MR1 to be retained in the ER and prevented surface expression (30). Importantly, unlike the other ligands, DB28 and NV18.1 were non-stimulatory to PBMC-derived TRAV1-2^{+} MAIT cells (30).

CHARACTERISTICS OF THE MAIT TCR REPERTOIRE

The classical MAIT TCRα rearrangements (*TRAV1-2-TRAJ33/20/12*) are highly conserved between genetically unrelated individuals, also referred to as public TCRα chain usage (3, 5). Collectively, these TCRα rearrangements account for the majority (~95%) of MAIT TCR clonotypes in human blood (7–9, 11, 62, 74). Nevertheless, variations of the classical MAIT TCR CDR3α region exist, typically at one or two non-germline-encoded residue positions (CAXXDSSYKLIF, CAVXXXDYKLSF, and CAXXDSNYQLIW for TRAJ12, TRAJ20, and TRAJ33 MAIT CDR3α rearrangements, respectively) (5, 11, 74). However, MR1-reactive clones responding to different microbes (bacteria and yeast) are characterized by more extensive non-germline-encoded differences within the TCRα chain, including what appeared to be microbe-specific MR1-reactive clonotypes in some instances (8). Similarly, systemic infection of healthy volunteers with *S. enterica* serovar Paratyphi A transiently altered the MAIT TCR repertoire within individuals at the clonotypic level (75). In this case, circulating, overrepresented MAIT clonotypes were reduced during infection but restored to homeostatic proportions at the resolution of infection (75). TCRs from MAIT clonotypes that expanded during infection showed greater stimulatory potential than TCRs from contracted MAIT clonotypes in response to synthetic riboflavin-based antigens or bacterial supernatant (*S. enterica* Paratyphi A or *E. coli*) (75), suggesting TCR-dependent but not microbe-specific differences in MAIT clonotypic responses. In mice, stimulation of circulating MAIT cells with the different riboflavin-based antigens induced activation of separate cell clusters based on their activated surface phenotype (66), perhaps involving preferential stimulation of clonal MAIT TCRs. Similarly, in mice, preferential accumulation of MAIT cells with divergent functional phenotypes occurs

in response to acute infection by different bacterial species (*S. enterica* serovar Typhimurium or *Legionella longbeachae*) (34, 46, 76), however, divergence in the functional response of MAIT cells has not been correlated with TCR usage in mice. Interestingly, the TCRα chain *TRAV1-2-TRAJ33* rearrangement is also found in some $CD161^{-}TRAV1\text{-}2^{+}$ T cells, and nearly one third of those *TRAV1-2-TRAJ33* rearranged TCRs express Tyr95α (74). Whether these cells are also MR1-reactive is not known, although it should be noted that other MHC-reactive T cells and a subset of CD1b-restricted T cells also utilize the *TRAV1-2* gene segment (77–79). A minority of MAIT TCRα *TRAV1-2* rearrangements occur with non-classical junctional regions (non-*TRAJ12/20/33*), some of which do not contain the Tyr95α residue yet still confer MR1-5-OP-RU reactivity (11). These observations indicate that other factors must account for recognition of 5-OP-RU among "MAIT TCR outliers," as in one case determined by crystallographic analysis (80).

Among MAIT cells, TCRβ chain composition provides the greatest source of variability within the TCR repertoire (7, 8, 10, 11, 28, 74). Despite this, *TRBV6* is a dominant *TRBV* gene segment used amongst MAIT TCRs, although some variations exist and are dependent on the TCRα junctional segment (7, 74). For instance, *TRAV1-2-TRAJ12* TCRα chains more commonly pair with *TRBV6* (>80%), compared to *TRAV1-2-TRAJ33* and *TRAV1-2-TRAJ20* rearranged TCRα chains which pair roughly evenly with TCRβ chains encoded by *TRBV6* and *TRBV20* family members (7, 74). Up to one quarter of paired MAIT TCRs express TCRβ chains that are not from *TRBV6* or *TRBV20* V segment family members (7, 74). In line with the less stringent nature of TCRβ chain usage, the CDR3β region among classical MAIT TCRs are non-germline-encoded and quite hypervariable, ranging from 9 to 19 amino acids in length and containing no discernible sequence motifs (9, 28, 74). A comparison of seven canonical MAIT TCRs (*TRAV1-2-TRAJ33* encoded TCRα chain paired with a *TRBV6-1* encoded TCRβ chain) with similar CDR3α loops and highly variable CDR3β loop length and composition revealed how CDR3β hypervariability impacts on MAIT TCR recognition of MR1 (28). All of the TCRs bound to MR1-5-OP-RU within a similar range of affinities (K_{deq} ~2 μM), with the exception of one TCR that bound MR1-5-OP-RU weaker (K_{deq} ~9.1 μM) (28). Structural analysis of four of the TCRs bound to MR1-5-OP-RU, including the weaker affinity TCR, demonstrated that the overall TCR footprint onto MR1 was conserved (28). Not surprisingly, the greatest variability in MR1 engagement was from contacts by the TCRβ CDR loops and framework regions that differed between TCRs; yet an overall similar buried surface area (BSA) at the interface was achieved (28). CDR3β loop sequence impacted on conformational flexibility of this loop as well as MR1 residues contacted. Importantly, in some structures the CDR3β loop made direct contact with 5-OP-RU, thus fine-tuning MR1 recognition in an antigen-dependent manner (28).

The MAIT TCR repertoire varies throughout the course of life (fetal, neonate, young, and old) and has now been examined in some detail, revealing consistent changes in MAIT cell frequency as we age (7, 9, 74, 80, 81). The overall frequency of MAIT cells in blood increases rapidly from low numbers early in life (11, 82), typically over the first two-to-three decades, declining slowly thereafter (83, 84). In addition, the diversity in clonotypes decreases as certain MAIT cell clones expand over time (84). This is most pronounced in older adults (>65 years), where a small number of private (non-shared) MAIT cell clonotypes dominate the MAIT cell pool (84). It is likely that expansion of naïve MAIT cells in young individuals is a consequence of microbial exposure following thymic egress (36, 45, 82, 85, 86). Interestingly, a large proportion of $CD161^{+}$ T cells found in cord blood express the canonical MAIT TCRα chain (TRAV1-2-TRAJ33) but do not recognize MR1-5-OP-RU tetramers (81). These non-reactive cells are not readily detectable in adults (11, 84), suggesting they do not expand akin to MR1-5-OP-RU-reactive MAIT cells. In this regard, some of these cells might feature a TCRβ chain which prohibits reactivity to MR1 presenting riboflavin-based antigens in favor of other antigens furnished by MR1, some of which may be less abundant or less stimulatory than the riboflavin-based antigens for MAIT cells expressing classical MAIT TCRs (28, 35, 80) or may trigger peripheral tolerance to avoid autoreactive or allergic responses (87). Accordingly, a pairwise comparison of MAIT TCR sequences in cord blood revealed much broader TCRβ diversity compared to those from MAIT cells from adult blood, further highlighting the effect of peripheral exposure on the narrowing of the MAIT TCR repertoire (84).

NON-RIBOFLAVIN BASED MR1 LIGANDS

In addition to the well-defined riboflavin (vitamin B2)-based MR1-ligands which are classified as pyrimidines (5-OP-RU and 5-OE-RU) and ribityllumazines (RL-6-Me-7-OH and RL-6,7-diMe), MR1 also presents other classes of small molecules. They include a range of pterins, namely the photodegradation product of folic acid (vitamin B9), known as 6-formylpterin (6-FP) (35), the synthetically acetylated form of 6-FP, acetyl-6-formylpterin (Ac-6-FP) (28), the photodegradation product 2,4-diamino-6-formylpteridine (2,4-DA-6-FP) of the drugs aminopterin and methotrexate which are synthetic derivatives of folic acid (29), and a synthetic derivative of Ac-6-FP, 2-acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal (33). See recent review for details on the photodegradation of folic acid (32). 6-FP, Ac-6-FP, and 2,4-DA-6-FP form a Schiff base with MR1 Lys43 based on crystal structures (28, 29, 35). Whilst the relevant formyl group of the synthetic derivative of Ac-6-FP, 2-acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal, is blocked (33), partial hydrolysis of the acetal might generate an aldehyde capable of reacting with Lys43 of MR1. Indeed the latter, just like all other listed pterins, caused cell surface upregulation of MR1 (26, 28, 29, 33, 35). In contrast, an equivalently modified version of 6-FP, 2-amino-4-hydroxy-6-formylpteridine dimethyl acetale, did not cause MR1 surface upregulation (33). In peripheral blood of some healthy donors, rare subsets of MR1-Ac-6-FP and MR1-6-FP reactive T cells have been described based on tetramer staining (80), as detailed in the next section. Also, some canonical MAIT TCRs form notable interactions with MR1-6-FP (27) and Ac-6-FP (28), involving direct molecular contact between TCR and Ac-6-FP (28) but not 6-FP (27). However, 6-FP and Ac-6-FP

typically do not stimulate MAIT cells (28, 35). Instead 6-FP and Ac-6-FP are mostly known as competitive inhibitors of activation by riboflavin-based antigens such as 5-OP-RU (27, 28). Also 2-acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal acts as a potent competitive inhibitor (33). So far Ac-6-FP remains the most potent competitive inhibitor of MAIT cell activation by 5-OP-RU and other pyrimidine antigens, both *in vitro* in human and mouse cell line assays and *in vivo* in mice (29). The acetyl group in Ac-6-FP which is absent in 6-FP forms additional van der Waals interactions with MR1 as well as a hydrogen bond, correlating with an 8°C increase in the stability of MR1-Ac-6-FP in comparison to MR1-6-FP (28). This, as well as other factors, such as solubility, molecule stability, and capacity for cellular uptake likely contribute to the potency of Ac-6-FP as a competitive inhibitor.

The same small molecule drug screen that identified three pyrimidines as potential MR1 ligands (described above) also identified 19 non-pyrimidine MR1 ligands, which belonged to one or more of the following diverse classes of molecules: aromatic aldehyde, aromatic carboxylate, phenol alinine, flavone, isoflavone, enone, quinone (29). Most of these non-pyrimidine ligands could not stimulate a MAIT TCR reporter cell line, and in crystal structures analyzed, no TCR contacts with the MR1 ligands were observed. All of the non-stimulatory ligands tested, including the salicylic acid analog 3-formylsalicylic acid (3-F-SA) (**Figure 1**), competitively inhibited MAIT cell activation to a similar or lesser extent as 6-FP. Some non-pyrimidine ligands were able to stimulate a MAIT TCR reporter cell line to varying degrees, of which diclofenac was most potent and whose activity was assigned to one of its metabolites, 5-OH-diclofenac (29) (**Figure 1**). Unlike the pyrimidine and ribityllumazine antigens which are contacted in a hydrogen bond by a conserved residue in the MAIT TCRα chain (Tyr95α), the 5-OH group of 5-OH-diclofenac was contacted in a hydrogen bond by Glu99 from the CDR3β loop. This matched the observation that 5-OH-diclofenac only activated one of a panel of MAIT TCR reporter cell lines, indicating that the MAIT TCRβ chain was able to "fine-tune" responsiveness to certain ligands (29), in line with an emerging concept that some MAIT cells are capable of discriminating between different classes of antigens based on differences in the MAIT TCRβ chain (28, 73, 80, 88, 89). A weak stimulator, 5-formyl-salicylic acid, also only activated one of a panel of MAIT TCR reporter cell lines, suggestive of a similar mechanism (29). Except for diclofenac/5-OH-DCF, all MR1 ligands, of which crystal structure bound to MR1 and complexed with a MAIT TCR were determined as part of this study, featured a Schiff base with Lys43 of MR1 (26, 27, 29) (**Figure 1**).

In summary, accumulated data thus far (26–29, 35) indicate that MR1 has the capacity to bind structurally diverse small molecules (150–400 Da). Most of the MR1 ligands (except for RL-6-Me-7-OH and Diclofenac/5-OH-diclofenac) form a Schiff base with Lys43 of MR1. However, regardless of the Schiff base, all ligands are located broadly within a similar location in the A'-pocket of MR1, although some are displaced; or oriented differently, involving mostly rotations with broadly similar depositions of the planes of the aromatic rings (26, 27, 29, 35). One notable exception is diclofenac/5-OH diclofenac, whose ring was not deposed in the same plane, rather, the central ring was essentially perpendicular to that of any of the other MR1 ligands (**Figure 1**) (29). Both in the context of bacterial infections (73, 88), cancer and steady-state (44, 90) there is evidence for the existence of additional MR1 ligands, including some that do not appear to be pyrimidines or ribityllumazines (73, 88), although the chemical identities of these remain to be defined. Some of these ligands appear to be recognized by T cells that do not necessarily share the phenotype of MR1-5-OP-RU specific MAIT cells, described in detail in the next section.

DIVERSE MR1-REACTIVE T CELLS

A range of MR1-antigen reactivity patterns have emerged for individual T cell clones and the TCRs they express. These reactivity patterns were identified based on (i) binding of T cell clones or TCR reporter cell lines to MR1-antigen tetramer (involving riboflavin-based antigens or other antigens) or mutated MR1 Lys43 to Ala (MR1-K43A) tetramers; and (ii) activation of T cell clones or TCR reporter cell lines in cellular assays with antigen presenting cells expressing physiological levels of MR1 or overexpressing MR1 or MR1-K43A. Notably, this is an active area of research and it is likely that additional reactivity patterns will emerge. Whilst the patterns observed so far are not always absolute, in that one reactivity might be less dominant, for simplicity they can be grouped as outlined below and illustrated in **Figure 3** and **Table 1**. Similarly, a particular T cell phenotype (TCR usage, surface markers, transcription factors) can be exclusively or more frequently associated with one particular antigen reactivity pattern, although in some cases significant overlaps in TCR usage (and other phenotypic characteristics) have been observed for TCRs with different MR1-antigen reactivity patterns.

(1) Reactivity to MR1-5-OP-RU/5-OE-RU (and less potent ribityllumazines) but not to other MR1-ligands. This reactivity is mediated by the population of cells referred to as MAIT cells, expressing the canonical MAIT TCR and phenotypic markers associated with the MAIT cell lineage in humans and mice (7, 24, 26, 28, 80). The same reactivity can also be mediated by a small fraction of human, TRAV1-2$^-$ T cells with diverse TCR usage (e.g., clone MAV36 that expresses a TRAV36$^+$ TCR), including some cells that feature a MAIT-like phenotype (CD161$^+$, IL-18Rα$^+$, CD218a$^+$, CD26$^+$) (80, 89). Notably, a follow-up study revealed that TRAV36$^+$ MR1 reactive T cells are possibly a second public TCR family, alongside MAIT cells, capable of specifically recognizing riboflavin-based antigens in the context of MR1 (80, 89). The *TRAV36* gene segment of these TCRs was not rearranged with the classical MAIT *TRAJ* gene segments (*TRAJ33, TRAJ12, TRAJ20*), and hence lacked a Tyr95α (80, 89). Reminiscent of the classical MAIT TCRα chain, they displayed a largely germline-encoded CDR3α loop. In stark contrast to canonical MAIT TCRs, these TCRs featured nearly invariant CDR3β loops of constrained length (14 amino acids) (89). Structural studies on the MAV36 TCR revealed the CDR1a loop was predominant

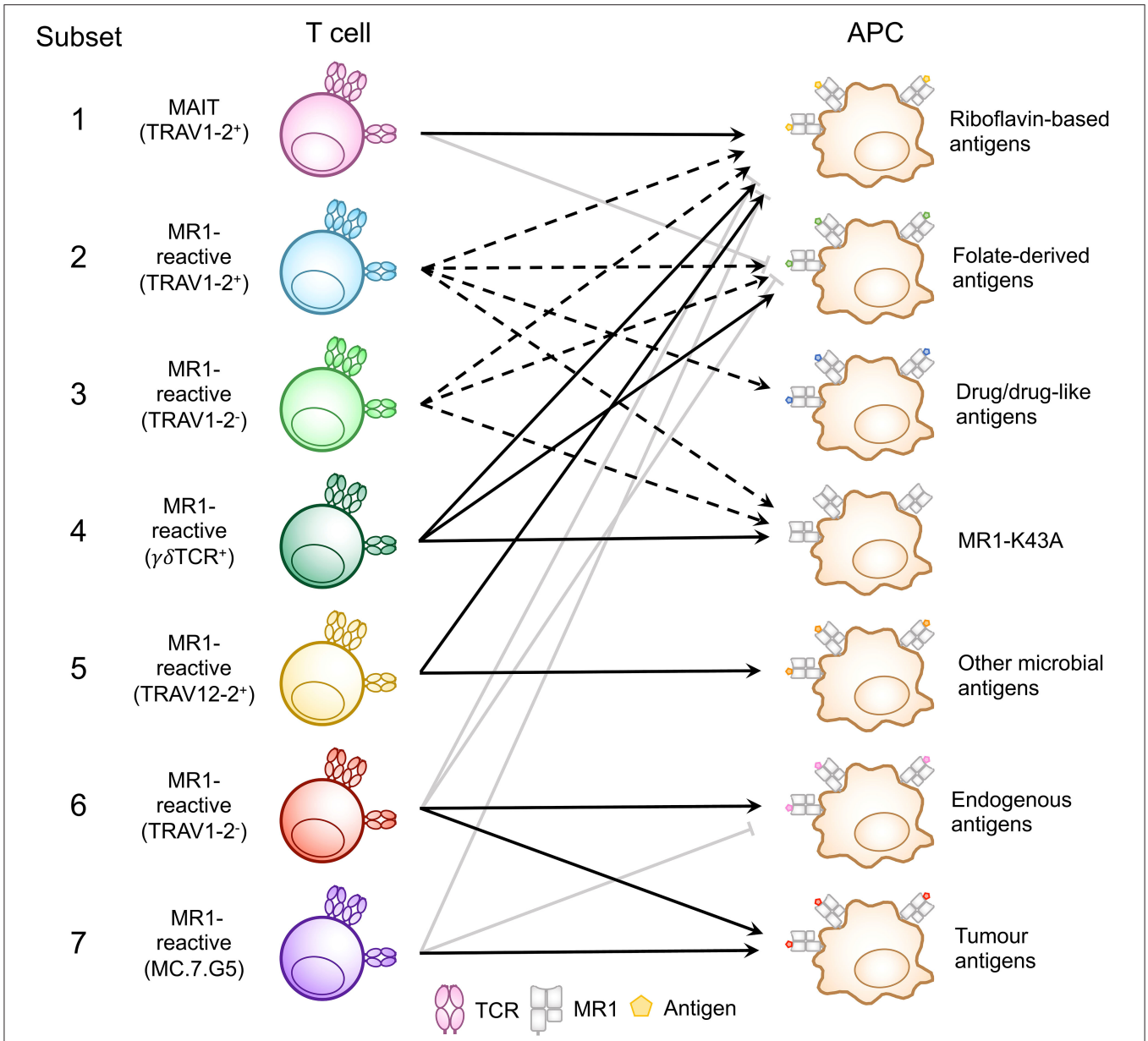

FIGURE 3 | Classification of known MR1-reactive T cells based on MR1-antigen reactivity pattern. MR1-reactive T cells bear an αβ or γδ T cell receptor (TCR) that recognizes one or more classes of antigen presented by MR1 on antigen presenting cells (APCs). Solid point arrows indicate that all T cells within the subset recognize the specified antigen, broken point arrows indicate that some T cells within the subset recognize the specified antigen and gray block arrows indicate no recognition of the class of antigen. **Table 1** outlines the frequency, TCR usage (where known) and surface characteristics of each subset using this classification. This schematic was inspired by **Figure 1** from a recent commentary piece (91).

at the MR1 interface, including contacting the antigen directly, analogous to the role of the CDR3α loop of classical MAIT TCRs (80). Not surprisingly, among TRAV1-2$^+$ T cells, MR1-5-OP-RU tetramer$^+$ cells predominate, the majority of which express a classical MAIT phenotype, while amongst TRAV1-2$^-$ cells, the frequency of MR1-5-OP-RU tetramer$^+$ cells is similar to that of MR1-pterin tetramer$^+$ cells [<0.2% of circulating T cells (80)], although it is perhaps higher among CD8$^+$ T cells (88). Regardless of antigen recognition pattern, generally TRAV1-2$^-$ MR1-reactive T cells are heterogeneous in surface markers and transcription factor profile, with the majority lacking CD45RO, PLZF, RORγt, and being heterogenous in T-bet expression, in line with developmental and functional differences compared to MAIT cells (80). Recently, novel MR1-reactive TCR rearrangements were identified from T cells in C57BL/6 mice deficient in the canonical MAIT junctional gene segment *TRAJ33* (89). In spite of genetic deletion of the *TRAJ33* gene segment, MAIT cells were detected using MR1-5-OP-RU tetramer, albeit at a dramatically reduced frequency (50-fold

TABLE 1 | Frequency, TCR usage (where known) and surface phenotype of each subset of MR1-reactive T cells as depicted in **Figure 3**.

Subset		Reactivity pattern	Frequency	TCR usage	Surface phenotype	References
1	MAIT (TRAV1-2$^+$)	1	Human: 3% of blood T cells Mouse: 0.1% of blood T cells, 0.1–5% in peripheral organs	Human: TCRα: TRAV1-2-TRAJ33, -TRAJ12, -TRAJ 20 TCRβ: TRBV6-1, TRBV6-4, TRBV20 Mouse: TCRα: TRAV1-TRAJ33, -TRA12, -TRAJ9, -TRAJ40 TCRβ: TRBV19, TRBV13	Human: TRAV1-2, CD161, ±CD4, ±CD8, IL-18R, CD26, ±CD56, CD45RO, CD218a, CD127 Mouse: ±CD4, ±CD8, ±NK1.1, CD44, ±CD103, CD127, CXCR6, IL-18R	(5, 7, 8, 10, 11, 24, 36, 38, 89)
2	MR1-reactive (TRAV1-2$^+$)	1, 2, 3	Human: <0.2% of blood T cells Mouse: ND	Human: TCRα: TRAV1-2-TRAJ33 TCRβ: TRBV6-2, TRBV6-4, TRBV20, TRBV4, TRBV5 Mouse: TCRα: TRAV1-TRAJ9 TCRβ: TRBV13-3	Human: TRAV1-2, ±CD161, ±CD45RO, CD8, ±CD56 Mouse: CD44	(29, 30, 80)
3	MR1-reactive (TRAV1-2$^-$)	1, 2, 3	Human: <0.2% of blood T cells Mouse: ND	Human: TCRα: TRAV36-TRAJ34 (lack Tyr95α), diverse TCRβ: TRBV28-TRBV2-5, diverse Mouse: TCRα: TRAV16, diverse TCRβ: TRBV13, diverse	Human: ±CD161, ±CD45RO, CD8, ±CD218a ±CD26 Mouse: CD44	(80, 89)
4	MR1-reactive (gd TCR$^+$)	3	Human: <0.1% of blood T cells	Human: TCRβ: TRDV1, TRDV3, TRDV5 TCRβ: TRGV8, TRGV5, TRGV4, TRGV3, TRGV2, TRGV9	Human: ±CD161, ±CD4, ±CD8	(96)
5	MR1-reactive (TRAV12-2$^+$)	2	Human: ND	Human: TCRα: TRAV12-2-TRAJ39 TCRβ: TRBV29-1, TRBJ1-5	Human: CD8, CD45, CD26, CD161$^-$	(73, 88)
6	MR1-reactive (TRAV1-2$^-$)	4	Human: <0.04% of blood T cells	Human: TCRα: diverse TCRβ: diverse	Human: ±CD8, ±CD161	(44)
7	MR1-reactive (MC.7.G5)	4	Human: ND	Human: TCRα: TRAV38-2/DV8-TRAJ31 TCRβ: TRBV25-1-TRBJ2-3	Human: CD8	(90)

ND, not determined.

less) compared to wildtype mice (89). MR1-reactive T cells from *TRAJ33* knockout (KO) mice formed two distinct subsets based on TCR usage: those that expressed classical MAIT TCRs (TRAV1, Tyr95α) with more diverse junctional gene segments; most notably *TRAV1-TRA12/TRAJ9/TRAJ40* rearrangements, or MR1-reactive T cells with more diverse TCR usage altogether (non-TRAV1) (89). T cell clones that expressed the classical MAIT TCRs, derived from the *TRAJ33* KO mice, recognized MR1-5-OP-RU tetramers but not MR1-Ac-6-FP tetramers, verifying their specificity for the riboflavin-based antigens. In contrast, amongst the more diverse TRAV1$^-$ MR1-reactive T cell clones, none were solely MR1-5-OP-RU specific (see recognition pattern 2) (89).

(2) Reactivity to MR1-5-OP-RU/5-OE-RU (and less potent ribityllumazines) and other MR1-ligands. This reactivity involves ligand-dependent crossreactivity, a well-known concept in T cell biology. Examples include the cross-recognition of pterins (6-FP and Ac-6-FP) and pyrimidines (5-OP-RU) by < 5% of human TRAV1-2$^+$ T cells (e.g., clones AM1, AM2, AM3) that expressed a classical MAIT TCR, typically featuring the *TRAV1-2-TRAJ33* rearrangement and no discernible TCRβ motifs (80). Only a very small fraction of MR1-reactive TRAV1-2$^-$ T cells (e.g., clone MAV14, TRAV14) in humans were identified to be crossreactive (80, 89). All of these TRAV1-2$^-$ T cells expressed TCRs with diverse usage and only one TCR sequence featured the Tyr95α (80). Differential antigen recognition was a common feature among both TRAV1-2$^+$ and TRAV1-2$^-$ crossreactive T cells, whereby some TCRs preferred antigens of a specific class (riboflavin-based or folate-derived), while others were capable of distinguishing between antigens of the same class (6-FP or Ac-6-FP) (80). A TRAV1-2$^-$ T cell clone (D462-E4, *TRAV12-2*) reacted to MR1-5-OP-RU tetramer and responded to RL-6-Me-7-OH but not RL-6,7-diMe and in addition to infection with *Streptococcus pyogenes* which lacks the riboflavin biosynthetic pathway, suggestive of an undefined riboflavin-independent MR1 antigen (88). The same clone also responded moderately to PLI, to a level similar as compared to RL-6-Me-7-OH (73). This clone did not express the MAIT cell marker

CD161, although it might have been downregulated as a result of T cell expansion with anti-CD3 (88). The same research team also identified another TRAV1-2$^+$ T cell clone (D481-C7) which also reacted with MR1-5-OP-RU tetramer, preferentially recognized RL-6,7-diMe over RL-6-Me-7-OH, and potently responded to PLI and PLIII (73). Some MAIT TCR expressing reporter cell lines and subsets of primary TRAV1-2$^+$ MAIT cells also displayed crossreactivity to chemically diverse drugs, drug metabolites and drug-like molecules (29, 30).

(3) Auto-reactivity to MR1, a more MR1-centric reactivity compared to antigen-centric reactivity. This type of reactivity was found amongst human TRAV1-2$^+$ T cells, where clone M33-64, which expressed a classical MAIT TCR, stained with MR1 tetramer loaded with 5-OP-RU or pterin antigens as well as MR1-K43A tetramer. A matching TCR reporter cell line displayed MR1-dose dependent activation and limited antigen dose dependency (80). Surprisingly, a mutation of Tyr95α to Phe in the M33-64 TCR did not abolish MR1-5-OP-RU reactivity, in stark contrast to all previously studied MAIT TCRs, which was attributed to a dominant role of the CDR3β loop in both stabilizing the TCR and in making considerable contacts with MR1 (80). Autoreactivity in the context of MR1 overexpressing cell lines was also observed in <15% of mouse hybridomas derived from TAP$^{-/-}$ C57BL/6 mice (clones 6C2, 6H2, 18G7, 8D12) (5, 6, 92–95) and <10% of hybridomas from Vα19Tg Cα$^{-/-}$ mice, all featuring canonical MAIT TCRs (94). Interestingly, based on extensive mutagenesis, the 6C2 hybridoma used overlapping but distinct TCR residues for the recognition of MR1 on *E. coli* infected cells vs. MR1 overexpressing cells in the absence of infection (95). Recently MR1-autoreactivity was also found to be mediated by γδ TCR$^+$ T cells, present in human PBMCs (<0.001 to 0.1% of CD3$^+$ T cells and from <0.1 to 5% of γδ T cells) and in tissues (liver, stomach, lung, and duodenum), the frequencies of which may be enriched in association with some diseases (96). Some MR1-reactive γδ TCRs bound underneath the antigen binding cleft (contacting primarily the α3 domain of MR1), while others interacted with the antigen binding cleft of MR1, akin to the classical MAIT αβ TCR. The former type was observed in blood of 6 out of 20 individuals. Activation of γδ TCR expressing reporter cell lines varied and was dependent on MR1 with some potential for modulation by MR1-bound antigen. MR1-reactive γδ TCRs were diverse in gene usage. Most (72%) used TRDV1; the remainders, aside from one TRDV5$^+$ clone, expressed TRDV3. Whilst all functional TRGV genes including TRGV2, 3, 4, 5, 8, and 9 were found amongst MR1-reactive γδ T cells, TRDV1$^+$ and TRDV3$^+$ TCRs predominantly paired with TRGV8. MR1-5-OP-RU tetramer$^+$ γδ T cells were mostly CD4$^-$CD8α$^-$ or CD8α$^+$ with variable CD161 expression. Thus, they resembled other cells of the γδ T cell lineage and appeared phenotypically diverse (96). Whether MR1-reactive γδ T cells exist in mice or other species remains to be investigated.

(4) Reactivity to other MR1-ligands but not MR1-5-OP-RU/5-OE-RU (and less potent ribityllumazines). Such reactivity pattern was first observed for the TRAV1-2$^-$ MAV21 TCR (TRAV21) which specifically recognized pterins but not 5-OP-RU (80). Recently, Crowther et al. identified another TRAV1-2$^-$ T cell clone in blood of a healthy individual (MC.7.G5, *TRAV38.2/DV8-TRAJ31, TRBV25.1 TRBJ2.3*) which specifically recognized an antigen/s that was expressed or upregulated in cancerous cells but not non-cancerous cells (resting, activated, stressed or infected), in an MR1-dependent manner (90). Interestingly, the observed T cell reactivity was donor-unrestricted, with multiple HLA-mismatched tumor cells recognized in an MR1-dependent manner, whilst not exerting allo-reactivity, making this T cell clone particularly interesting for immunotherapies (90). Potent activation, including cytotoxicity, was achieved with physiological levels of MR1 and high effector (T cell) to target (APC) ratios. Both Ac-6-FP and bacterial riboflavin-based antigens were not recognized and acted as competitive inhibitors of activation. Whilst a clone with a similar reactivity pattern was also isolated from a second donor, the frequency of similar clones within an individual and their prevalence in the general population and in the context of cancers are unknown. Also, the phenotype of the clones (other than the TCR of one of them) was not described which may provide insights into its developmental origin. Previously, Lepore et al. (44) had identified a population of T cells in healthy individuals that comprised 1 in 2,500–5,000 of circulating T cells. Designated "MR1T" cells, clones of these cells recognized, in the context of physiological levels of MR1 and dependent on MR1, endogenous cellular antigens from various tissues, including cancer tissues, and thus were not cancer specific. One MR1T cell clone was TRAV1-2$^+$ (clone DGA4), but most were TRAV1-2$^-$, expressing diverse TCRα and β chains (e.g., clone DGB129; *TRAV29/DV5-TRAJ23*; and *TRBV12-4-TRBJ1-1*) (44). Like the MC.7.G5 clone, MR1T cell clones (e.g., DGB129) did not recognize 6-FP and their activation was inhibited by 6-FP. However, unlike the MC.7.G5 clone, which did not bind MR1-K43A tetramer or respond to MR1-K43A overexpressing cells (90), MR1T cell clones equally recognized wild type and MR1-K43A overexpressing cells. This suggested to the authors that relevant antigens that were recognized did not depend on Schiff base formation with MR1 for TCR recognition but may also suggest an MR1-centric recognition or MR1 autoreactivity by some MR1T cells for which 5-OP-RU/ribityllumazine are permissive antigens but pterins are not. At least two clones (DGB129 and DGB70) did however, differentially recognize fractions of cell/tumor lysates, demonstrating differential antigen specificity for at least some clones. With the exception for one MR1T clone, all TRAV1-2$^-$ clones were CD161$^-$ and all, except for one, were CD8α$^+$ (DGB129, TRAV1-2$^-$, CD161$^-$, CD8α$^+$; DGB70, TRAV1-2$^-$ [TRAV5], CD161$^-$, CD8$^-$). DGA4, the TRAV1-2$^+$ clone, was CD161$^+$, CD8$^-$. Phenotypic and functional characterization of MR1T cell clones showed multiple chemokine receptor expression profiles

and secretion of diverse effector molecules, suggesting functional heterogeneity within this population that was also distinct from that of MAIT cells. In a T-helper like function, clone DGB129 induced maturation of monocyte derived dendritic cells and another clone suggested the possible contribution of MR1T cells to intestinal epithelial barrier homeostasis.

DISCUSSION

We are beginning to appreciate the diversity of microbial and endogenous (including cancer) antigens presented by MR1 and the heterogenous populations of T cells which recognize them. Independent and repeated identification of these cells has led to the classification "MR1-reactive T cells," illustrated in **Figure 3** and **Table 1**, inclusive of MR1-restricted MAIT cells, and more phenotypically and functionally heterogenous T cells, some of which are MAIT-like in their phenotype, grouped together as "other MR1-reactive T cells" (44, 80, 89, 91). Whilst TRAV1-2$^+$ T cells are dominant amongst MAIT cells, other MR1-reactive T cells mostly include TRAV1-2$^-$ αβ TCR$^+$ T cells (44, 73, 80, 89, 90) as well as γδ TCR$^+$ T cells (96). Thus, the current stage of research suggests that most MR1-reactive T cells, namely MAIT cells, feature a biased TCR α-chain, and react with a limited repertoire of pyrimidine and ribityllumazine antigens derived from riboflavin biosynthesis. There are also several examples of TCR β-chain usage influencing antigen preference and allowing for antigen crossreactivity by classical MAIT TCRs, and future work might identify additional examples of physiological relevance. The emerging descriptions of other MR1-reactive T cells highlight that the classical MAIT TCR α-chain is not the only solution that warrants MR1-reactivity, however, more work is needed to validate and corroborate the TCR usage and antigen specificities of other, more diverse T cells. In particular, determining the chemical identities and biosynthetic origins of the tumor-associated and endogenous antigens recognized by some of the other MR1-reactive T cells will allow the generation of tetramers which will greatly assist in determining the prevalence of these cells as well as their potentially important role in anti-tumor immunity and other immune-functions. It will also help to better characterize the phenotype (including the TCR usage) and of this diverse set of T cells including public as compared to private existence of individual clones, recently described for TRAV36$^+$ MR1-reactive T cells (89). Another interesting question concerns the lineage development of other MR1-reactive T cells that feature a non-MAIT phenotype. Is it possible that these cells might have originated from another T cell lineage and cross-react across MHC families, including classical MHC molecules (MHC-I and MHC-II) and MHC-I-like molecules? In TCR α-chain transgenic mice that lack MR1 (*Vα19iCα*$^{-/-}$*MR1*$^{-/-}$) MR1-5-OP-RU tetramer$^+$ T cells make up ∼32% of T cells in the spleen as compared to ∼50% in mice proficient in MR1 (*Vα19iCα*$^{-/-}$) (97). The population of these cells mediated both MR1-dependent and MHC-I (H2-K^b/H2-D^b) dependent reactivity *in vitro* (97), although it was not investigated whether these specificities were mediated by single T cells and single TCRs. More recently, MR1-5-OP-RU tetramer$^+$ T cells featuring the canonical MAIT TCRα chain (*TRAV1-TRAJ33*) but not the typical TRBV13 bias were also found in very low percentages in the thymus of non-transgenic mice, *Mus musculus castaneus* (CAST mice), modified to lack MR1 (86). Thus, MR1-5-OP-RU tetramer$^+$ T cells can develop in the absence of MR1, inferring selection may occur on other MHC molecules. Indeed, MR1 is highly conserved with HLA-A2 (39% sequence conservation), suggesting mimicry as a potential mode of crossreactivity by these cells. So far evidence of peripheral T cells that crossreact between classical MHC molecules and MHC-I-like molecules remains elusive.

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MS and SE wrote and revised the manuscript, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: SE is an inventor on patents describing MR1 antigens and MR1 tetramers.

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MAIT Cells at the Fetal-Maternal Interface During Pregnancy

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One of the main functions of the human placenta is to provide a barrier between the fetal and maternal blood circulations, where gas exchange and transfer of nutrients to the developing fetus take place. Despite being a barrier, there is a multitude of crosstalk between maternal immune cells and fetally derived semi-allogeneic trophoblast cells. Therefore, the maternal immune system has a difficult task to both tolerate the fetus but at the same time also defend the mother and the fetus from infections. Mucosal-associated invariant T (MAIT) cells are an increasingly recognized subset of T cells with anti-microbial functions that get activated in the context of non-polymorphic MR1 molecules, but also in response to inflammation. MAIT cells accumulate at term pregnancy in the maternal blood that flows into the intervillous space inside the placenta. Chemotactic factors produced by the placenta may be involved in recruiting and retaining particular immune cell subsets, including MAIT cells. In this Mini-Review, we describe what is known about MAIT cells during pregnancy and discuss the potential biological functions of MAIT cells at the fetal-maternal interface. Since MAIT cells have anti-microbial and tissue-repairing functions, but lack alloantigen reactivity, they could play an important role in protecting the fetus from bacterial infections and maintaining tissue homeostasis without risks of mediating harmful responses toward semi-allogenic fetal tissues.

Keywords: MAIT cells, placenta, pregnancy, decidua, intervillous blood

INTRODUCTION

During pregnancy, the maternal immune system is confronted with foreign antigens derived from the semi-allogenic fetus and placenta. A challenging task is therefore to display tolerance toward the HLA-disparate fetus and at the same time maintain anti-microbial responses. Feto-maternal tolerance is retained due to several mechanisms, including physical barriers, a diminished expression of polymorphic HLA molecules on fetal trophoblast cells, and production of immunosuppressive factors from fetally derived cells including trophoblasts, as well as maternally derived cells including both stromal cells and immune cells (1, 2). However, it is evident that the

maternal immune system not only detects but also reacts toward fetal antigens. For instance, it has been shown that women during early pregnancy transiently increase T cell-mediated responses toward tumor-associated antigens that are highly expressed by fetal trophoblasts in the placenta, including HER2 and WT1 (3). Furthermore, fetal DNA and fetal immune cells are detected in the maternal circulation (4), and anti-HLA antibodies are often developed during pregnancy (5).

PLACENTAL STRUCTURE AND FETAL-MATERNAL INTERFACE

A main function of the placenta is to provide the developing fetus with nutrients and gas exchange through an intricate placental blood circulation system. The maternal placental circulation is gradually established during the first trimester (6), and from the second trimester until birth, maternal arterial blood delivers oxygen, IgG antibodies and nutrients over a thin membrane of fetally derived cells to the fetal blood circulation via the umbilical cord (7) (**Figure 1A**). Maternal immune cells are in close contact with semi-allogeneic fetal trophoblast cells in two anatomically different parts of the placenta; in the decidua and in the intervillous space (**Figure 1B**). The decidua is a specialized tissue emanating from the uterine endometrium, which functions to prepare for and accommodate pregnancy. The decidua is invaded by both maternal immune cells and fetal extravillous trophoblasts during early pregnancy. The extravillous trophoblasts play an important role in the remodeling of the spiral arteries (8), thereby securing the maternal blood flow into the intervillous space from the second trimester, where nutrients and gas exchange to the fetus takes place over the syncytiotrophoblast layer of the chorionic villi (**Figure 1B**).

MATERNAL IMMUNE CELLS AT THE FETAL-MATERNAL INTERFACE

The composition of maternal immune cells in the decidua has been characterized both in early and term pregnancy, showing that first trimester decidua is dominated by $CD56^{bright}$ NK cells with few T cells, whereas the proportion of T cells increases substantially at term (9). Macrophages in the decidua maintain their proportion during pregnancy and display immune regulatory actions (10). Maternal decidual stromal cells are tissue resident cells that can suppress immune activation (11, 12). It has been suggested, based on murine studies, that these cells prevent activated maternal T cells from entering the decidua in early pregnancy by silencing of the T cell-attracting chemokines CXCL9 and CXCL10 (13). In humans, the mechanisms for the relative low proportion of T cells in the first trimester decidua are not known. Regulatory T cells are enriched in the decidua (14), and both γδ T cells and $CD8^+$ T cells make up larger portions relative to $CD4^+$ T cells compared with blood (2). γδ T cells have been suggested to have a protective role during early pregnancy by producing IL-10 and promote trophoblast survival and invasion (15). Invariant NKT cells are also enriched in the decidua relative to peripheral blood (16). Activation of NKT cells with the CD1d agonist αGalCer promotes pregnancy loss in murine models (17). Single cell analysis of the early fetal-maternal interface in the decidua has further identified predicted regulatory interactions between maternal immune cells and fetal trophoblasts that prevent harmful immune reactions (18). For instance, extravillous trophoblasts highly express the genes encoding PD-L1 and CD155, which could inhibit cytotoxic responses by T cells and NK cells via PD-1 and TIGIT ligation, respectively.

In contrast to the decidua, very little is known about the composition and function of maternal immune cells in the intervillous space, in which fetal villous tissue bathes in maternal blood (**Figure 1B**). The general notion has been that the blood volume in the intervillous space is replaced 2–3 times every minute to provide gas exchange (7), suggesting that the intervillous blood cell composition reflects that of peripheral blood. However, others (19, 20) and our own recent studies (21–23) show that NK cells and certain T cell and B cell subsets are enriched in the intervillous blood, indicating that particular cell types are sequestered in the intervillous space, which is discussed in more detail below. Similar to the spleen and liver, in which a proportion of the circulating blood is shunted into the low-pressure pools in the sinusoids, it is likely that maternal blood constituents entering the intervillous space are retained inside the placenta. Mucosal-associated invariant T (MAIT) cells are one type of immune cell subset that is relatively enriched in intervillous compared to peripheral blood at term pregnancy (21, 22) and MAIT cells are also present in decidual tissues (24).

MAIT CELLS AT THE FETAL-MATERNAL INTERFACE AND IN UTERINE ENDOMETRIUM

In contrast to conventional T cells, which need to get their peptide antigen presented on highly polymorphic MHC molecules, MAIT cells are restricted to the monomorphic MHC-like receptor 1 (MR1) molecule (25). MAIT cells express the semi-invariant T cell receptor alpha chain Vα7.2 (TRAV1-TRAJ33) (26), and respond to vitamin B2 metabolites in an MR1-dependent manner (27). These non-peptide ligands are produced by microbes with a functional riboflavin biosynthesis pathway (27–31), including many commensal and pathogenic bacterial and fungal species. Inflammatory cytokines, such as IL-12 and IL-18, can also partially activate MAIT cells without the need for TCR-ligation (32), which broadens their capacity to also be involved in anti-viral and inflammatory responses (33). The majority of MAIT cells are $CD8^+$, but a subset of MAIT cells lacks the expression of both CD4 and CD8 (double-negative, DN), and a minor fraction expresses CD4 (34–36). MAIT cells display a memory phenotype and they respond quickly by producing cytotoxic molecules and inflammatory cytokines upon activation. Moreover, there is emerging evidence suggesting that MAIT cells also express tissue repair signatures upon TCR-ligation (37–40). Thus, MAIT cells are anti-microbial and tissue-repairing T cells that lack the capacity to respond to allogeneic HLA molecules, which could be ideal traits of effector cells at the fetal-maternal

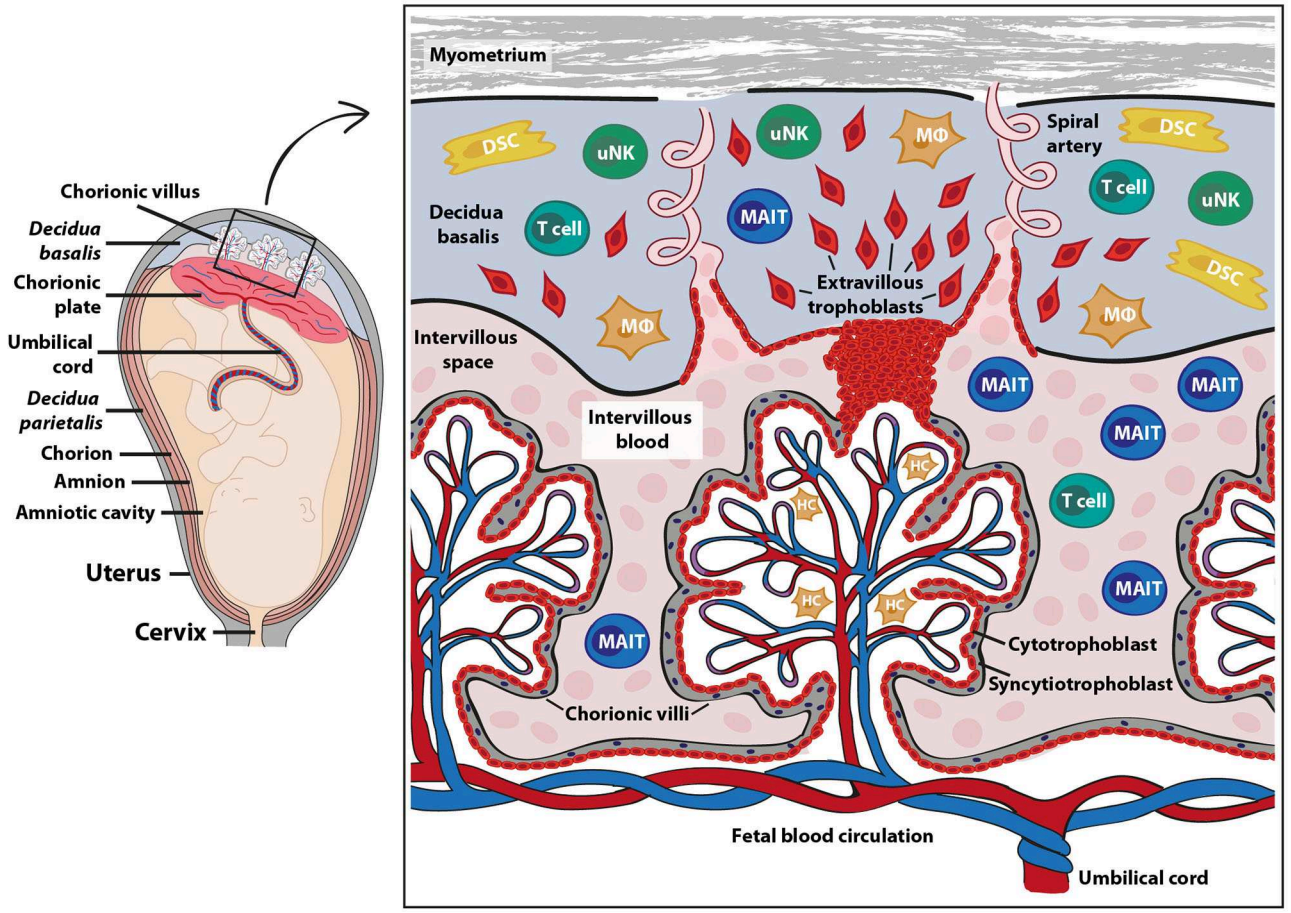

FIGURE 1 | Fetal-maternal interface and immune cells at term pregnancy. The placenta serves to ensure exchange of nutrients and gases between the maternal and fetal blood circulation. The fetal part of the fetal-maternal interface consists of chorionic villi that extend from the chorionic plate **(A)** into the intervillous space and bathe in maternal intervillous blood **(B)**. On the maternal side, the decidua parietalis and decidua basalis are in direct contact with fetal membranes (amniochorion) and the invading fetal extravillous trophoblasts, respectively **(A)**. The intervillous blood enters the intervillous space through spiral arteries **(B)** and leaves this compartment through uterine veins (not shown). Maternal immune cells in the intervillous blood are in direct contact with the fetal syncytiotrophoblast and decidual immune cells can interact with extravillous trophoblasts **(B)**. DSC, decidual stromal cells; HC, Hofbauer cells (fetal macrophages); MAIT, mucosal associated invariant T cells; MΦ, macrophages; uNK, uterine natural killer cells. The pink cells in the intervillous space depict erythrocytes.

interface. On the other hand, since MAIT cells respond quickly and are capable of inducing prominent inflammation, they must also be kept under strict control.

In healthy term pregnancies, pregnant women had lower proportions of MAIT cells in the circulation compared to non-pregnant women, suggesting that MAIT cells home to the placenta (22). Indeed, the proportion of MAIT cells among both $CD3^+$ T cells and total $CD45^+$ cells is approximately 2-fold higher in placental intervillous blood compared to peripheral blood in healthy term pregnancies (21). Ravi et al. showed that proportions of peripheral MAIT cells were unaltered during the course of pregnancy (41), and since MAIT cell proportions were not investigated before pregnancy it can be speculated that MAIT cells localize to the placenta or other tissues already during early gestation. Although leukocyte counts increase during pregnancy, the lymphocyte concentration shows a slight decrease from early to late pregnancy, which can be attributed to the hemodilution that takes place because of the physiological increase in plasma volume during pregnancy (42). However, since the proportion of T cells in blood does not change during pregnancy (43) and since most studies report MAIT cell frequencies as proportion of $CD3^+$ T-cells, early and late pregnancy can safely be compared.

Intervillous MAIT cells exhibit a stronger IFN-γ and granzyme B expression compared to paired peripheral MAIT cells in response to riboflavin-producing *Escherichia coli* (21). However, in a resting state, intervillous and peripheral MAIT cells express similar levels of the activation markers HLA-DR

and CD69, while intervillous MAIT cells express lower levels of CD25 and PD-1 (21, 22). Intervillous MAIT cells consist of a higher proportion of DN MAIT cells compared to peripheral MAIT cells. This is in accordance with the reported decrease in peripheral DN MAIT cells from the first to the third trimester (41), which could potentially reflect their localization to the intervillous space of the placenta. Together, these data suggest that maternal MAIT cells in the intervillous space of the placenta at term pregnancy display an increased inflammatory response to riboflavin-producing bacteria and that there are phenotypic differences between peripheral and intervillous MAIT cells. It remains to be determined if the elevated inflammatory response of intervillous MAIT cells is due to intrinsic properties or whether extrinsic effects, such as antigen presentation or soluble factors, are involved in potentiating the response compared to peripheral MAIT cells.

MAIT cells are present also in the endometrium and cervix of the genital tract of non-pregnant women, but the endometrium contains lower frequencies of MAIT cells out of $CD3^+$ T cells compared to peripheral blood (44). After fertilization, the endometrium undergoes decidualization to form the decidua. The part of the decidua underlying the placental disc, which is perfused by spiral arteries to provide the intervillous space with maternal blood, is termed decidua basalis (**Figures 1A,B**). The decidua parietalis refers to the decidual layer that is attached to the fetal membrane, consisting of the fused chorion and amnion which create the amniotic sac. For early pregnancy, it is known that MAIT cells are present in the decidua (18), but there is no information on their relative abundance, phenotype or location. In contrast to the non-pregnant endometrium, which contains fewer MAIT cells compared to peripheral blood, the proportion of MAIT cells in term pregnancy decidua parietalis is similar to peripheral MAIT cells, and MAIT cells are even more abundant in the decidua basalis compared to the decidua parietalis (24). This may suggest that MAIT cells to some degree home to the decidual mucosa at term pregnancy.

Decidual MAIT cells at term express high levels of CD69, consistent with a tissue-residency phenotype (21, 24). MAIT cells in decidua parietalis express higher levels of PD-1, CD38 and CD25 compared to MAIT cell in decidua basalis, indicating a more activated phenotype (24). While endometrial MAIT cells are biased toward IL-17 and IL-22 expression, with less production of IFN-γ and granzyme B (44), decidual MAIT cells produce higher levels of granzyme B and similar levels of IFN-γ in response to *E. coli* as compared to peripheral MAIT cells (21). It is not yet known if decidual MAIT cells also have a propensity to produce IL-17 and IL-22. Mucosal production of IL-17 and IL-22 is important for anti-bacterial and anti-fungal responses and mucosal barrier function, respectively (45, 46), suggesting that it would be an advantage also for decidual MAIT cells to possess this function. It remains to be determined how decidual MAIT cells in early and late pregnancy are polarized in terms of cytokine production, but in contrast to genital tract MAIT cells it appears that IFN-γ and cytotoxic molecule secretion from term decidual MAIT cells are comparable to that of peripheral MAIT cells, indicating that pregnancy may affect the functional responses of uterine MAIT cells.

CHEMOKINE-INDUCED ATTRACTION OF MAIT CELLS TO THE PLACENTA

Intervillous MAIT cells do not express the proliferation marker Ki67, suggesting that they are in a non-cycling state (21). It can therefore be speculated that the increased proportion of MAIT cells in the intervillous space is due to recruitment and retention by chemotactic factors. The fetal placenta and its trophoblasts produce a wide array of chemokines (22, 47), and maternal platelets in the intervillous space may also contribute to local chemokine release (48). Interestingly, the chemokine pattern in intervillous plasma is clearly different compared to paired peripheral plasma, with higher levels of several chemokines, including macrophage migration inhibiting factor (MIF), CCL2, CCL25, CXCL9, and CXCL10 (22). Other chemokines are instead lower in intervillous compared to peripheral plasma, including CCL21 and CCL27 (22). MAIT cell proportions in intervillous blood and in decidua are positively associated to levels of MIF and CCL25 in intervillous plasma. Migration assays have shown that conditioned medium from term fetal placental tissues attracts effector memory T cells in general and MAIT cells in particular, and that MIF is one of the factors involved in attracting MAIT cells (22). MIF is a chemokine-like cytokine that binds to CXCR4 and CXCR2 (49). MAIT cells express high levels of CXCR4 but low levels of CXCR2 (22, 34), suggesting that CXCR4 is an important receptor for MIF-mediated homing of MAIT cells. $CD8^+$ and DN MAIT express similar proportions of CXCR4 (50), and both subsets migrated to the same extent toward placental conditioned medium (22). However, $CD8^+$ MAIT cells have been described to express higher levels of CCR6 compared to DN MAIT cells (51).

In contrast to MAIT cells, proportions of conventional $CD8^+$ effector memory T cells, which are also enriched in intervillous blood of term placentas, showed no correlation to MIF levels but to the CXCR3-ligands CXCL9, CXCL10, and CXCL11 in intervillous plasma (22). Moreover, the levels of the CCR6-ligand CCL20 is correlated to proportions of mature naïve B cells in intervillous blood (23). This suggests that different kinds of chemokines are involved in attracting and retaining distinctive immune cell subsets to the placenta, but it is likely a combination of different chemokines that shapes the composition of immune cell subsets in the intervillous space. It should also be noted that other chemokines could play a more prominent role in attracting MAIT cells to other types of tissues. For instance, MAIT cells have been suggested to home to ascites in liver cirrhosis patients by CXCR3-CXCL10 ligation (52) and to the liver by CXCR6 and CCR6 and their ligands CXCL16 and CCL20, respectively (53).

Interestingly, both syncytiotrophoblasts and extravillous trophoblasts highly express the chemokine decoy receptor D6/ACKR2, which can decrease chemokine availability to control leukocyte migration (54). D6 internalizes and degrades inflammatory CC chemokines which are ligands to the classical chemokine receptors CCR1-CCR5 (55). It can be speculated that this atypical chemokine receptor with CC chemokine scavenging function can play a role in regulating the number and position of maternal immune cells at the fetal-maternal interface. It remains

to be determined if D6 is involved in shaping the immune cell composition with increased accumulation of MAIT cells, effector memory T cells and mature naïve B cells in the intervillous space.

ANTIGEN-PRESENTING MOLECULES ON CELLS IN THE PLACENTA

The syncytiotrophoblasts, which line the fetal villi and are in immediate contact with maternal blood in the intervillous space (**Figure 1B**), appear to lack expression of the MR1 molecule both at early second trimester and at term (21). This is in line with the absence of HLA molecule expression on syncytiotrophoblasts from the second trimester (56). Thus, this lack of antigen presenting molecules could prevent any MR1- or HLA-mediated cytotoxicity toward the fetal syncytiotrophoblasts by maternal MAIT cells and T cells, respectively. It is not yet known if extravillous trophoblasts express MR1, but they do express HLA-C and the non-classical oligomorphic HLA-E, HLA-G and HLA-F at varying intensities during gestation (56). Extravillous trophoblasts also express CD1d in early pregnancy, suggesting that NKT cells may interact with fetal cells in the decidua (16). Fetal macrophages (also called Hofbauer cells) in the fetal villi (**Figure 1B**) express MR1 both at second trimester and at term, indicating that they can function as antigen-presenting cells to MAIT cells if the barrier of the fetal syncytium is broken (21). $CD8^+$ maternal T cells can be detected inside the villi in villitis of unknown origin, a non-infectious condition that is associated with fetal growth restriction (57). Whether maternal MAIT cells are present in the villi during this condition is not yet known. $MR1^+$ cells are also detected in term decidua and some of these cells are macrophages, as assessed by CD68 expression (21), indicating that decidual macrophages have the potential to present antigens to MAIT cells.

CAN MAIT CELLS ENCOUNTER THEIR ANTIGENS IN THE PLACENTA?

It has long been thought that the placenta is devoid of microbes in healthy pregnancies. This perception was challenged by studies suggesting that the placenta has its own microbiome (58, 59), but emerging evidence indicates that the detection of the placental microbiome may have been caused by contamination during the analysis process (60–62). However, De Goffau et al. observed that approximately 5% of placentas contained *Streptococcus agalactiae*, which was concluded to not be due to contamination (63). *S. agalactiae* is associated with commensal carriage, but is also a neonatal pathogen since it can cause neonatal sepsis (64). *S. agalactiae* strains possess riboflavin operons (65), suggesting that they have the potential to produce MR1-ligands and act as MAIT cell targets. Seferovic et al. used 16S *in situ* hybridization to visualize bacteria in healthy placental tissues and found that microbes were present at low abundance and preferably were localized to the villous parenchyma and syncytiotrophoblast layers. Thus, the current literature suggests that bacterial cells occasionally are present in the healthy placenta. Intervillous and decidual MAIT cells could play a role in preventing bacteria from crossing the fetal-maternal barrier.

TISSUE-REPAIRING CAPACITY OF MAIT CELLS

Apart from mediating pro-inflammatory responses upon infection, MAIT cells have recently been described to express a functional gene signature of tissue repair (37–40) and to have tissue protective capacities in murine models of inflammation (66, 67). The tissue repair function of MAIT cells is dependent on TCR-triggered activation, indicating that activation of this pathway is dependent on the presence of riboflavin-producing bacteria. The activating bacterial MR1-ligand 5-OP-RU can cross epithelial barriers (68), and it could potentially be present in organs devoid of infection, including the placenta. It is possible that intervillous MAIT cells are involved in maintaining barrier integrity to protect the fetal villi from barrier disruption and other placental lesions. Discontinuities in the syncytiotrophoblast layer with fibrin deposits are common in term villi (69). Fetal macrophages have been described to aggregate around injured regions of villous tissue in *ex vivo* models (70) and could potentially interact with maternal intervillous MAIT cells to assist tissue repair. Since MAIT cells express several genes encoding proteins involved in tissue repair and fibrin formation, including thrombospondin-1, furin and thrombin receptors (37, 38, 40), it is possible that they play a role in repairing the syncytiotrophoblast layer. It can also be speculated that MAIT cells could be involved in accelerating wound repair when the placenta is detached from the uterine wall and the spiral arteries are disrupted at birth. However, further studies are needed to increase our knowledge in the intriguing area of MAIT cells and tissue repair.

MAIT CELLS IN PREGNANCY COMPLICATIONS

An insufficient invasion of extravillous trophoblasts leads to a poor development of spiral arteries and, hence, to an impaired maternal blood circulation in the intervillous space. This is one of the causal factors of preeclampsia. Preeclampsia affects 3–5% of pregnant women and is a leading cause of maternal and perinatal morbidity and mortality worldwide (71). Immunological factors are likely involved in the pathogenesis of preeclampsia, including an imbalance in $CD4^+$ T cell subsets with increased proportions of inflammatory Th17 cells and less regulatory T cells (72) and elevated systemic inflammation (73), but the mechanisms remain to be defined. It was recently shown that the proportion of peripheral MAIT cells was lower in mothers with early-onset preeclampsia compared to healthy pregnancies (74). No investigation of placental MAIT cells was performed, and a low MAIT proportion in peripheral blood could account for homing to tissues as discussed above. Peripheral MAIT cells from preeclampsia patients also

displayed a lower expression of PD-1, but higher expression of CD69 and perforin, compared to healthy pregnancies. Whether MAIT cells play an active role in preeclampsia remains to be determined.

Spontaneous preterm birth (PTB), i.e., birth before gestational week 37, is one of the leading causes of childhood morbidity and mortality. Similar to preeclampsia, there is an association with immunological factors also in PTB (75). For instance, maternal T cell infiltration is observed in chronic chorioamnionitis, the most common placental lesion leading to late spontaneous PTB, and increased influx of cytotoxic effector memory cells has been associated with preterm labor and birth (76). Although the overall frequencies of peripheral MAIT cells were unaltered during the course of healthy pregnancy, in HIV-infected pregnant women, and in women with subsequent PTB, MAIT cells subsets were altered with a higher proportion of $CD8^+$ MAIT cells in first trimester in women with PTB compared to term birth (41). HIV-infection, which entails a higher risk of PTB, was associated with a higher proportion of $CD8^+$ MAIT cells compared to HIV-negative women. In healthy pregnancies the proportion of $CD8^+$ MAIT cells increased during the course of pregnancy. Functional differences in MAIT cell subsets have been described (51, 77), and $CD8^+$ MAIT cells express more IFN-γ, granzyme B and perforin compared to DN MAIT cells (77) and $CD4^+$ MAIT cells (51). It is possible that an imbalance in the different MAIT cell subsets during early pregnancy could be involved in immunological aberrations associated with PTB, but the putative importance of MAIT cells and different subsets of MAIT cells in PTB and other pregnancy complications still needs to be defined.

CONCLUDING REMARKS

Several questions remain regarding the function of MAIT cells during pregnancy and the data available so far derive solely from observational studies on human pregnancies. It is not known if MAIT cells are enriched in the placenta throughout pregnancy or if they are retained in the intervillous space only at term. Selective enrichment of MAIT cells in the intervillous space but not in adjacent decidual tissue signify separate immunological entities which deserve more attention in future research. The enhanced functional response of term placental MAIT cells could indicate a putative role in placental inflammation and dysregulated MAIT cell responses could be involved in pregnancy complications. However, since MAIT cells have anti-microbial and tissue-repairing functions, but lack alloantigen reactivity, they could play an important role in protecting the fetus from bacterial infections and maintaining homeostasis at the fetal-maternal interface.

AUTHOR CONTRIBUTIONS

JR prepared the figure. All listed authors made a substantial intellectual contribution and approved the manuscript for publication.

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REVIEW
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Antigen Recognition by MR1-Reactive T Cells; MAIT Cells, Metabolites, and Remaining Mysteries

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Mucosal-associated Invariant T (MAIT) cells recognize vitamin B-based antigens presented by the non-polymorphic MHC class I related-1 molecule (MR1). Both MAIT T cell receptors (TCR) and MR1 are highly conserved among mammals, suggesting an important, and conserved, immune function. For many years, the antigens they recognize were unknown. The discovery that MR1 presents vitamin B-based small molecule ligands resulted in a rapid expansion of research in this area, which has yielded information on the role of MAIT cells in immune protection, autoimmune disease and recently in homeostasis and cancer. More recently, we have begun to appreciate the diverse nature of the small molecule ligands that can bind MR1, with several less potent antigens and small molecule drugs that can bind MR1 being identified. Complementary structural information has revealed the complex nature of interactions defining antigen recognition. Additionally, we now view MAIT cells (defined here as MR1-riboflavin-Ag reactive, TRAV1-2$^+$ cells) as one subset of a broader family of MR1-reactive T cells (MR1T cells). Despite these advances, we still lack a complete understanding of how MR1 ligands are generated, presented and recognized *in vivo*. The biological relevance of these MR1 ligands and the function of MR1T cells in infection and disease warrants further investigation with new tools and approaches.

Keywords: mucosal-associated invariant T cell, MR1, antigen, ligand, MR1T, MAIT

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INTRODUCTION

The enormous diversity of possible T cell receptors, combined with the presentation of antigens on polymorphic MHC molecules, enables the detection of a vast number of foreign or altered-self molecules by T cells. Most well-characterized is the specific recognition of peptide antigens by conventional CD4$^+$ and CD8$^+$ T cells, when presented on MHC Class II and Class I molecules, respectively. Recently, there have been significant advances in understanding antigen recognition by unconventional T cells, in particular natural-killer T (NKT) cells, which recognize lipid-based antigens in the context of CD1 molecules, as well as γδ-T cells, most of which respond to phosphoantigens from infected cells and cancer cells in the context of butyrophilin molecules (1–3). For NKT cells, which share many characteristics with MAIT cells, the first antigen was described

in 1997 to be α-galactosylceramide (α-Galcer), derived from a marine sponge (4). Since then, it has become clear that greater diversity exists both in the array of ligands presented by CD1 molecules, and the subsets of NKT cells capable of recognizing these (5, 6).

MAIT cells are a highly conserved unconventional T cell subset, which are abundant in humans and recognize antigens in the context of MR1. MAIT cells express a semi-invariant TCR, comprising TRAV1-2-TRAJ33/12/20 α-chains, paired with a limited array of TCR-β chains (typically TRBV6-1, TRBV6-4, or TRBV20) in humans, and homologous receptors (TRAV1-TRAJ33 paired with TRBV19 or TRBV13) in mice (7–9). The constrained nature of the MAIT TCR repertoire and monomorphic antigen presentation molecule suggested a more limited array of antigens than for conventional T cells. Similar to other unconventional T cell subsets, the MR1T-MR1 axis is being revealed as more complex than initially believed. The first MR1-ligand, the non-agonist 6-formyl pterin (6-FP) was identified in 2012 (10), then some transitory pyrimidine-based MAIT cell antigens were identified in 2014 (11), among which 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) represents the most potent MAIT cell agonist to date. Since then several more MR1 ligands have been described, as well as an increased definition of subsets of MR1-reactive cells, beyond the recognition of riboflavin-based antigens by MAIT cells. Thus, MR1-antigen recognition and MR1-reactive T cell responses in immunity are emerging fields. Like conventional and other unconventional T cell subsets, there is a great promise for developing MR1T-cell-based therapies in several contexts. The definition of the scope of antigens that they recognize, the fine detail of their antigen specificity, and the factors that govern their activation and function will be crucial for achieving this goal.

MAIT CELL RECOGNITION OF A NEW CLASS OF T CELL ANTIGEN

MAIT cells were initially described over 25 years ago as an abundant $CD4^{-}CD8^{-}$ (DN) T cell subset in human blood (8), and later dubbed Mucosal-associated Invariant T (MAIT) cells, due to their conserved TCR usage and enrichment in mucosal tissues, such as the small intestine, in mice and humans (9, 12, 13). It was subsequently shown that MAIT cells reside broadly in tissues like conventional T cells (14) and are restricted to MR1 (12, 15), which has been highly conserved through evolution (16). For several years the antigens recognized through the MR1-MAIT axis were unknown, although early studies suggested they were non-peptide-based molecules (9, 17). The key breakthrough; MR1-ligand identification, was triggered by two key publications from 2010, which demonstrated that MAIT cells could be activated, in an MR1 dependent manner, by a wide range of bacteria and yeasts, but that viruses were non-stimulatory (18, 19). This suggested that the potent activating ligands may come from a conserved biosynthetic pathway and, after careful detective work, this source of antigen was discovered to be microbial biosynthesis of riboflavin (vitamin B2) (10). For a detailed history of the discovery of vitamin B based MAIT cell antigens, readers are referred to previous review articles (20–22) and original research publications (10, 11).

Riboflavin synthesis is a highly conserved biosynthetic pathway, which is essential for many bacteria and yeasts (23, 24). Some organisms cannot synthesize riboflavin but have transporters to take it up from their environment (24), and importantly, several of these microbes (including *Enterococcus faecalis* and *Listeria monocytogenes*) are non-stimulatory for MAIT cells (18, 19). A series of enzymes drive each step in the riboflavin biosynthetic pathway, with *ribA* and *ribG* (alternatively named *ribD* in some microorganisms) being essential for the production of a key intermediate 5-amino-6-D-ribitylaminouracil (5-A-RU). Condensation of 5-A-RU with small carbon metabolites, including glyoxal and methylglyoxal, results in the formation of highly potent pyrimidine MAIT cell antigens 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-OP-RU, respectively (11) (**Figure 1**). These pyrimidine antigens are highly unstable, thus are further converted to lumazine derivatives unless trapped by MR1. The derived lumazines, RL-6,7-diMe and RL-6-Me-7-OH, are also capable of activation of human and murine MAIT cells, albeit with reduced potency (10, 25) (**Table 1**). Studies that identified these novel small molecule antigens utilized an MR1-capture approach, in which recombinant human MR1 was refolded, with human β2 microglobulin, in the presence of culture supernatant from bacteria, such as *Salmonella* Typhimurium, or media controls. Importantly, these metabolite antigens could be detected by liquid chromatography-mass spectrometry (LC-MS) of MR1 refolded in the presence of supernatant from riboflavin-producing bacteria capable of activating MAIT cells, but not from the MAIT cell non-stimulatory bacteria *Enterococcus faecalis*, or *Lactococcus lactis* mutants lacking individual *rib* enzymes (11).

The derivation of the potent antigens, 5-OP-RU and 5-OE-RU, from a metabolic intermediate in a conserved biosynthetic riboflavin pathway, 5-A-RU, appeared to answer the question of why the MR1-MAIT axis has been so highly conserved through mammalian evolution (12, 16, 31) since it suggested that MAIT cells would play an important immune role in protecting against diverse microbial pathogens. The idea that MAIT cells may have evolved to directly detect pathogens (by sensing 5-A-RU-derived molecules) and rapidly respond to metabolically active microorganisms that breach the mucosal barriers was then pursued by many researchers in the field, including our own group. Indeed, the presence of MAIT cells has now been shown to contribute to protective immunity against several pathogens capable of riboflavin synthesis (32–34). More recently, roles in barrier function and tissue repair have also been described (35–40), and this would be consistent with the possible sensing of antigens from microflora, which may indicate a breach of barrier function.

Although the most potent MAIT cell agonist known to date, 5-OP-RU, is often studied or cited in isolation, several MR1 ligands, including both MAIT cell agonists and non-agonists, have now been described (**Table 1**). Even in the initial discovery

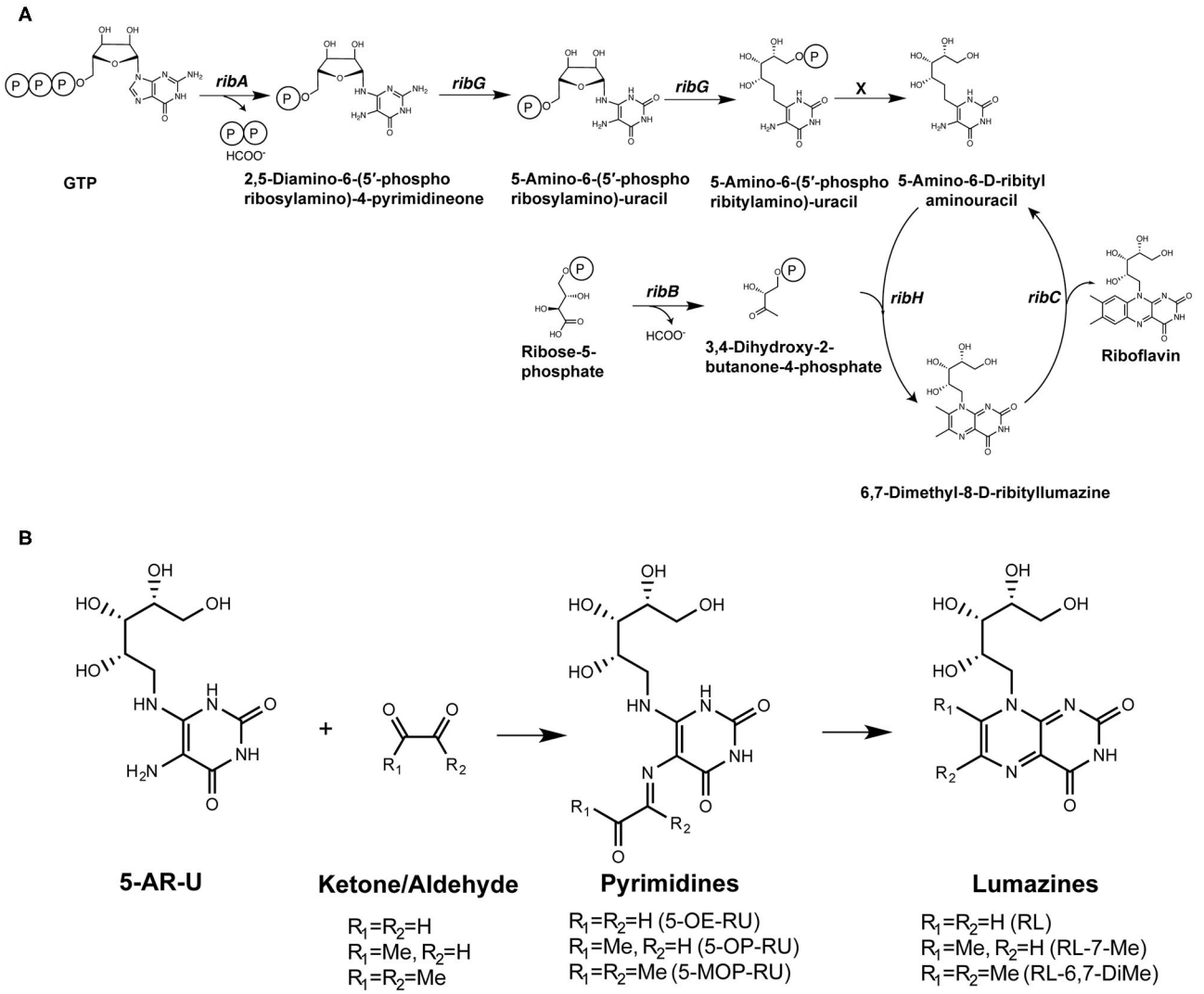


FIGURE 1 | Formation of riboflavin based MAIT antigens. **(A)** Riboflavin biosynthesis pathway. **(B)** The riboflavin biosynthesis intermediate 5-A-RU non-enzymatically reacts with small metabolites to form pyrimidine antigens 5-OP-RU and 5-OE-RU. These can be captured by MR1, or alternatively cyclize to form lumazines, some of which are also weakly antigenic [modified from (11)].

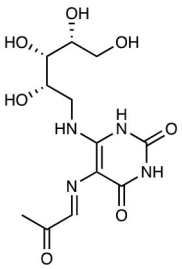
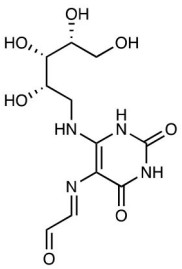
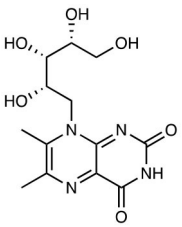
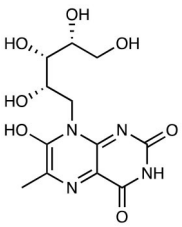
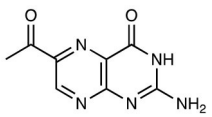
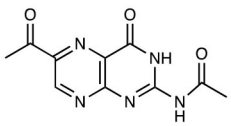
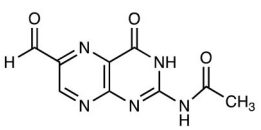
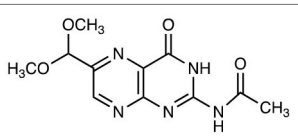
studies it was evident that, like for conventional T cells and other unconventional T cells, there was not just one antigen, but a family of related molecules that could bind MR1 and potentially interact with MAIT cells. The question of just how large this MR1-ligand family is, remains. MR1 bound antigens are recognized by MAIT cells through their TCR, which is conserved, but not completely invariant. Thus, it has been hypothesized that different antigens are differentially recognized by MAIT cells expressing different TCRs. Indeed structural studies have demonstrated a role for TCRβ chain in antigen recognition, suggesting that certain subsets of MAIT cells may be enriched in response to different antigens (27). We will address both the diversity of MR1 ligands and the recognition of MR1-antigens by MAIT cells and other MR1-reactive T cells in the following sections.

EXPANSION OF THE MR1 LIGAND FAMILY

The first identified MR1-ligand, 6-FP, is a photosynthetic breakdown product of folic acid. This pterin-based ligand is non-stimulatory for most MAIT cells (10) and exhibits a competitive inhibitory effect on MAIT cell activation by 5-OP-RU (25, 27, 28, 41). Acetyl-6-FP (Ac-6-FP) and acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal, synthetic derivatives of 6-FP, are similarly capable of binding to MR1 (as judged by an increase in cell surface expression), but do not activate MAIT cells and, like 6-FP, competitively inhibit activation by 5-OP-RU (25, 27, 28, 41) or *E. coli* (25, 42).

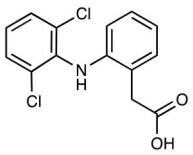
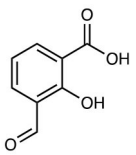
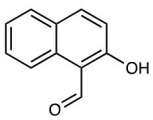
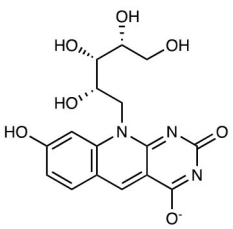
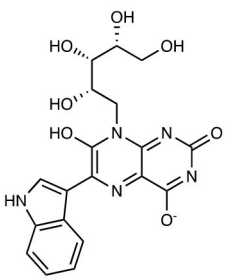
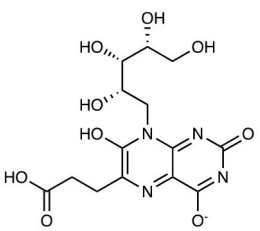
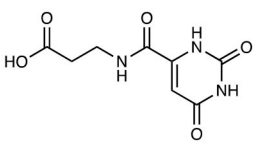
Recently, the family of MR1 ligands has grown significantly, with several groups identifying compounds capable of binding

TABLE 1 | MR1 ligands identified to date.

Compound name	Chemical structure	MAIT activation or inhibition	References
5-OP-RU		Potent activation of human and mouse MAIT cells EC50 = 1-8 pM	(11)
5-OE-RU		Potent activation EC50 = 510 pM	(11)
RL-6,7-diMe		Weak activation	(10)
RL-6-Me-7-OH		Weak activation EC50 = 25 μM	(10)
6-FP		MR1 upregulation of surface expression Competitive inhibition Activation of TRAV1-2$^-$ "atypical" MAIT cells	(10) (26)
Ac-6-FP		MR1 upregulation of surface expression Competitive inhibition *in vitro* and *in vivo* Activation for TRAV1-2$^-$ "atypical" MAIT cells	(25, 27, 28) (26)
2-acetylamino-4-hydroxy-6-formylpteridine		MR1 upregulation Competitive inhibition	(25)
2-acetylamino-4-hydroxy-6-formylpteridine dimethyl acetal		MR1 upregulation of surface expression Competitive inhibition	(25)

(Continued)

TABLE 1 | Continued

Compound name	Chemical structure	MAIT activation or inhibition	References
Diclofenac (shown) 5-hydroxy diclofenac 4-hydroxy diclofenac Others including: Benzbromarone Chloroxine Floxuridine Galangin Mercaptopurine		Weakly antigenic with some TCR specificity	(28)
3-formyl salicylic acid (3-FSA) (shown) 5-formyl salicylic acid (5-FSA)		MR1 upregulation of surface expression Competitive inhibition *in vitro* and *in vivo*	(28)
2-Hydroxy-1-naphthaldehyde (2-OH-1-NA) Others including: 1,4 Naphthoquinone 5-Hydroxy-1,4-naphthaldehyde Apigenin Mefenamic acid Menadione		MR1 upregulation of surface expression	(28)
7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO)		Inhibition of MR1T clone response to *M. smegmatis* supernatant	(29)
6-(1*H*-indol-3-yl)-7-hydroxy-8-ribityllumazine (photolumazine III)		Activation of MR1T clones (blockable by 6-FP)	(29)
6-(2-carboxyethyl)-7-hydroxy- 8-ribityllumazine (photolumazine I)		Activation of MR1T clones (blockable by 6-FP)	(29)
3-[(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)formamido] propanoic acid (DB28)		MR1 downregulation of surface expression	(30)

to MR1 and/or stimulating MAIT cells (**Table 1**). Using *in silico* screens of chemical libraries followed by *in vitro* functional testing, Keller et al. showed that over 20 compounds, with different chemical scaffolds, could bind to MR1 and modulate MAIT cell activity (28). Interestingly, these included some common drugs and drug metabolites such as diclofenac and the salicylates 3-formylsalicylic acid and 5-formylsalicylic acid. This study also demonstrated competitive inhibition of MAIT cell activation *in vivo*, suggesting potentially important physiological effects. Additionally, there was some selectivity in the ability of diclofenac metabolites to activate cell lines expressing MAIT TCRs with the same TCR α-chain but different β-chains, consistent with the role of the β-chain in antigen recognition shown in an earlier study (27).

It is considered likely that additional riboflavin-related antigens may exist. Soudais et al. showed that, as well as methylglyoxal and glyoxal, the small molecule di-hydroxy acetone (DHA) in combination with 5-A-RU, caused activation of mouse MAIT cells, *in vitro*. However, it is unclear whether this was due to the generation of a novel antigen, or conversion of DHA to methylglyoxal (25). In contrast, the same small molecules, glyoxal, methylglyoxal, and DHA, when mixed with 5-nitro-6-D-ribitylaminouracil (5-N-RU) did not significantly activate mouse MAIT cells (25). In 2018, Harriff et al. identified several additional MR1 ligands. These included photolumazines I and III, each capable of activating TRAV1-2$^+$ T cell clones and, more weakly, a TRAV1$^-$ T cell clone in an MR1-dependent manner and 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO), which could competitively inhibit activation. They also described the capture by MR1 of the plant flavonoid hesperidin, although this did not appear to either activate or inhibit MAIT cells (29). The reduced potency of these MR1-ligands for MAIT cells (or reporter cells expressing MAIT TCRs) (**Table 1**), and the selective activation of subsets of MR1T cells, mean it is currently unclear what the physiological role of T cell detection of such molecules plays in immunity.

Most recently, using an *in silico* screen based on the MR1 binding pocket, Salio et al. identified an intriguing effect of a novel MR1 ligand (3-[(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)formamido]propanoic acid "DB28," which appeared to decrease, rather than increase cell surface expression of MR1, as well as competitively inhibiting activation of MAIT cells by agonist ligands (30).

Although many do not appear to be antigenic to MR1-5-OP-RU reactive MAIT cells, it is now widely accepted that the MR1 ligands encompass more than those originally described. Intriguingly, crystal structures (10, 11, 27, 41, 43, 44) show that the known ligands do not fully occupy the MR1 antigen-binding cleft, suggesting that, in addition to small variations, much larger and structurally different scaffolds of MR1-ligands may be possible. Thus, it is likely that the list of described MR1 ligands will continue to grow, and these may also represent antigens for MAIT cells and other MR1T cells.

UNDERSTANDING MAIT CELL RECOGNITION OF MR1-PRESENTED RIBOFLAVIN-BASED ANTIGENS

A remarkable characteristic of the potent pyrimidine antigens, 5-OP-RU and 5-OE-RU, and the pterin-based ligands, such as 6-FP, is that these are capable of forming a covalent bond to the MR1 antigen-presenting molecule via a Schiff base formed between the ligand and a Lys residue at position 43 of MR1, which sits in the base of the antigen-binding pocket (10, 11, 45). Whilst several MR1-ligands have now been described, the ability to form this linkage appears critical for stable binding to MR1, as detected by egress of loaded MR1 molecules from the ER and upregulation on the cell surface (44–46). The lower potency of the related lumazine antigens also appears to be due to their inability to form a Schiff base with the Lys 43 residue. Interestingly, the MR1 downregulating ligand, DB28, was unable to form a Schiff based with the Lys 43 residue (30). Within MAIT TCRs, there was the conservation of key residues including a highly conserved Tyr at position 95 of the CDR3 loop of the TCR α-chain. X-ray crystallographic analyses of MAIT TCR-MR1 complexes revealed that this Tyr95α "reaches" down toward the small molecule antigens, potentially explaining the selective TCR usage among MR1-5-OP-RU reactive MAIT cells (11, 27, 41, 47), with the MAIT TCR β chain also playing a role in antigen recognition (27).

Despite these advances, our understanding of the high potency of 5-OP-RU remains incomplete. A panel of 20 analogs of 5-OP-RU was recently developed (44, 48) (**Figure 3**) in order to better understand the intricate factors for MR1 and TCR binding. These "altered metabolite ligands" (AML) are equivalent to altered peptide ligands (APL) which have been instrumental in defining the rules governing classical MHC I and II peptide recognition by conventional T cells. Using the complementary approaches of chemical, functional and high-resolution structural analyses, Ler et al. established a set of molecular rules governing ligand binding to MR1 and interactions with the TCR, driving activation. The impact of various modifications to the antigen correlated with the extent to which they disrupted the formation of an "interaction triad," a network of hydrogen bonds between the conserved Tyr95α in the CDRα loop of MAIT TCRs, the ribityl moiety of the antigen and the Tyr152 residue of MR1. MAIT cell activation potency was found to be orchestrated by dynamic compensatory interactions within this "interaction triad". Among all tested ligands, 5-OP-RU, the most potent MAIT agonist identified to date, was found the most capable of establishing a strong and stable interaction triad. Even small modifications to the ribityl chain resulted in profoundly reduced potency in cellular assays.

Similar conclusions were reached by a second group, who produced a partially overlapping set of 5-OP-RU analogs (**Figure 2A**) by different methodologies (49). In both studies, removing the terminal hydroxyl group of the ribityl chain significantly reduced the ability of the ligand to engage the TCR and activate MAIT cells. With this interaction removed, the ligands could act as competitive inhibitors (49). In another study, sugar analogs of the weaker lumazine ligands RL-6-Me-7-OH were also shown to bind MR1 and tetramers loaded with these

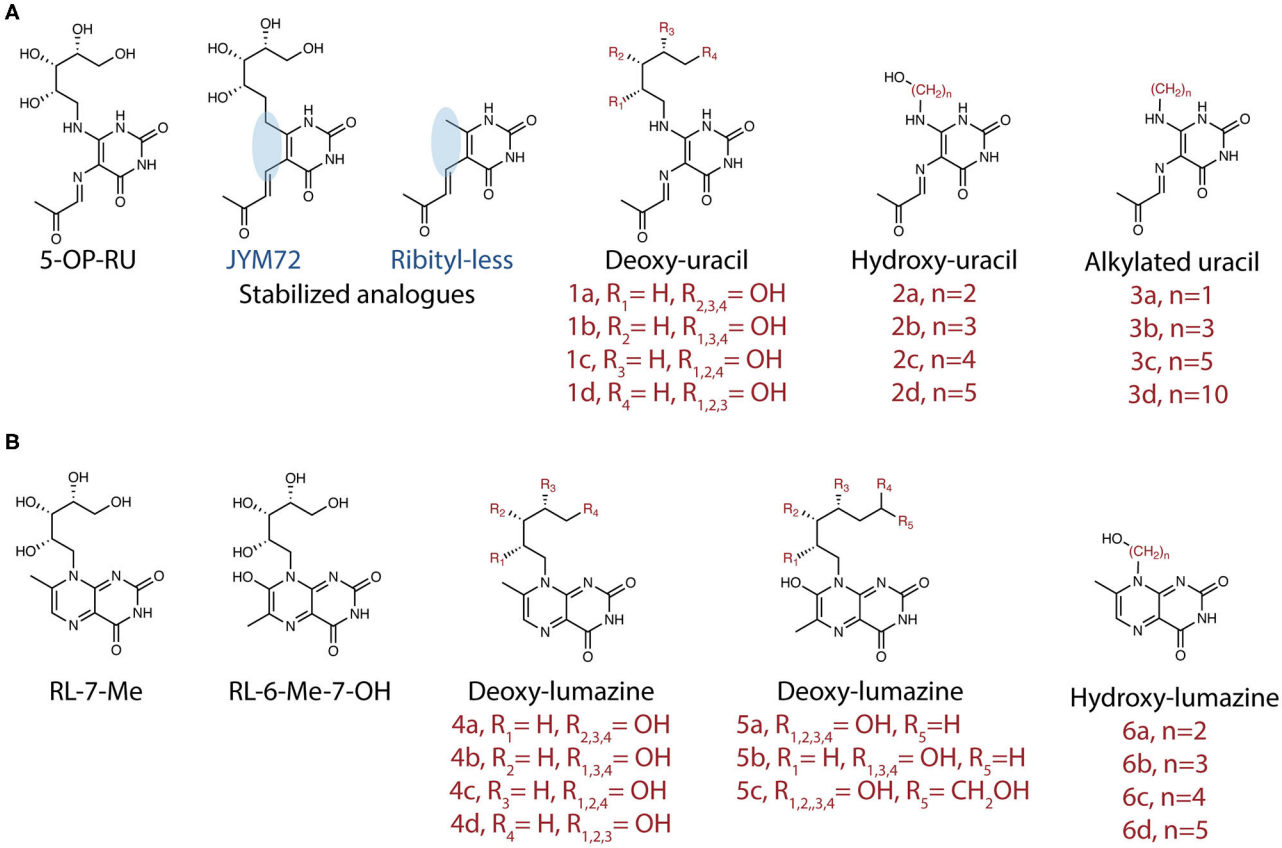


FIGURE 2 | Synthetic analogs of **(A)** pyrimidine antigen 5-OP-RU or **(B)** lumazines RL-7-Me and RL-6-Me-7-OH have been created to understand how different modifications impact MR1 binding and MAIT cell activation or inhibition.

analogs could stain a cell line expressing a MAIT TCR (50) (**Figure 2B**). However, their potency in stimulating bone-fide MAIT cells has not yet been examined.

MORE THAN MAITs; THE INCREASING DIVERSITY OF MR1-REACTIVE T CELLS

MAIT cells were initially described as a large population of cells with restricted TCR α- and β-chain usage (8, 9). This, together with the high conservation of MR1 (51) suggested a capacity for recognition of a single or limited set of antigens. However, as described above, the array of MR1-ligands that may serve as T cell antigens is now acknowledged to be larger than first believed (**Table 1**). Development of MR1-tetramers in 2013 (K43A mutant MR1) and 2014 (WT mouse and human MR1) enabled sorting and TCR sequencing of MR1-5-OP-RU reactive cells. This revealed greater heterogeneity of TCR usage than previously described (7); a finding later supported by other studies (52–54). We define MAIT cells here as TRAV1-2$^+$ MR1-riboflavin-Ag-reactive T cells (**Figure 3**). These cells can be detected using MR1-5-OP-RU tetramers, are restricted to MR1, acquire the hallmark promyelocytic leukemia zinc finger (PLZF) molecule during thymic development (55), and display an effector-memory phenotype (CD44^{hi} and CD62L^{lo}).

In addition to the riboflavin-reactive TRAV1-2$^+$ MAIT cells, populations of TRAV1-2$^-$ MR1-reactive T (MR1T) cells have been described by several groups. In 2016, Gherardin et al. described "atypical MAIT cells," which were variably riboflavin- or folate- reactive or MR1 autoreactive cells (26), with a role for the CDR3β loops of the TCRs in determining reactivity. These cells were relatively rare, comprising <0.1% of αβ-T cells in human blood. Additionally, more diverse TRAV1-2$^-$ cells were described, which were phenotypically distinct from TRAV1-2$^+$ MAIT cells. X-ray crystallographic analyses of one TRAV1-2$^-$ (TRAV36-TRBV28) TCR in complex with-MR1-5-OP-RU revealed a different mode of recognition, whereby the TCR docked more centrally on MR1 compared to the TRAV1-2$^+$ TCRs [reviewed in detail (56)] (**Figure 3**). Broadly consistent with these studies, another group reported the existence of MR1-5-OP-RU reactive TRAV1-2$^-$ cells, with at least one clone instead expressing TRAV12-2, which lacks the Tyr95 residue previously found to be conserved in TRAV1-2$^+$ MAIT cells (57). Furthermore, this clone displayed distinct antigen specificity, detecting infection with *Streptococcus pyogenes* (group

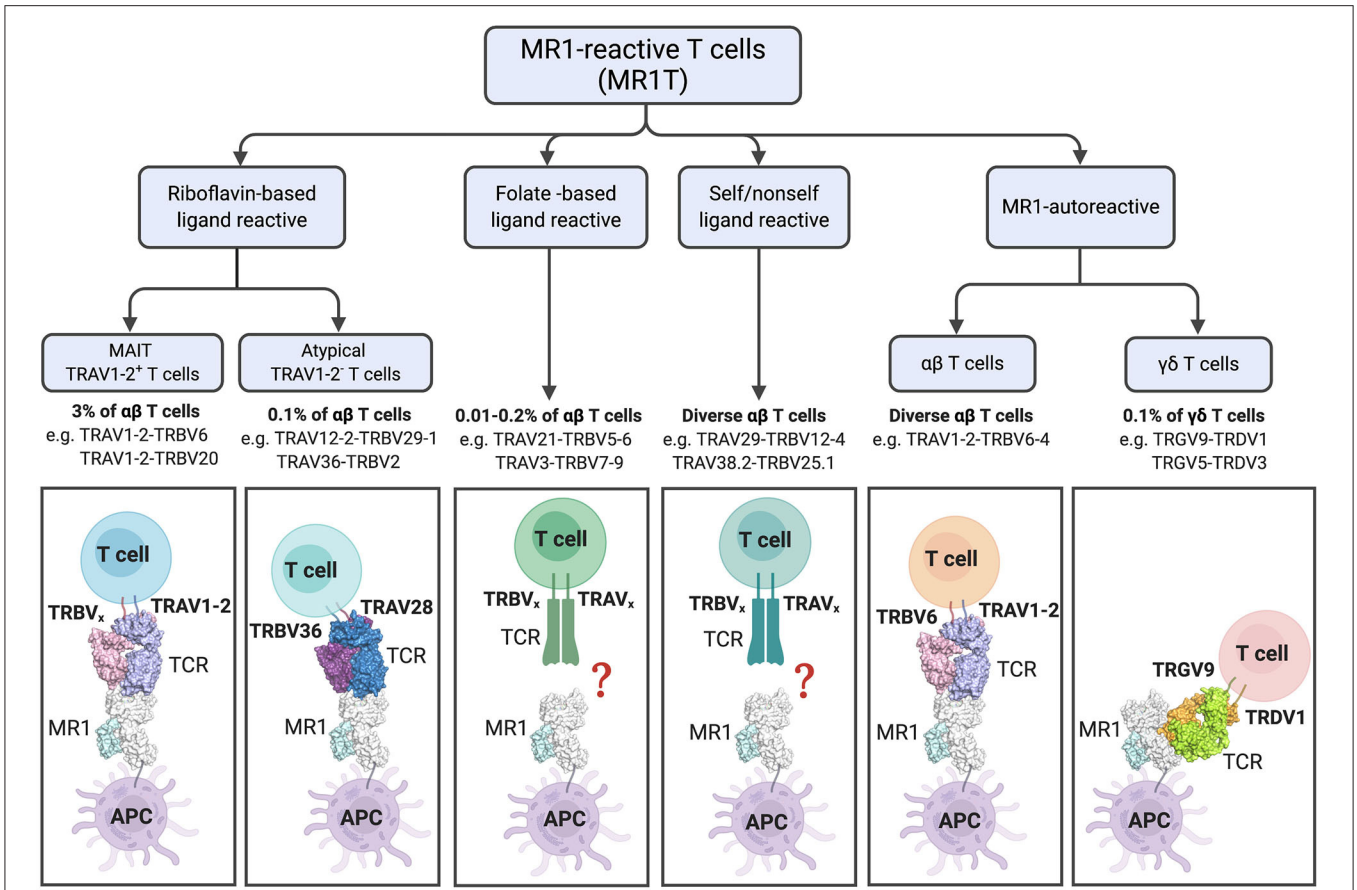


FIGURE 3 | Diversity and characteristics of MR1-reactive T cells. % refer to those reported in human PBMCs. For subsets with diverse TCRs examples are listed. The lower panel shows cartoon representations of the crystal structures of ternary TCR-MR1-Ag complexes: The typical MAIT A-F7 TRAV1-2-TRBV6-1 TCR-MR1-5-OP-RU (PDB; 6PUC); the atypical TRAV36-TRBV28 TCR-MR1-5-OP-RU (PDB; 5D7L); MR1-autorective M33.64 (TRAV1-2/TRBV6-4) TCR-MR1-5-OP-RU (PDB; 5D5M); and G7 γδ TCR with MR1-5-OP-RU complex (PDB; 6MWR). The MR1 and β2-microglobulin molecules are colored white and pale-cyan, respectively. TRAV1-2 TCRα, light-blue; TRAV6 TCRβ, light-pink; TRAV36 TCRα, violet-purple; TRBV28 TCRβ, sky-blue; TRGV9 TCRγ; lemon, TRDV1 TCRδ, orange. Structural illustration was created using PyMOL Molecular Graphics System, Version 1.8.6, Schrodinger and BioRender.com.

A strep), which does not have the capacity to produce riboflavin, suggested recognition of microbial non-ribityl-based antigens (57). Similarly, a proportion of cells staining with MR1-tetramers generated with *E. coli*-derived ligands were TRAV1-2^{-}, again indicating that not all MR1T cells are TRAV1-2^{+} MAIT cells (29). Lepore et al. and Koay et al. have also identified MR1T cells in healthy individuals, which were capable of reacting to MR1-expressing cells in the absence of microbial ligands. These MR1T cells were found to have a diverse TCR repertoire and functional capacity (58, 59).

In addition to αβ-T cells, γδ-T cell populations displaying reactivity to MR1 have recently been described. Interestingly, a distinct population of these γδ-T cells recognized MR1 via a novel binding mode, whereby the TCR binds the underside of the MR1 molecule (60) (**Figure 3**). Thus, in addition to its role in presenting microbial antigens for specific recognition by MAIT cells, it appears that MR1 may act as a pattern recognition receptor, with its cell surface upregulation potentially triggering a more innate-like immune response. It is unclear whether MR1 molecules are present at the cell surface in an empty form, but it is considered likely that they contain endogenous (self) ligands. Indeed, recent studies suggest MR1 can present endogenous or tumor antigens to MR1T cells other than MAIT cells (58, 61). However, these antigens have not yet been identified.

Thus, our definition of MR1-reactive T cells has expanded to encompass not just MR1-5-OP-RU reactive TRAV1-2^{+} MAIT cells, but folate-reactive and autoreactive atypical MAIT cells, as well as γδ-T cells (**Figure 3**). Thus, the term MR1T cells is now used to denote all MR1-reactive cells, of which MAIT cells represent the majority. In contrast, other subsets of MR1T identified to date are PLZF negative. While the co-evolution of MR1 with the TRAV1 invariant TCR alpha chain (16, 62) suggests that presentation of microbial signature Ag to MAIT cells is the primary function of MR1, questions remain regarding the thymic development, functional capability and physiological relevance of the other MR1T cell populations. Although some subsets, such as the folate-reactive "atypical MAIT cells" represent only a very small percentage, as pointed out by Gherardin et al.,

these are present in similar numbers in humans to type I NKT cells, and may be expanded in disease settings (26). The selective recognition of antigens by various subsets of MR1T cells expressing different TCRs suggests that these unconventional T cells may generate display specific memory cell pools similar to conventional T cells. Indeed, a recent study by Loh et al. demonstrated that older adults have large clonal expansions of MAIT cells, similar to those seen in conventional virus-specific $CD8^+$ T cells (63). Clonal expansion of MAIT cells has also been reported (64) and these may suggest differential recognition of diverse MR1-ligands, or expansion of higher affinity clones in some individuals, perhaps due to a history of infection. A recent study of a small cohort of patients with multiple sclerosis reported oligoclonality, with limited TCRβ repertoires, which remained stable over 3 years (65). Stable oligoclonality was confirmed in healthy individuals by Howson et al., who also found in six individuals the MAIT TCRβ repertoire maintained oligoclonality following *S.* Paratyphi infection, but with expansion and contraction of particular clonotypes, resulting in an altered composition. Cell lines expressing TCRs from expanded clones were more responsive to MAIT cell riboflavin-based antigens (66) suggesting preferential expansion of activated MAIT cells. The TCR repertoire of expanded cells has not yet been assessed in experimental settings such as in mouse models using defined antigens. Since the selective recognition of MR1 ligands by subsets of MR1T cells, potentially with functional differences, present opportunities for selective targeting, more research is needed into this area.

MR1-ANTIGEN RECOGNITION IN PHYSIOLOGICAL SETTINGS

Although several advances have been made in defining MR1-ligands and their recognition by MR1T cells, many questions remain about the physiological relevance of each antigen. As outlined recently by Ler et al. (44), the ability of ligands to generate a MAIT cell response is the result of the combination of several factors, including chemical properties of the ligands, the ability to bind strongly to MR1 resulting in egress to the cell surface, and the avidity of T cell recognition. The expression and regulation of MR1-ligand presentation remain incompletely understood, and this will impact the activation of MR1-reactive T cells *in vivo*. It is clear from studies comparing different natural ligands or altered metabolite ligands (AML), that the recognition and subsequent response depends on many factors, including chemical stability, binding to MR1 (significantly dependent on the ability to form a Schiff base covalent bond) and TCR recognition (27, 41, 44, 46). To date, 5-OP-RU is the most potent MAIT antigen in any assay, activating MAIT cells even at pmol concentrations. It has also been shown to be relevant to MAIT cell thymic development and activation of MAIT cells *in vivo*. On the other hand, a key question remaining in the field is whether any of the less potent antigens, antigens that activate smaller subsets of MR1T cells, or MR1 ligands that inhibit MAIT cell activation, are physiologically relevant. Here, we will focus not on the MRIT cell response in these settings, but clues to the *in vivo* production and MR1-presentation of antigens in different contexts.

Physiological Expression and Impact of MAIT Antigens

Riboflavin-based antigens that stimulate MAIT cells include the potent pyrimidine-based compounds 5-OP-RU and 5-OE-RU, have now been identified from several bacterial species, including *Salmonella* Typhimurium, *Escherichia coli*, *Lactococcus lactis*, and were not detected from riboflavin deficient bacteria (*E. faecalis*) (10, 11). Indeed, bacteria with deletions in the *rib* genes necessary for this biosynthetic pathway are incapable of a full *in vivo* response, as MAIT cells accumulated in the lungs after intranasal infection with *Salmonella* Typhimurium but not a *ribDH*-deficient mutant (67). Many other microbes possessing the riboflavin pathway have now been shown to activate MAIT cells [summarized in a previous review (20)] and the requirement for the presence of the riboflavin pathway in this activation was confirmed for *E. coli* (25) and *S. pneumoniae* (68), as well as in a study screening 47 microbiota-associated bacterial species. In the latter study, differences in activation capacity were observed between different phyla, and levels of riboflavin production correlated with MAIT cell activity (69). The presence of shared antigens, 5-OP-RU and 5-OE-RU by these microbes is likely, although so far these have only been detected for a handful of bacterial species. Additionally, the physiological relevance of the less potent, but related, lumazines particularly during infection, has not been fully addressed.

MAIT cells have been analyzed in several mouse models of bacterial infection, demonstrating their role in protecting against pathogenic organisms such as *Francisella tularensis* (33, 70), *Klebsiella pneumoniae* (32), *Mycobacterium bovis* BCG (54), and *Legionella longbeachae* (34). Riboflavin-based antigens are likely produced not only by pathogenic microorganisms during infection, but also by commensal microorganisms. Indeed, the microbiota is important for the development of MAIT cells, as they are almost completely absent in the periphery in germ-free mice (12, 55). The re-introduction of bacteria, even with single strains, was shown to restore MAIT cells (19). Recently it was demonstrated that the ability of microflora to support thymic development of MAIT cells was dependent on riboflavin biosynthesis, and that providing exogenous 5-OP-RU was sufficient to reconstitute MAIT cell development (71). Recent studies also demonstrate the importance of gut microbiota and the presence of *rib* genes, on MAIT cell reconstitution after allogenic haematopoietic cell transplantation in humans (72, 73). Dysbiosis in viral infections or changes to the gut microbiota have been shown to be associated with impaired MAIT cell responses (74) and it is hypothesized that dysbiosis in metabolic conditions also affects MAIT cells, altering their capacity to promote barrier integrity, and instead triggering a pathogenic, inflammatory MAIT cell response (75). Understanding microenvironmental factors controlling the production of antigen by both pathogens and commensal microorganisms will be important for a complete understanding of MAIT cell responses.

Despite an abundance of information on the regulation of riboflavin production in several bacteria and fungi (23, 24), and the identification of 5-A-RU as the key chemical building block of these molecules, the *in vivo* production of riboflavin-based antigens, including 5-OP-RU and 5-OE-RU (11), remains poorly understood. For example, recently Kurioka et al. showed that the *S. pneumoniae* riboflavin operon genes, which are highly conserved between *Streptoccci*, were upregulated with heat stress, followed by later downregulation (68). It is thus probable that MR1-antigen production, and the resultant MAIT cell activation, may be enhanced under different conditions that may be encountered during the course of infection. Schmaler et al. showed that the growth of bacteria in conditions designed to resemble the human colon (e.g., low oxygen) or on different sugar sources affected bacterial metabolism and the subsequent ability of bacterial samples to activate MAIT cells (76). Intriguingly, the ability of *E. coli* to activate $CD8^+$ human peripheral blood MAIT cells, *in vitro*, was increased by treatment with the pesticides chlorpyrifos and glyphosate, which also were found to cause changes in the riboflavin and folate biosynthesis pathways (42). Moreover, folate producing bacteria that didn't stimulate MAIT cells (*Lactobacillus reuteri* and *Bifidobacterium adolescentis*) were shown to inhibit activation of human MAIT cells by *E. coli*, as measured by the production of IFNγ and TNF (42), suggesting the balance of activating and inhibitory MR1-ligands may be important for the generation of MAIT cell responses, *in vivo*.

In humans, MAIT cell responses during infection have been suggested by studies of MAIT cells in patient cohorts, including in the blood and an increasing selection of tissues, paired with subsequent *in vitro* analysis. These studies included patients with tuberculosis, *Helicobacter* infection, *cholera*, and undefined infections such as sepsis and community-acquired pneumonia (77–80). MAIT cells were also activated in human volunteers infected with live *Salmonella enterica* Paratyphi A vaccine (66). The role of human MAIT cells in infection and diseases has been reviewed elsewhere (14, 81, 82) so will not be covered in detail here.

In addition to the setting of infection with pathogens producing riboflavin-based antigens, MAIT cells have been studied in the context of viral infections, autoimmune diseases including diabetes, multiple sclerosis systemic lupus erythematosus and arthritis, and have most recently gained attention as potentially important in cancer. There have been several recent reviews covering the findings in these areas as well as animal models developed, which allow the study of MAIT cells in these settings (75, 83–87). The function of MAIT cells in these settings is complex and differs from the direct anti-bacterial role seen in infection models. For example, in the non-obese mouse model of diabetes, deletion of MR1, and thus MAIT cells, resulted in a loss of gut integrity, indicating a protective function, but MAIT cell alterations, including increase in cytotoxic effectors were also observed in mice and human type-1 diabetic patients at the onset of disease suggesting a pathogenic role (35). A role for MAIT cells in tissue repair or inflammatory conditions such as inflammatory bowel disease would be consistent with their enrichment at barrier sites where they may encounter metabolically active pathogenic or commensal microorganisms. However, the recognition of riboflavin-based or other MR1-ligands in these settings has not yet been confirmed.

MR1 Expression and Regulation

MR1 is highly conserved and shows low genetic polymorphism (16). The MR1 protein is expressed, along with β2m, at low to undetectable levels on a range of cell types (88). MR1 is ubiquitously expressed at the transcript level in mouse and human cells (31, 89), although recent studies point to different levels of expression in different tissues (90, 91). Like MHC Class I molecules, MR1 forms stable complexes on the cell surface only when stably folded in the presence of a ligand (88, 92) and upon ligand binding MR1 is released from the ER and traffics to the cell surface (45), a characteristic exploited for detecting the presence of ligands in cellular assays of MR1 upregulation (10, 11, 45). However, MR1 remains difficult to analyse due to its low expression, particularly in primary murine cells, despite evidence for an its function both in MAIT cell development (12) and impressive MAIT cell accumulation *in vivo* (67).

Several cell types have been used as MR1 antigen-presenting cells (APCs) in *in vitro* assays. *In vivo*, one recent study showed that both bone marrow-derived professional APC and non-bone marrow-derived cells (epithelial or stromal cells) can present antigen to activate a MAIT cell response, with the relative importance determined by the biology of the infecting pathogen (93). Thus, it remains to be fully understood which cells are responsible for MAIT cell activation in physiological settings such as during bacterial infection.

Downregulation of MR1 by HSV-1 and CMV suggests that TCR-dependent MAIT cell activation may also be impaired in the context of viral infection (94). Given that MAIT cell numbers and function are altered in human cohorts including bacterial infection or sepsis, viral infection and autoimmune disease, it remains to be shown whether there is a direct link to MR1 expression. Seshadri et al. identified a single nucleotide polymorphism in an intronic region of MR1, which was associated with susceptibility to tuberculosis in a Vietnamese adult cohort. This SNP was also associated with MR1 expression in cell lines, and thus may act to modulate the MR1-dependent response of MAIT cells (95). MR1 also exists in multiple isoforms (31) but the biological relevance of these is unclear.

THE CONTEXT OF ANTIGEN RECOGNITION DETERMINES MAIT CELL RESPONSES

MAIT cells have been described as "innate-like" rapid responders, with a memory-like phenotype. However, like conventional T cells, MR1-antigen alone is insufficient to drive an optimal MAIT cell response, with co-stimuli including cytokines and/or surface co-receptors required (67) and able to enhance MAIT responses to antigen stimulation (96–99), although *in vitro* findings have not always translated to *in vivo* studies (70). Additionally, polarizing effects on MAIT cell function have been demonstrated. In particular, priming with IL-7 or treatment with

IL-23 significantly increases IL-17 secretion, which otherwise is minimally induced in MAIT cells (93, 100, 101). IL-7 has been linked to effects on MAIT cell function and polarization in disease settings such as multiple sclerosis (102) and primary biliary cholangitis (103). Recently, it was shown that ICOS and IL-23 are important for MAIT cell *in vivo* responses in mouse infection models with *Salmonella* and *Legionella* (93). In genetically deficient mice lacking these co-stimulators, expansion of MAIT cells was impaired and the profiles of responding pulmonary MAIT cells shifted from RORγt$^+$ to T-bet$^+$ phenotypes (93).

Different cytokine signals synergize to control MAIT responses (104), and thus, the MAIT cell functional capacity may differ in tissue settings compared to blood MAIT cells (105). In humans, most peripheral blood MAIT cells display type-1 responses upon stimulation, whereas in tissues a IL-17-secreting population is more readily detected (80, 105), suggesting there may be functional differences. Following infection with *S.* Paratyphi, the MAIT cell response was partially IL-12-dependent, suggesting the quality and quantity of MAIT cell immunity is driven by the combination of pathogen signals and host cytokines (66). The augmenting effects of cytokines may act via increasing expression of the MAIT TCR (101) or upregulating cellular components related to TCR signaling (100).

In the absence of TCR-dependent antigen recognition, MAIT cells can be activated by cytokines, including IL-12 and IL-18 (54, 106–108), and by super-antigens (109). In one study, the MAIT cell response in group A streptococcus (GAS) infection appeared to contain both TCRβ-dependent (super-antigen) and independent activation (110). Cytokines likely drive the MAIT cell activation observed in viral infections and contribute to their response during bacterial infection (111). Importantly, there are qualitative and quantitative differences in the MAIT cell responses triggered by TCR-dependent and TCR-independent mechanisms (38). However, the implications of these differences in various immune settings are yet to be determined.

In settings of chronic inflammation, infection or cancer, MAIT cells display altered phenotypes (112), can produce Th2 cytokines (113), and can contribute to pathology (114). MAIT cell function may be impaired in settings such as chronic infection (115–117) and cancer (118). Various studies have implicated immune checkpoints (116, 119, 120), cytokines such as IL-10 (121) and suppression of MAIT cell function by hydrogen peroxide released from neutrophils (122). Thus, in addition to recognition of MR1-bound antigen, the context of other signals in different setting is important for MAIT cell activation and function.

TOOLS FOR THE ASSESSMENT OF MR1 LIGAND PRODUCTION AND RECOGNITION

The discovery of a new class of antigens, while providing exciting opportunities, also presents challenges when seeking to understand their biological significance. The study of metabolite-based ligands presents new and different challenges to the better-known peptide and lipid-based antigens. The MR1-binding and MAIT cell activation by metabolites of drugs such as diclofenac (28), as well as the recently described breakdown of 5-A-RU in air (123), highlights the importance of confirming the precise identity of MR1 captured ligands by a combination of assays such as high accuracy mass spectroscopy, X-ray crystallography, NMR spectroscopy, functional assays and ultimately *in vivo* studies. Identification of 5-OP-RU and 5-OE-RU was originally achieved by mass spectrometric analysis of recombinantly expressed MR1 protein refolded in the presence of bacterial supernatant to "capture" the small molecule ligands, in combination with X-ray crystallography and functional validation (10, 11, 20). These techniques do not easily lend themselves to the detection of MR1-bound ligands from complex biological samples, and to date, the field has not developed a simple assay to confirm antigen identity. Thus, many studies did not confirm the identity of the antigens involved. Evidence of riboflavin pathway involvement provided by detecting or deleting *rib* genes has been used as an indirect confirmation. However, it is possible that 5-A-RU recombines with other small molecules to form alternate antigens. For example, Harriff et al. suggest that novel antigens photolumazines I and III may form from 5-A-RU and α-ketoglutarate, the availability of which may be altered in nitrosative stress during intracellular infection (29).

The MR1 autoreactivity shown by some MR1T cells also reveals the importance of validating reactivity with specific controls (26, 60). In this section we present a summary of the basic tools and approaches used to develop our current understanding of the immune response to metabolite antigens.

Detection of MAIT Cells Using Antibodies or Tetramers

The precise identification of MAIT cells was hampered for several years, particularly in mice and other non-human mammals, by the lack of specific reagents for their detection. Human MAIT cells can be identified by co-staining with a panel of antibodies. Typically, CD3, CD161, and TRAV1-2 (Vα7.2) are used (124, 125), although some groups have also used the high expression levels on MAIT cells of surrogate markers such as CD26 and IL-18R (13, 57, 126, 127). The CD4$^+$, CD8$^+$, and double-negative (DN) MAIT cell subsets have been variably included or excluded. However, it is now clear that MAIT cells contain all of these subsets (128). Complicating these approaches, it is apparent that the expression of some markers may change in different physiological settings. For example, CD161 expression decreased in rheumatoid arthritis (129) and HIV infection (121). In mice, there are currently no available antibodies recognizing the TCR Vα19 utilized by mouse MAIT cells. This, combined with the low MAIT cell numbers in naïve laboratory mice, prevented the systematic assessment of MAIT cells in wild-type laboratory mice prior to the generation of MR1-tetramer reagents (7, 11, 54, 67, 130).

The first generation of MR1-tetramers (7) utilized a modified human MR1 containing a Lys to Ala mutation at position 43 at

the base of the MR1 A' binding pocket. This mutation enabled the refolding of "empty" MR1-β_2m molecules in the absence of an added ligand, which would otherwise be needed to stabilize the MR1-β_2m complex. These K43A MR1-β_2m monomers could then be loaded with an antigen of choice and tetramerised with fluorochrome-coupled streptavidin, enabling the detection of MAIT cells in human blood and intestinal cell preparations (7). The elucidation of the formation of 5-OP-RU and 5-OE-RU from 5-A-RU and methylglyoxal or glyoxal, respectively, enabled the subsequent generation of mouse and human [and later macaque (131)] wild-type MR1 tetramers loaded with each of these molecules. These were formed by refolding the MR1 and β_2m proteins in the presence of the precursor molecules, followed by biotinylation and tetramerization by standard methods (11). The resultant new generation tetramers were far more stable and easier to use in standard flow cytometry protocols. These reagents have now enabled the detection and study of MAIT cells in many contexts, similarly to the use over many years of MHC Class I (132) and CD1d tetramers (133). MR1 tetramers loaded with 6-FP and Ac-6-FP were similarly generated. These are typically used as control reagents but have been shown to detect a small number of reactive or MR1-autoreactive cells (26), the biological relevance of which is currently unclear.

Despite the clear overlap between MAIT cells detected using MR1-5-OP-RU tetramers and using antibodies to TRAV1-2 [∼95% in human blood (11)], these reagents will not detect all MR1T cells, since some of these may have different antigen specificities. This may be particularly true in tissues, or in different mammalian species, where analysis has been less extensive. Thus, similar to the methods for detecting MR1-ligands discussed above, the generation of MR1-tetramers loaded with different sources of metabolite molecules (29) will likely be useful in understanding MR1T cells other than 5-OP-RU-reactive MAIT cells. For small modifications to 5-OP-RU, such as with the altered metabolite ligands discussed above (44), the identified populations almost completely overlap, suggesting that either MR1T cells recognizing ligands other than 5-OP-RU are rare, or were excluded from this analysis by prior gating. Thus, removing the assumption of shared markers, and the use of tetramers loaded with more complex sources of potential ligands is expected to broaden the scope of detection of MR1T cells.

In vitro Cellular Assays

In order to identify MR1-bound ligands that are recognized by MAIT or other MR1T cells, several groups have developed cellular assays for screening. These utilize either T cell clones or T cell lines (such as Jurkat, SKW3, or mouse 6C2) engineered to express a MAIT TCR, which accordingly acts as a reporter cell line. These are co-cultured with cell lines expressing or overexpressing MR1, which act as antigen-presenting cells. Again, various cell lines have been utilized by different groups, in some cases with MR1 deficient cell lines acting as controls (25, 34, 49, 57, 76, 134). Alternatively, gated MAIT cells within PBMCs are analyzed after stimulation, either alone, or with cell lines or dendritic cells as APCs (11, 28, 46, 124, 135–137). Although a number of studies have examined the *in vitro* activation responses of MAIT cells sourced from human tissues (77, 100, 138, 139) to our knowledge these have not been used for MR1 ligand identification purposes. The activation of cells in these assays has been assessed by upregulation of markers [e.g., CD69, CD25, CD137(41-BB)], down-regulation of CD3 and cytokine production. These readouts give an indication of the presence of activating MR1-ligands, but do not reveal their identity. The presence of MR1-ligands is also indicated by the upregulation of cell surface MR1, as detected by conformational monoclonal antibodies 26.5 or 8F2.F9 (15, 140). However, this measure has some caveats, including lower sensitivity than MAIT cell activation, and the increase in surface expression can be missed depending on the timing of the assay due to the instability of the compounds or MR1-ligand complexes.

Interestingly, 5-A-RU, which is not believed to be a MAIT cell antigen in its own right, can activate MAIT reporter cells in culture, indicating the formation of antigens, such as 5-OP-RU, by reacting with aldehydes or ketones from the medium (such as methylglyoxal) or within cells (11). This highlights the danger of inferring antigen identity when using whole-cell assays to test for MAIT cell activation. To limit the complexity relative to the cell systems, plate-bound MR1 has also been utilized in a similar activation assay (141). The direct elution of MR1-bound ligands from immunoprecipitated cell surface MR1 for identification by mass spectrometry is technically challenging, but has been shown using C1R cells overexpressing MR1 (C1R.MR1 cells) for Ac-6-FP, 3-F-SA and diclofenac metabolites (28).

Chemical and Biochemical Investigations of MR1 Antigens and Analogs

The small molecules 5-OP-RU and 5-OE-RU are formed as chemical intermediates in the reaction of 5-A-RU with methylglyoxal and glyoxal, respectively. These open-ring compounds rapidly cyclise to form relatively weak lumazine antigen RL-7-Me, and RL (**Figure 1**). Their original identification as MR1 ligands was achieved by refolding recombinantly expressed MR1 in the presence of synthetic compounds or complex sources of antigen, such as bacterial culture supernatant (10, 11, 28), which was demonstrated to be sufficient to stimulate a MAIT cell response (47). This method enabled the MR1 molecules to "fish out" ligands that were capable of binding in a relatively unbiased approach, for subsequent analysis by LC-MS, which was complemented by high-resolution crystal structures. Bacteria with *rib* gene deletions (*S.* Typhimurium, *L. lactis*) were generated to confirm the involvement of the riboflavin synthesis pathway (10, 11). The relatively short half-life of 5-OP-RU in aqueous solution [$t_{1/2}$ half-life time ($t_{1/2}$) ∼1.5 h, 37°C] (11, 46) suggests that related compounds, if they exist in nature, may be similarly difficult to identify. More recently the Lewinsohn group used a modified procedure to assess the capture of MR1-ligands from mammalian or insect cells infected with *E.coli* and *M. smegmatis* (29). LC-MS combined with molecular networking analysis revealed many candidate ligands, with a

subset of these functionally validated using MR1T cell clones (29) (**Table 1**).

The chemical instability of MAIT antigens poses difficulties for the potential development of therapies targeting MAIT cells. On the other hand, this also provides us an insight into an important aspect of the biology of these cells. MAIT cells appear to be precisely poised to detect metabolically active microbes by recognizing antigens that are swiftly captured by MR1. Nevertheless, a few groups have attempted to develop tools in the form of more stable analogs, with the hope that these may better stimulate MAIT cell responses. Mak et al. reported that 5-OP-RU synthesized in DMSO had improved stability. Stabilized compounds (**Figure 3**) could be generated by replacing the exocyclic nitrogen atoms with carbon atoms (44, 46). However, whilst replicating a similar MAIT recognition and response, this analog was ∼1,000-fold less efficient than 5-OP-RU in boosting MAIT cells numbers when delivered intranasally with CpG (46). This again, tells us something of the exquisite specificity of MR1-MAIT recognition, selected through the co-evolution of mammalian hosts and pathogenic or commensal microorganisms.

Using a different approach to tackle the chemical instability of MAIT antigens, the Painter group recently created a stabilized 5-A-RU "pro-drug" which releases 5-A-RU upon enzymatic cleavage in the recycling endosomes and which was active *in vivo* (123). Another group reported that the stability of the precursor 5-A-RU, long known as a chemically unstable precursor of riboflavin synthesis is increased by synthesizing and storing it as its HCl-salt (142). The recent availability of 5-A-RU from commercial sources should further facilitate its study in this context.

Mouse Models

The ability of compounds to bind MR1, and to activate or inhibit MAIT cells, has been tested in various assays. However, understanding their physiological relevance requires *in vivo* models. Historically, the study of MAIT cells in mice has been difficult, due to their low numbers, and lack of specific reagents. Four main types of mice have been employed for their study; gnotobiotic (germ-free) mice, in which MAIT cells are barely detectable (12, 55), $MR1^{-/-}$ mice, which do not develop MAIT cells (12), and conversely, transgenic mice expressing the Vα19*i* invariant TCR α chain (12, 143, 144), and CAST/EiJ mice (145), which have higher numbers of MAIT cells. The use of these and other tools to study MAIT cells in mice has been previously reviewed (83). With our recent understanding that MR1T cells exhibit greater diversity than originally believed, it is important to note that many studies use $MR1^{-/-}$ mice as a "MAIT-cell deficient" model, but these will also lack MR1-dependent responses by non-MAIT MR1T cells. Additionally, the Vα19*i*Tg mice, are not a clear-cut "MAIT cell transgenic." In these mice, MR1-tetramer-reactive cells were of high frequency, however about one third of these cells did not express the promyelocytic leukemia zinc finger (PLZF) protein, a hallmark of classical MAIT cells. Additionally, a high frequency of MR1-tetramer-reactive cells could be detected even in the absence of MR1 (Vα19*i*Tg.$MR1^{-/-}$ mice) (54), and presumably these were selected by conventional MHC molecules during their development in the thymus. More recently, it has been described that MAIT cells are deficient in TCR Jα18 knockout mice (146), and also in Jα33 knockout mice, which revealed a residual population of MAIT-like TRAV1-2^{+} or TRAV1-2^{-} cells that were responsive to antigen or bacterial infection (59).

Models of infection with bacterial pathogens in mice (32–34, 54) and non-human primates (147) have revealed much about MAIT cell biology and their role in protective immunity. The development of MR1-5-OP-RU-tetramer reagents (7, 11) has enabled the specific assessment of MAIT cells in naïve SPF-housed mice (67, 130, 148), in disease-relevant models including infection, autoimmune disease (35, 149, 150), transplantation (151) and cancer (152), and MAIT cell development (55, 71, 153). MR1-tetramers also allow the assessment of the ability of defined compounds to act as antigens, with 5-OP-RU (67, 93), premixed 5-A-RU and methylglyoxal (25, 123) and a pro-drug designed to release 5-A-RU (123) all shown to boost MAIT cell numbers in mice, in the presence of TLR agonists or other co-stimuli. However, the direct assessment of MAIT cell responses to MR1 ligands *in vivo* is still a relatively under-explored area with more research needed.

CONCLUSIONS

The MR1-MR1T cell field has progressed rapidly in the short time since the discovery of the first MR1-ligands. We now understand that both MR1-ligands and MR1-reactive T cells are more diverse than first believed. MR1T, particularly MAIT cells, have been studied in physiological settings such as homeostasis, infection, autoimmune disease and cancer, sometimes with contradictory findings in both human cohorts and animal studies. There remain several questions and challenges in the field. For example, we do not fully understand the production of ligands from microbes, how these enter cells, how they are processed and presented, or the full range of signals determining the quality and quantity of the TCR-MR1-dependent response. Deciphering the complexity of antigen presentation and recognition, and the relative importance of other signals contributing to the MR1T response, particularly within the local tissue environment, is essential to understanding their biology. For instance, how do these cells differentiate between pathogens and commensal microorganisms allowing them to play diverse roles including aiding the clearance of pathogens, tissue repair and gut homeostasis? It is possible that there is a threshold amount of antigen that needs to penetrate the mucosal barriers before they will respond, and thus the abundant microbes in the intestines, which can synthesize riboflavin, are normally ignored. However, we consider it likely that the context of antigen and other signals drives different responses, including in a tissue-specific manner, whereby these cells contribute to homeostasis or inflammation and immune protection. Answering these questions will require the analysis of clearly defined MR1T subsets, and likely new tools to combat the issues of chemical instability and ligand identification. Additionally, the definition

of an increasing number of MR1T subsets with different Ag-recognition and phenotypic features raises many questions about the physiological relevance and therapeutic potential of these cells. MR1T cells offer great opportunities as attractive targets in vaccination (154) and immunotherapy (61). We hope that further advances in understanding T cell recognition of MR1-ligands using definitive tools and approaches such as those described above, will enable the MR1-MR1T axis to be harnessed to combat infection and disease.

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WA and AC generated the figures and tables. All authors wrote and edited the manuscript.

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REVIEW
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Covering All the Bases: Complementary MR1 Antigen Presentation Pathways Sample Diverse Antigens and Intracellular Compartments

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The ubiquitously expressed, monomorphic MHC class Ib molecule MHC class I-related protein 1 (MR1) presents microbial metabolites to mucosal-associated invariant T (MAIT) cells. However, recent work demonstrates that both the ligands bound by MR1 and the T cells restricted by it are more diverse than originally thought. It is becoming increasingly clear that MR1 is capable of presenting a remarkable variety of both microbial and non-microbial small molecule antigens to a diverse group of MR1-restricted T cells (MR1Ts) and that the antigen presentation pathway differs between exogenously delivered antigen and intracellular microbial infection. These distinct antigen presentation pathways suggest that MR1 shares features of both MHC class I and MHC class II antigen presentation, enabling it to sample diverse intracellular compartments and capture antigen of both intracellular and extracellular origin. Here, we review recent developments and new insights into the cellular mechanisms of MR1-dependent antigen presentation with a focus on microbial MR1T cell antigens.

Keywords: antigen presentation, MR1, MAIT cell, ligands, endosomal trafficking

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INTRODUCTION

The immune system is traditionally thought of as dichotomous. On the one hand, the innate immune system is activated by broadly conserved pathogen-associated molecular patterns (PAMPs) detected by germline-encoded pattern recognition receptors. By contrast, the adaptive immune response relies on somatic re-arrangement of antigen receptor genes to generate the diversity and specificity needed to sense extensively processed peptide antigens in the context of highly polymorphic major histocompatibility complex (MHC) molecules. However, it is increasingly appreciated that these categories rather represent the extremes of a spectrum with non-classical immune cell subsets such as innate-like, donor-unrestricted T cells (DURTs) defying a binary classification (1–3). Instead, DURTs have attributes of both adaptive and innate immunity. For example, while they express somatically re-arranged T cell receptors (TCRs), their TCR repertoire is limited, and in many cases semi-invariant. Moreover, although these TCRs recognize their cognate antigen in the context of antigen presenting molecules, they are restricted by highly conserved, monomorphic proteins displaying primarily non-peptidic ligands (1, 3). One of these is

the MHC class Ib molecule MHC I-related protein 1 (MR1). First identified in 1995 as an MHC I-related gene encoded outside the MHC locus (4), MR1 was later found to be the restricting element of the innate-like mucosal-associated invariant T (MAIT) cells (5). Like other non-conventional T cell subsets, these cells express a limited TCR repertoire and rapidly exert effector functions upon activation [recently reviewed in (6)]. While classical MAIT cells are defined by expression of the TRAV1-2 TCRα chain, more recent work has identified TRAV1-2^{-} T cells that are activated in an MR1-dependent manner, expanding the family of MR1-restricted T cells (MR1Ts) (5, 7–11). The first MAIT cell-activating MR1 ligands identified were intermediates produced during the microbial biosynthesis of riboflavin (vitamin B2) (12, 13). Since mammalian cells do not express the enzymes of this biosynthetic pathway, riboflavin precursors represent a microbe-derived molecular pattern (14). Ongoing ligand identification efforts have revealed many more microbial and non-microbial MR1 ligands which comprise both agonists and antagonists of MR1T cell activation (9, 15–17). Importantly, the number of microorganisms that synthesize riboflavin or other putative MR1 ligands is large and includes many commensal species in addition to pathogens (18, 19). This, together with the frequency of MR1Ts and the ubiquitous expression of MR1, likely necessitates tight regulation of MR1 antigen presentation to prevent inappropriate activation (20).

Classical peptide antigen presentation relies on a division of labor on the molecular scale: ER-resident MHC class I molecules bind and present peptides derived from intracellular protein synthesis whereas MHC class II molecules survey endosomal compartments where they encounter extracellular material taken up by endo- or phagocytosis. Although there are exceptions to this paradigm such as cross-presentation of exogenous or particulate antigen on MHC class I molecules, broadly speaking, immune surveillance of endogenous and exogenous peptide antigen is achieved by compartmentalization of two different antigen presenting molecules with distinct intracellular trafficking patterns (21). By contrast, MR1 is the only known metabolite-presenting molecule, placing the burden of sampling both intracellular and exogenous sources of antigenic metabolites on a single molecule. Accordingly, it is becoming increasingly clear that MR1T antigens are presented through multiple specialized presentation mechanisms likely reflecting the biochemical properties as well as the abundance and intracellular distribution of the antigen (20, 22).

In this review, we will discuss recent advances in our understanding of both the expanding repertoire of MR1 ligands and current models for distinct pathways by which these ligands are presented to MR1Ts.

TOWARD DEFINING THE MR1 LIGANDOME

Canonical MAIT Cell Antigens: Lumazines, Pterins, and Pyrimidine Neoantigens

The identification of microbial riboflavin metabolism as a source of MAIT cell-activating ligands marked a major breakthrough for the MR1 field (12, 13). The first microbial MAIT cell antigens to be identified were ribityllumazine metabolites upstream of riboflavin (vitamin B2) biosynthesis. These MR1 ligands include 6,7-dimethyl-8-D-ribityllumazine (DMRL), 7-hydroxy-6-methyl-8-D-ribityllumazine (HMRL), and reduced 6-hydroxymethyl-8-D-ribityllumazine (rRL) (13). In the same report, Kjer-Nielsen et al., described the MAIT antagonist ligand 6-formylpterin (6-FP), which derives from folate (vitamin B9). 6-FP has the same bicyclic ring structure as the ribityllumazines but lacks the ribityl tail, which is critical for recognition by the MAIT TCR (23). A subsequent report described the formation of the pyrimidine neoantigens 5-(2-oxo-propylidenamino)6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), which form upon the spontaneous reaction of the riboflavin precursor 5-aminoribityluracil (5-A-RU) with methylglyoxal or glyoxal, respectively (12). 5-OP-RU and 5-OE-RU have a single ring structure, but still feature the ribityl moiety. In a unique mode of antigen binding, both 6-FP and the pyrimidine neoantigens covalently bind to MR1 by forming a Schiff base with lysine residue 43 (K43) at the bottom of the MR1 antigen binding groove (12, 13). The ribityllumazine ligands, on the other hand, non-covalently associate with MR1, which correlates with lower antigenicity (12, 16, 24). More recently, we identified the additional MR1T-activating ribityllumazine ligands photolumazine I (PLI) and photolumazine III (PLIII), as well as the antagonistic ligand 7,8-didemethyl-8-hydroxy-5-deazariboflavin (FO) (15). All of these ligands could have 5-A-RU as a precursor metabolite, suggesting that it may be a key molecule in the synthesis of MR1T antigens. In support of this, modulation of riboflavin biosynthesis correlates with MR1T activation (25) and deletion of the enzyme responsible for the synthesis of 5-A-RU abrogates MR1T recognition of some microbes (12, 13, 25, 26). Microbial vitamin B metabolites comprise the most potent and well-characterized MAIT cell agonists to date. However, recent studies described below clearly demonstrate that a much broader range of small molecule metabolites can bind to MR1 and activate MR1Ts.

Beyond Vitamin B Metabolites: Evidence for Additional MR1 Ligands

Aspects of both MR1 itself and the MR1T TCRs support the hypothesis that the MR1 ligand repertoire includes other classes of molecules in addition to those in the vitamin B family. MR1 is structurally similar to other MHC class I molecules in that its heavy chain consists of three extracellular domains (α_1-α_3), a transmembrane domain, and a small cytosolic tail. Like MHC class Ia, MR1 non-covalently associates with β_2-microglobulin (β_2m) to form a heterodimer (27). The MR1 antigen binding cleft is formed by the α1 and α2 domains of the heavy chain and consists of an A' and an F'-pocket (23, 24, 28, 29). The canonical antigens described above bind in the A'-pocket, which consists primarily of hydrophobic amino acids (13, 28). The non-polar nature of these residues accommodates organic ligands such as the vitamin B metabolites (30) and there are various other classes of small molecules with chemical properties consistent with

binding in this groove (15, 16, 30). Furthermore, the unoccupied space remaining in the MR1 ligand binding groove outside of the A' pocket leaves open the possibility for binding of additional ligands or chaperones (31). Recent advances in defining the TCRs restricted by MR1 support this notion. MAIT cells were originally defined by their semi-invariant TCR, which consists of the TRAV1-2 α chain paired with a limited number of β chains, and features a signature CDR3α sequence (11). However, numerous studies continue to expand the MR1T TCR repertoire [reviewed in: (6, 32)]. While many of the MR1Ts with non-canonical MAIT TCRs recognize the vitamin B metabolite ligands, there are others that do not (9, 10, 33). Additionally, even among those TCRs that do recognize vitamin B metabolites, there is differential recognition of individual ligands by distinct TCRs (7, 15, 34). Combined with the conformational plasticity of the MR1 binding groove (13, 28), the increasingly recognized diversity in MR1-restricted TCRs suggests the repertoire of ligands is likely to be much broader than the vitamin B metabolites.

New Classes of MR1 Ligands: Synthetic Compounds, Riboflavin-Deficient Bacteria, and Cancer Metabolism

Inspired by the reasoning presented above, ongoing ligand identification efforts have discovered a number of non-vitamin B-derived MR1 ligands, both microbial- and non-microbial. The first non-vitamin B-derived ligands were identified through *in silico* modeling of putative MR1 interactions with synthetic molecules in chemical compound libraries (16). These ligands include the synthetic drug compounds diclofenac, an aspirin analog (3-formylsalicylic acid), and a methotrexate derivative (2,4-diamino-6-formylpteridine). Like the ribityllumazines and pyrimidines, these drugs are small cyclic compounds, some of which are MAIT cell agonists and some of which are antagonists (16). The role these ligands may play in drug-induced immune modulation through MAIT cell activation or inhibition is not yet clear. Using a similar *in silico* screen, Salio et al., recently expanded the library of MR1 ligands, including the first molecule that prevents MR1 egress from the ER (17). Intriguingly, this ligand binds in the MR1 A'-pocket in a non-covalent fashion and prevents MR1 surface translocation and MAIT cell activation in response to canonical ligands (17).

In addition to these synthetic molecules, we have found evidence for the existence of non-vitamin B metabolite microbial MR1 ligands. For example, we identified a TRAV12-2$^+$ MR1T clone that responds to an unidentified ligand from *Streptococcus pyogenes* (*S. pyogenes*), a bacterium that does not express the enzymes of the riboflavin biosynthetic pathway (10). We also performed mass spectrometry on MR1 molecules purified from cells infected with *Escherichia coli (E. coli)* or *Mycobacterium smegmatis* (*M. smegmatis*) (15). While the canonical ribityllumazine and pyrimidine ligands were the most common ions bound to the MR1 purified from *E. coli*-infected cells, these ligands had relatively low abundance in the molecules purified from *M. smegmatis*-infected cells. MR1 preparations from either infection contained ions with an ionization pattern not consistent with ribityllumazine molecules (15). Together, these data suggest a distinct class of small molecules metabolite ligands that is likely to be more prevalent in *M. smegmatis*. Consistent with this notion, Corbett et al., reported that some MR1 ligands were differentially abundant in *E. coli* compared to *Salmonella typhimurium* (*S. typhimurium*) (12). Together with data demonstrating the differential recognition of ligands by distinct MR1T TCRs (15), evidence that infection with different microbes drives the expansion of MR1Ts with distinct β chains (35) further supports the idea that different bacterial species express different ligands.

Moreover, a number of studies have recently provided indirect evidence for non-microbial self-ligands for MR1 (9, 15, 33). In the same study describing novel microbial MR1 ligands by mass spectrometry, we also identified numerous unique ions associated with MR1 purified from uninfected insect cells. We hypothesize that some of these ions represent novel endogenous MR1 ligands, whereas others may originate from chaperones or cellular co-factors associated with MR1 loading and trafficking (15). Others have demonstrated the existence of putative self-ligands in the context of tumor cell lines and primary cancers. For example, Lepore et al., identified a population of TRAV1-2$^-$ MR1Ts that recognize molecules derived from tumor cells and not microbes or cell culture medium (9). Similarly, Crowther et al. generated a non-MAIT MR1T clone specifically responding to cancer cell lines and primary cancer cells (33). Interestingly, the tumor-associated antigens reported by Lepore et al. did not form a Schiff base with MR1 like the pyrimidines and the pterins but were more similar to the ribityllumazine ligands in their non-covalent interaction with the antigen presenting molecule. The chemical identity of these MR1T antigens remains to be determined. Crowther et al. hypothesized that the ligand recognized in their system was derived from the altered metabolism characteristic of neoplastic transformation but did not report its identity. Since all remain to be identified, it is still unknown whether any of these potential self-ligands are present in healthy cells and may play a role as chaperone-like molecules such as Ii, serve as MR1T antigens that contribute to inflammation, or constitute regulatory MR1 ligands involved in immune modulation and tolerance.

DISTINCT AND COMPLEMENTARY MR1 ANTIGEN PRESENTATION PATHWAYS

MR1 at Steady State: ER and Vesicular Pools but Not Much at the Cell Surface

While MR1 has been consistently found to localize to the ER, it has also been reported to co-localize with late endosomal proteins (30, 36, 37). We have observed a vesicular distribution of MR1 even in the absence of exogenously provided ligands (36), indicating that constitutive egress from the ER is possible. However, since endogenous MR1 is hardly detectable at the cell surface of most cell lines and primary cells, these molecules are likely very transiently expressed at the cell surface and either sequestered in intracellular stores or rapidly degraded upon internalization (38) (**Figure 1**, "steady state"). This hypothesis is supported by the ability of an anti-MR1 antibody to stabilize transiently expressed MR1 molecules at the cell surface (39) and the observation that MR1 detection by flow cytometry

is cumulative when cells are incubated with antibody under conditions that allow internalization of MR1-antibody complexes (40). Of note, microbe-induced upregulation of MR1 surface expression was independent of MR1 ligand in the same study. Instead, toll-like receptor (TLR) signaling increased MR1 surface levels in some but not all antigen presenting cells and both this modulation as well as steady state MR1 surface expression were dependent on NF-κB (40). Importantly, pre-treatment with a TLR2 agonist that induced upregulation of MR1 surface expression increased MR1-dependent antigen presentation in the same report (40), providing evidence that increased anterograde flux of MR1 could feed into an "exchange pathway" (**Figure 1**, discussed below). Furthermore, McWilliam et al. showed that incubation with 5-OP-RU led to the detection of a small number of MR1-5-OP-RU complexes even at 4°C, supporting the existence of loadable MR1 molecules at the cell surface at steady state (41). MR1 requires ligand binding for stable association with β_2m and acquisition of EndoH resistance, a marker of ER egress (41). Therefore, we expect that any MR1 molecules in subcellular compartments other than the ER carry a ligand. The two most likely sources of this molecule are derivatives of folate and riboflavin contained in the culture medium or an endogenous self-ligand (discussed above). Alternatively, a small proportion of ER-resident MR1 molecules may stochastically acquire a conformation that allows them to leave the ER as a result of a conformational equilibrium, as proposed by McWilliam and Villadangos based on similar concepts in MHC class Ia folding (42–44). Regardless of how different MR1 molecules reach their respective intracellular locations, the existence of ER, vesicular, and cell surface pools of MR1 conceivably contribute to the sampling of different intracellular environments harboring different sources of MR1T antigens (20). This notion is not without precedent as different pools of MHC class Ia molecules similarly survey different subcellular compartments. Specifically, nascent MHC class Ia molecules present antigens loaded in the ER whereas a subset of recycling molecules is thought to be loaded with exogenous antigen in other compartments in the context of cross-presentation [reviewed in (45)]. Notably, microscopic localization studies of MR1 so far have relied on overexpression of GFP-tagged versions of the molecule (36, 41). It would be extremely informative to directly investigate the intracellular distribution of endogenous MR1 but this has so far been prevented by the prohibitively low abundance of the protein in WT cells.

The ER Pathway: Surface Translocation in Response to Exogenous Ligand

The use of defined model antigens such as 5-OP-RU in both *in vitro* and *in vivo* models has enabled valuable insights into the mechanisms of MR1-mediated presentation of soluble ligands administered exogenously. These studies have elucidated an "on-demand" mode of presentation for MR1 in this context (38, 41). Here, MR1 resides primarily in the ER in a partially folded, ligand-receptive state. Only when exogenous ligand neutralizes the positive charge on the K43 residue in the MR1 binding groove can the protein associate with β_2m and acquire the ability to leave the ER and translocate to the cell surface (38, 41) (**Figure 1**, "ER pathway"). MR1 loading in this model is thought to take place in the ER, although the mechanism by which the MR1 ligand reaches this compartment is still unclear (22, 38). This pathway has been the subject of excellent previous reviews to which we refer the reader [Example: (43)].

The Exchange Pathway: Swapping Out Ligands on Recycling MR1 Molecules

Recent work by our group suggests that ligand exchange plays an important role in MR1-mediated presentation of exogenous antigen (31). In this study, pre-incubation of a bronchial epithelial cell line with 6-FP overnight enhanced the presentation of exogenous ligand but did not affect MR1T activation in response to *Mycobacterium tuberculosis* (*Mtb)* infection. This supports a model in which pre-incubation with MR1-stabilizing ligand brings MR1 to the cell surface and from there into an exchange compartment where it can be re-loaded with exogenous antigen [(20, 22); **Figure 1**, "exchange pathway"]. This model is consistent with work from McWilliam et al., who showed that re-loading of 6-FP-bound molecules was possible at 37°C but not on ice, indicating a requirement for internalization and recycling for ligand exchange to occur (41). Similarly, presentation of a set of novel MR1 ligands was reduced in cells over-expressing GPI-linked MR1 compared to those transduced with the WT protein, suggesting that a motif in the MR1 cytoplasmic tail may be required for ligand exchange and loading of some ligands (17). The notion of post-ER loading of MR1 molecules is further supported by the observation that pre-incubation with 6-FP rendered the subsequent surface expression of MR1-5-OP-RU complexes less sensitive to Brefeldin A (BFA) (41). Importantly, there was still a contribution of ER-derived molecules in this system as BFA partially reduced the MR1 surface levels (41).

Intriguingly, a shorter pre-incubation with 6-FP was previously shown to abrogate presentation of *M. smegmatis* supernatant (36), seemingly contradicting the hypothesis of an exchange pathway supplied with MR1 molecules that leave the ER bound to endogenous or exogenous antagonist ligands. These two observations could, however, be reconciled by a time-dependent model of MR1 trafficking. In this scenario, the majority of the MR1 molecules are occupied by 6-FP and localized to the cell surface after 2 h while overnight incubation allows enough time for internalization and recycling to the cell surface to occur. Consequently, ligand exchange is only observed upon the longer pre-incubation. McWilliam et al. reported much faster recycling kinetics for MR1, but these measurements were made in the hematopoietic cell line C1R (41) whereas our exchange studies were carried out in epithelial cells (31, 36). Thus, recycling kinetics might be different between cell lines, particularly since C1R cells are phagocytic professional antigen presenting cells whereas epithelial cells are not. Alternatively, the different outcomes following short compared to long 6-FP pre-incubation could be explained by the presence or absence of the antagonist during antigen presentation. Specifically, 6-FP was present for the duration of the ELISPOT after the short pre-incubation whereas the antagonist was washed off before

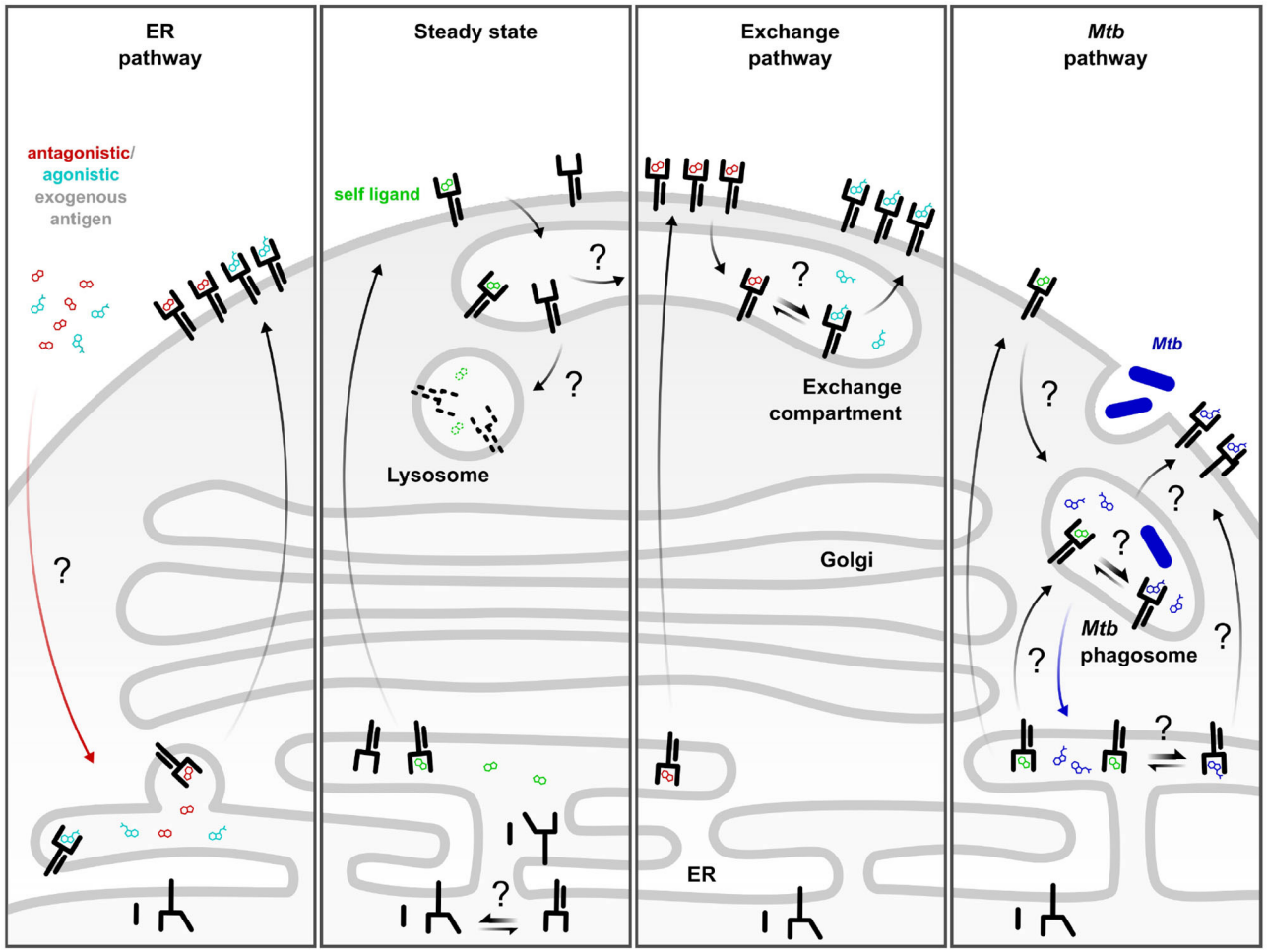

FIGURE 1 | Complementary MR1 antigen presentation pathways. ER pathway: Defined antagonistic and agonistic exogenous MR1 ligands including 6-FP and 5-OP-RU access incompletely folded MR1 in the ER and trigger its translocation to the cell surface. This pathway is dependent on neutralization of the K43 residue. Steady state: At baseline, a small fraction of MR1 molecules constitutively leaves the ER, potentially as a result of the conformational equilibrium of MR1 or through binding to an unknown self-ligand or a ubiquitous environmental ligand. These molecules are rapidly internalized and degraded in the absence of exogenous ligand or microbial infection. Exchange pathway: Alternatively, the self-ligand may be exchanged for exogenous, antigenic ligand in an exchange compartment from where the re-loaded MR1 can return to the cell surface. This process can be amplified by TLR stimulation or by pre-incubation with MR1-stabilizing antagonist ligand which increases the pool of post-ER MR1 available for exchange. *Mtb* pathway: Presentation of mycobacterial MR1T antigens generated upon intracellular infection are likely loaded in a *Mycobacterium tuberculosis* (*Mtb*)-specific phagosomal compartment. MR1 molecules may be delivered to the *Mtb* phagosome directly or *via* internalization from the cell surface. Alternatively, *Mtb*-derived ligand may reach the ER and induce MR1 surface translocation. Black arrows denote movement of MR1. Colored arrows denote movement of ligand. Straight double arrows indicate equilibria. Question marks denote hypothetical steps which have not been defined mechanistically.

co-culture with T cells after overnight incubation with 6-FP (31, 36). Thus, 6-FP was present during initial MR1 loading in both cases but was only present during presentation to MR1Ts in the 2 h pre-incubation experiment. While the rate of MR1 internalization from the cell surface is independent of ligand binding (41), the efficiency of ligand exchange or other aspects of MR1 antigen presentation could be differentially affected in the presence of the antagonist. Since exchange likely depends on the relative concentrations of the alternative ligands, the continuous presence of 6-FP in the first study may have shifted the equilibrium toward MR1 occupied by the antagonist.

The Mtb Pathway: A Requirement for Intracellular Infection and Many Open Questions

The intracellular pathogen *Mtb* was one of the first to be discovered to produce MR1T antigens (18, 46). Nevertheless, how MR1 ligands are loaded in the context of intracellular microbial infection is less well defined compared to soluble, exogenous ligands. In fact, accumulating evidence demonstrates that the molecular machinery required for the presentation of microbe-derived antigen differs from that involved in the loading of exogenous ligand (20, 22, 38). For example, presentation

of whole fixed *E. coli* bacteria is reduced upon inhibition of lysosomal acidification whereas MAIT cell activation by bacterial supernatant is not (40). In the same study, presentation of exogenous bacterial supernatant correlated with MR1 expression whereas presentation of MR1T antigen from intact bacteria was less dependent on MR1 over-expression (40). Similarly, we showed that Stx18 and VAMP4 both affect *Mtb* presentation but only Stx18 also affects surface translocation of MR1 in response to the stabilizing ligand 6-FP (36). This is consistent with early work by Huang et al., who showed that MR1 surface levels do not necessarily correlate with the ability to activate MAIT cells and that these two read-outs have different requirements for endosomal trafficking (47). Of note, these early experiments were carried out in the absence of bacterial infection or defined MR1T antigen.

Using transwell assays, we demonstrated that intracellular infection of the antigen presenting cell was required for the activation of MR1Ts in response to *Mtb* (48). By contrast, the supernatants of other bacteria such as *E. coli*, *M. smegmatis,* and *S. pyogenes* contain MR1 ligands capable of activating MR1Ts without the need for bacterial infection (10, 31, 36, 40). This difference could be explained by a comparatively low abundance of MR1 ligands produced by *Mtb*. As a consequence, containment of the microbe in an endosomal compartment might be necessary to achieve sufficiently high local concentrations of the antigen for MR1 loading. As mentioned above, gene expression levels of key enzymes of riboflavin synthesis correlated with the extent of MAIT cell activation in different *Streptococcus pneumoniae* isolates (25). Thus, it is plausible that such differences exist at the species level also. Alternatively, *Mtb* may not produce secreted MR1 ligands and liberation of MR1T antigens may require endosomal processing (31). Supporting a role for intracellular infection for optimal MR1 presentation of other intracellular pathogens, Le Bourhis et al. showed that rendering *Shigella flexneri* incapable of invading HeLa cells drastically reduced its ability to activate MAIT cells (49). Similarly, a *S. typhimurium* mutant unable to actively invade non-phagocytic cells did not elicit a MAIT cell response *in vivo* (50). However, supernatant from this mutant still activated an MR1-restricted Jurkat T cell clone *in vitro*, highlighting different mechanistic requirements for *in vitro* presentation of exogenous antigens compared to microbial infection under physiological conditions (50). Interestingly, administration of 5-OP-RU alone was not sufficient to induce MAIT cell accumulation in murine lungs although MAIT cells were activated as measured by CD69 expression (51). Accumulation of MAIT cells in this model was dependent on TLR signaling and could be achieved by co-administration of 5-OP-RU with a riboflavin-deficient bacterium or purified TLR ligands (51). This may either highlight a need for bacterial infection for optimal MR1 antigen presentation *in vivo* or indicate that MAIT cell expansion requires TLR-induced cytokine production at the site of infection. In another *in vitro* study, addition of fixed bacteria incapable of producing riboflavin did not increase MAIT cell activation in response to exogenously applied *E. coli* supernatant, indicating that both bacterium and ligand have to be present in the same compartment for optimal presentation in the context of MR1 in this model (40). Overall, the requirement for intracellular infection as well as the molecular mechanisms employed for antigen loading and presentation likely depend on the specific features of the infecting microbe. The metabolic state of the bacterium, the identity and stability of the MR1 ligands it produces, and the biochemical conditions it encounters in the intracellular environment are all likely determinants of the cellular mechanisms required for efficient MR1-mediated antigen presentation.

As mentioned, the requirement for intracellular infection with *Mtb* may indicate that endosomal processing is needed to generate and/or load mycobacterial antigen. While the cell surface can be a source of antigen presenting molecules that are loaded in endosomal compartments (45, 52), other theoretical possibilities include direct recruitment of MR1 molecules to the *Mtb* phagosome or delivery of *Mtb*-derived MR1 ligands to the ER (**Figure 1**, "Mtb pathway"). Indeed, both classical and non-classical MHC class I molecules have been detected in purified *Mtb* phagosomes (53). One way to account for the presence of these and other ER-resident proteins in phagosomes is the fusion of ER and phagosomal membranes (54, 55). Correspondingly, early evidence suggested that the ER endomembrane could contribute to phagosome membranes (54) although this has remained a point of contention [reviewed in (55)]. More recently, membrane contact sites (MCS), defined as points of close physical proximity between organelles which allow the exchange of lipids and ions without membrane fusion, have emerged as a potential explanation for the detection of ER material in phagosomal preparations (55). Alternative explanations for the presence of a subset of ER proteins in phagosomes include ER-to-phagosome vesicular trafficking and delivery of MHC-I from recycling endosomes (45, 55, 56). Both have been extensively studied in the context of MHC class I-mediated cross-presentation and although many details remain to be elucidated, multiple studies have implicated the ER SNARE Sec22b and its interaction partner Stx4 in the delivery of ER proteins directly to endosomal compartments (45, 57, 58). In our hands, knock down of Sec22b resulted in reduced presentation of *Mtb*-derived MR1T antigens (36) whereas Stx4 knock down specifically affected the presentation of *M. smegmatis* supernatant without inhibiting responses to *Mtb* infection (31). Thus, the extent of mechanistic overlap between MHC class I cross-presentation and MR1-mediated antigen presentation remains to be determined. In fact, MR1 also associates with MHC class II chaperones under certain circumstances (47), although it is not dependent on these as evident from the observation that epithelial cells, which do not express MHC class II machinery, can present MR1T antigens (20, 36, 48). While the MR1 antigen presentation pathway(s) may intersect with both MHC class I and class II pathways, we expect that specialized machinery exists to allow for the juxtaposition of MR1 and microbe-containing compartments and look forward to their identification.

By contrast, it is more difficult to envision a scenario in which mycobacterial antigens or even entire microbes should gain access to the ER. Although recent work by Legoux et al. implies that 5-OP-RU can not only rapidly cross lipid bilayers but even traverse skin and organs to reach the thymus when topically applied to mouse ears (59), the mechanism

of transport remains to be identified. We hypothesize that dedicated molecular machinery is in place to capture, stabilize, and shuttle MR1 ligands between organelles and, potentially, across longer distances on micro- and macro-anatomical scales. The identification of these chaperones for MR1 ligands is of high priority for the MR1T field.

OUTSTANDING QUESTIONS

Taken together, the current literature supports a model in which redundant and complementary pathways allow MR1 to sample discrete antigens from a variety of subcellular compartments [(20, 22, 38); **Figure 1**]. The chemical nature of the antigens may be critical to understanding these pathways, as different classes of ligands may be generated and presented through different pathways. The existence of previously identified neoantigens (e.g., 5-OP-RU), the observation of clusters of unidentified ligands that may represent novel neoantigens, and the evidence for self-ligands, demonstrate a need to continue working toward defining the MR1 ligandome. While there is overwhelming evidence that 5-OP-RU is a potent ligand for MR1Ts, antigens of the highest potency may not necessarily be those that are the most protective. The identification of additional ligands would also provide better tools to investigate how different sources and types of MR1T antigens relate to differential TCR recognition and clonal expansion. As such, a key outstanding question is whether and how ligand diversity contributes to memory formation. Finally, a pragmatic question regarding ligand diversity is whether ligands can be modified in order to improve stability, biosynthetic capability, bioavailability, deliverability, and other features that will be requirements if MR1Ts are to be targeted for vaccine or therapeutic development. In this respect, a number of groups have recently generated new synthetic versions of the known ligands, including glyco-analogs (60), monodeoxyribityl and monohydroxyalkyl analogs (61), and pro-drug analogs (62). A better understanding of ligand diversity will be required as work to modify ligands for therapeutic purposes moves forward.

The outstanding questions regarding MR1-mediated antigen presentation primarily center on the intracellular trafficking of both the antigen presenting molecule and its ligands. Firstly, it remains puzzling why endogenous surface levels of MR1 are extremely low, yet surface expression readily increases upon over-expression of the molecule even if cells are cultured in medium devoid of folate (41). This would indicate that ligand availability is not the only limiting factor and implicates MR1 protein abundance, too. It is tempting to speculate that there might be an active retention mechanism at play (20), similar to the extensive quality control governing the release of loaded MHC class I molecules (63, 64). Indeed, although it has been shown that neutralization of K43 in the MR1 ligand binding groove facilitates ER egress (41), how this is detected on a molecular level is not known. Interestingly, MR1 seems to be able to breach cellular quality control mechanisms and translocate to the cell surface in a fully folded state upon incubation at 26°C (65). This is consistent with the idea that the molecular feature, likely a specific conformation, that releases MR1 from the ER, can be achieved without addition of exogenous ligand. We hypothesize that a key determinant of MR1 surface translocation is the extent of conformational plasticity in the heavy chain, as has been postulated in the context of peptide antigen presentation (63, 66, 67). Neutralization of K43 may be one of multiple ways to restrict conformational flexibility in a way that enables ER egress. Related questions pertain to the stability of partially folded MR1 in the ER and how stabilizing ligands reach this compartment. Moreover, the increasing evidence for multiple presentation pathways, including the notion of ligand exchange in endosomal compartments, opens the door to numerous questions concerning the molecular mechanisms governing exchange of MR1 ligands. Since Schiff bases are more labile in acidic environments (41), one possibility is that exchange occurs simply when internalized MR1 molecules reach a point in the endocytic pathway where the pH is sufficiently low to destabilize the covalent bond between MR1 and its ligand. As a result, the original ligand is released, and a new ligand can be bound to "empty" MR1 molecules. In this scenario, the equilibrium between MR1 molecules bound to each ligand is determined by the pH of the exchange compartment and the relative concentrations of the available ligands (**Figure 1**, "exchange pathway"). Alternatively, exchange could be an active process catalyzed by dedicated exchange chaperones, which have been described for other MHC molecules. Examples include TAPBPR for MHC class I (64), HLA-DM for MHC class II (68), and lipid transfer proteins for CD1 molecules (69). Taken together, it is becoming clear that different MR1 antigen presentation pathways enable the MR1-MR1T axis to sample various intracellular compartments while avoiding inappropriate MR1T cell activation. The relative contributions of these complementary pathways to protective MR1T cell immunity as well as the molecular machinery underlying the individual mechanisms remain to be established.

AUTHOR CONTRIBUTIONS

MH and CK wrote and edited the manuscript. CK generated the figure. EK and DL provided intellectual contribution to the topics covered and edited the manuscript. All authors contributed to the article and approved the submitted version.

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MAIT Cells Display a Specific Response to Type 1 IFN Underlying the Adjuvant Effect of TLR7/8 Ligands

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Mucosal-associated invariant T (MAIT) cells constitute a highly conserved subset of effector T cells with innate-like recognition of a wide array of bacteria and fungi in humans. Harnessing the potential of these cells could represent a major advance as a new immunotherapy approach to fight difficult-to-treat bacterial infections. However, despite recent advances in the design of potent agonistic ligands for MAIT cells, it has become increasingly evident that adjuvants are required to elicit potent antimicrobial effector functions by these cells, such as IFNγ production and cytotoxicity. Indeed, TCR triggering alone elicits mostly barrier repair functions in MAIT cells, whereas an inflammatory milieu is required to drive the antibacterial functions. Cytokines such as IL-7, IL-12 and IL-18, IL-15 or more recently type 1 IFN all display an apparently similar ability to synergize with TCR stimulation to induce IFNγ production and/or cytotoxic functions *in vitro*, but their mechanisms of action are not well established. Herein, we show that MAIT cells feature a build-in mechanism to respond to IFNα. We confirm that IFNα acts directly and specifically on MAIT cells and synergizes with TCR/CD3 triggering to induce maximum cytokine production and cytotoxic functions. We provide evidences suggesting that the preferential activation of the Stat4 pathway is involved in the high sensitivity of MAIT cells to IFNα stimulation. Finally, gene expression data confirm the specific responsiveness of MAIT cells to IFNα and pinpoints specific pathways that could be the target of this cytokine. Altogether, these data highlight the potential of IFNα-inducing adjuvants to maximize MAIT cells responsiveness to purified ligands in order to induce potent anti-infectious responses.

Keywords: MAIT cells, type 1 interferons, co-stimulation, cytokines, vaccines

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INTRODUCTION

Mucosal-associated invariant T cells (MAIT) represent one of the largest subsets of innate T cells in humans, making up on average 3% of circulating T cells in healthy subjects (1, 2). Their semi-invariant TCR repertoire is hard-wired to bind conserved microbial ligands associated with the monomorphic MHC-related 1 (MR1) molecule (3–7). The most potent ligands identified thus far

are pyrimidines produced from the vitamin B2 (or riboflavin) derivatives, such as 5-OP-RU (8, 9). A large number of yeasts and bacteria species are riboflavin producers and therefore synthetize these ligands, making them targets for MAIT cells (10, 11). Upon activation, MAIT cells quickly gain the ability to kill infected targets in a perforin- and granzyme/granulysin-dependent manner, as well as to produce inflammatory cytokines such as IFNγ, TNFα, GM-CSF, and IL-17 (1, 12–15). Although the network of cells interacting with, or influenced by, MAIT cells remains to be detailed, they probably also exert indirect protective functions by increasing dendritic cell (DC) maturation and B cell activation and by recruiting other immune effectors (16–18). The "innateness" of MAIT cells (19) relies both on a specific intra-thymic differentiation program, leading to acquisition of a memory phenotype and expression of specific transcription factors associated with effector functions (such as PLZF), as well as with a gradual peripheral expansion and seeding into anatomical locations directly (gut and lung mucosae) or indirectly (liver) interfacing with the environment (20–29). Importantly, MAIT cell activation by pathogens has been shown *in vivo* in human infections and could be linked to disease outcome in some conditions (30–35). Thus, MAIT cells act as innate-like T cells, recognizing highly conserved, broadly expressed microbial ligands at primary sites of pathogen infection and dissemination, and display both direct antimicrobial functions as well as the ability to influence subsequent innate and adaptive responses. The observation that all human subjects analyzed thus far dedicate a significant proportion of their T cell compartment to this specific microbial metabolite recognition system in a MHC-unrestricted manner has prompted a major interest in their potential use as targets of immune intervention in major, life-threatening infectious diseases such as tuberculosis (36).

Virtually all circulating MAIT cells display an effector-memory ($CD45RA^{-}CCR7^{-}$) phenotype and, as such, display rapid effector functions upon TCR activation. However, in contrast with other T cells with similar phenotypes, their response is blunted both *in vitro* and *in vivo* (37, 38). TCR triggering with anti-CD3 mAbs or MR1 ligands is not sufficient to induce significant IFNγ production and cytotoxic functions (39). In fact, in contrast with conventional memory CD8 T cells, resting MAIT cells express low levels of perforin and almost few granzymes, with the exception of granzyme A (5, 12, 15, 40). In contrast, activation of MAIT cells with bacteria induces full effector functions, suggesting that TLR ligands and their downstream signaling are crucial for MAIT cell activation (12, 41). Indeed, co-administration of 5-OP-RU with TLR ligands is necessary to activate and recruit MAIT cells in mice (37, 42). In humans, TLR8 ligands have been identified as potent co-activators of MAIT cells through the release of IL-12 and IL-18 by TLR-activated monocytes (43). Several laboratories have shown the potency of IL-12 + IL-18 as MAIT cells co-stimulators, but other cytokines may have similar effects, such as IL-7 (15, 39, 44–47). It is likely that these requirements for co-stimulation are the result of some kind of tolerogenic process to avoid overt stimulation of MAIT cells by the microbiota-derived metabolites in the absence of danger (48–50). Nevertheless, this is an issue when considering the prospect of immune intervention targeting MAIT cells for protection. Further, a thorough description of the cellular and molecular requirement for potent MAIT cell activation is also important to our understanding of their contribution to natural immunity against pathogens, especially for microorganisms able to evade the immune system, such as *Mycobacterium tuberculosis*.

In this report, we reassessed *in vitro* the human MAIT cells response to TLR7/8 ligands. We show that type 1 IFN play a major role in the co-stimulation of MAIT cells and provide strong evidences that these cells display a specific signaling and transcriptional program upon IFNα stimulation.

MATERIALS AND METHODS

Blood Samples

Blood samples were obtained from buffy coats of healthy donors under an agreement with the Etablissement Français du Sang (EFS)—Midi-Pyrénées, in accordance with the EFS ethical guidelines. PBMC were isolated after centrifugation in a density gradient (Pancol, PAN Biotech) and frozen in DMSO before use. Experiments were performed after thawing except for phospho-flow and microarray analyses where fresh cells were used.

Ethics Statement

Blood samples from anonymous healthy donors were obtained from Etablissement Français du Sang (EFS, the French National Blood Agency). Sample use for scientific purposes was carried out in accordance with convention between EFS and Centre de Physiopathologie Toulouse-Purpan. According to French law, no agreement from a local ethic committee was required.

Cell Stimulations

Cell stimulations were performed in RPMI 1640 supplemented with antibiotics and 10% FCS. PBMC were plated at 5×10^6 cells/ml in tissue culture-treated 96-well plates. R848 (10 μg/ml), gardiquimod (1 μg/ml) (both from Invivogen), IFNα2b (1000 IU/ml; Schering-Plough), IL-12 (100 ng/ml; Peprotech), and IL-18 (100 ng/ml; Peprotech) were added to the cells for 3 h before addition of OKT3 (10 ng/ml; Muromonab, Janssen-Cilag). For blocking experiments, anti-IFNαβR chain 2 (Merck Millipore), anti-IL-12 (BD Biosciences), anti-IL-18 (RD systems) or an isotype control (RD systems) was incubated with the cells 1 h before any stimulation. For the detection of intracellular cytokines, 3 μg/ml of Brefeldin A (Thermo Fisher Scientific) was added 1 h after OKT3. After 16 h of incubation, cells were harvested and processed for flow cytometry. For phosphoflow and imaging cytometry experiments, cells were incubated with 10^4 IU/ml of IFNα2b for 15 min before processing.

Cell Subset Isolation

For QRT-PCR experiments and functional studies, PBMC were stained with anti-TCRVα7.2-PE (Miltenyi Biotec), washed, and incubated with anti-PE microbeads (Miltenyi Biotec). After washing, cells were processed for positive selection with the autoMacs Pro Separator (Miltenyi Biotec) and the positive

TCRVα7.2 fraction was collected. These cells were further stained with anti-CD5, anti-CD8, anti-CD45RA, and anti-CD161, and the $CD5^+CD8^+TCRV\alpha7.2^+CD45RA^-CD161^+$ MAIT cells and the $CD5^+CD8^+TCRV\alpha7.2^+CD45RA^-CD161^-$ conventional memory CD8 were Facs-Sorted. For microarray analysis, untouched CD8 T cells from 4 healthy donors were purified from fresh PBMC with the CD8 T cell Isolation Kit (Miltenyi Biotec), according to the manufacturer's instructions. The purified fractions were stained with anti-TCR Vα7.2, CD45RA and CD161, and the $TCRV\alpha7.2^+CD45RA^-CD161^+$ MAIT cells and the $TCRV\alpha7.2^-CD45RA^-CD161^-$ memory CD8 T cell fractions were electronically sorted to a purity >98%. Cell sorting was performed with a FacsAria SORP equipped with four lasers (488 nm, 633 nm, 405 nm, and 375 nm) (Becton Dickinson).

Flow Cytometry

Extracellular stainings were performed by incubating cells with the appropriate concentration of antibodies for 15 min at + 4°C, washing, and resuspending cells in PBS with 1% FCS. For MAIT cell identification, we chose to use the CD5 (sometimes together with CD2) marker as a mean to avoid artifacts resulting in CD3 downregulation induced by OKT3 stimulation. Intracellular stainings for granzyme B, perforin, and cytokines were performed after extracellular stainings and fixation and permeabilization with the Cytofix/Cytoperm (BD Biosciences), by incubating cells for 1 h with the appropriate concentrations of antibodies. For phosphoflow analyses, fresh cells were first stained with anti-CD161, washed, fixed with Max Buffer Phosflow (BD Biosciences), and permeabilized with Perm Buffer III (BD Biosciences). Cells were then stained with anti-CD8, anti-CD45RA, and either an anti-pSTAT4 or IgG2a isotype control for 1 h, before washing and analysis. The following antibodies were used: anti-TCRVα7.2 PE, anti-TCRVα7.2 FITC, anti-TCRVα7.2 PeCy7 (clone 3C10, Biolegend), anti-IFNγ FITC (clone 45-15), anti-CD8 VioBlue (clone B135/80), anti-CD107a FITC, anti-CD107a PE (clone H4A3), anti-CD8 PE (clone BW135/80), anti-CD45RA Viogreen, anti-CD45RA PE Vio770 (clone REA562), anti-CCL4 PE, anti-CCL4 APC (REA511), anti-CD161 PE Vio770, anti-CD161 APC (clone 191B8), anti-Granzyme B PE (clone REA226), anti-CD5 APC Vio770, anti-CD5 VioBlue (clone UCHT2), anti-CD2 Percp Vio700 (clone LT2), anti-TNFα FITC, anti-TNFα PE (clone cA2) (all from Miltenyi Biotec), anti-STAT1 (pY701) eFluor450 (clone KIKSI0803, eBiosciences), IgG1 isotype control eFluor450 (P3.3.2.8.1, eBiosciences), anti-STAT4 (clone 38/p-Stat4, pY693) AF488 (BD Biosciences), and IgG2a isotype control AF488 (BD Biosciences).

Data acquisitions were performed on a MacsQuant (Miltenyi Biotec), and data were analyzed with FlowJo (BD Biosciences).

Imaging Flow Cytometry

Untouched CD8T cells were enriched from the PBMC of healthy donors by magnetic depletion with the CD8 T Cell Isolation Kit (Miltenyi Biotec). Cells were stimulated for 15 min with 1000 IU/ml IFNα in FCS/ATB-supplemented FCS and washed. Surface staining was performed with anti-CD5 APC Vio770, CD8 VioBlue, and CD161 APC, followed by fixation and permeabilization as described for the phosphoflow experiments, and stained with either anti-pSTAT4 (Y693) AF488 or IgG2a Isotype control. Data were acquired on the Amnis Image Stream X Mark II and analyzed with IDEAS software (Merck Millipore).

QRT-PCR

mRNA was extracted from isolated MAIT and memory CD8 T cells using the microRNA easy plus kit (Qiagen), according to the manufacturer's instructions. Protein contamination was excluded by quantification of the sample absorbance at 260/280. mRNA samples were reverse transcribed with the SuperScript III First-Strand Synthesis System for RT-PCR (Thermo Fisher Scientific). QRT-PCR was performed with the SYBR Green Real-Time PCR Master Mix (Thermo Fisher Scientific), at a hybridation temperature of 58°C, on a LightCycler 480 (Roche). The following primers were used: STAT4 5′-GGCAATTGGAGAAACTAGAGG-3′ and 5′-AGGGTGGGTTGGCATACAT-3′; STAT1: 5′-TCACATTCA CATGGGTGGAG-3′ and 5′-CAAAGGCATGGTCTTTGTCA-3′; GAPDH: 5′-ATCTTCTTTTGCGTCGCCAG-3′ and 5′-ACGACCAAATCCGTTGACTCC-3′.

Microarray Analysis

Gene expression analysis was performed on paired purified MAIT and conventional memory CD8 T cells from healthy donors (n = 4), either unstimulated or stimulated with 2 000 IU/ml of IFNα for 90 min at the GeT facility (INSA, Toulouse, France). RNA was extracted with microRNA easy plus kit (Qiagen), according to the manufacturer's instructions. The quality of RNA was determined with an automated electrophoresis tool (Agilent Technologies, 2100 Bioanalyzer system) and the RNA quantity with a UV–Vis spectrophotometer (Thermo, NanoDrop 2100) at 260 nm/280 nm/230 nm absorbance. 500 pg of total RNA was used for sample preparation with the GeneChip Pico Reagent Kit. Following fragmentation, 5.5 μg of cRNA was hybridized for 16 hr at 45°C on human GeneChip Clariom S Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450. Then, GeneChips were scanned using the GeneArray Scanner 3000 7G and images were analyzed using Command Console software to obtain the CEL files with raw data (values of fluorescent intensity). Microarray analysis was carried out by the Transcriptome Analysis Console (TAC, version 4.0) software certified by Affymetrix. Raw data were transformed in log2, and Affymetrix Gene microarrays were normalized with the "Signal Space Transformation-Robust Multichip Analysis" (SST-RMA) method. After summarization of probes, a unique value for each gene was obtained. To remove false positives, the false discovery rate correction was applied with a p-value-adjusted threshold of 0.05.

Statistical Analysis

Statistical analyses were performed with the GraphPad Prism 6.0 software. Paired or unpaired, two-tailed, and non-parametric Mann–Whitney tests were used to assess statistical significance. A p-value < 0.05 was considered significant.

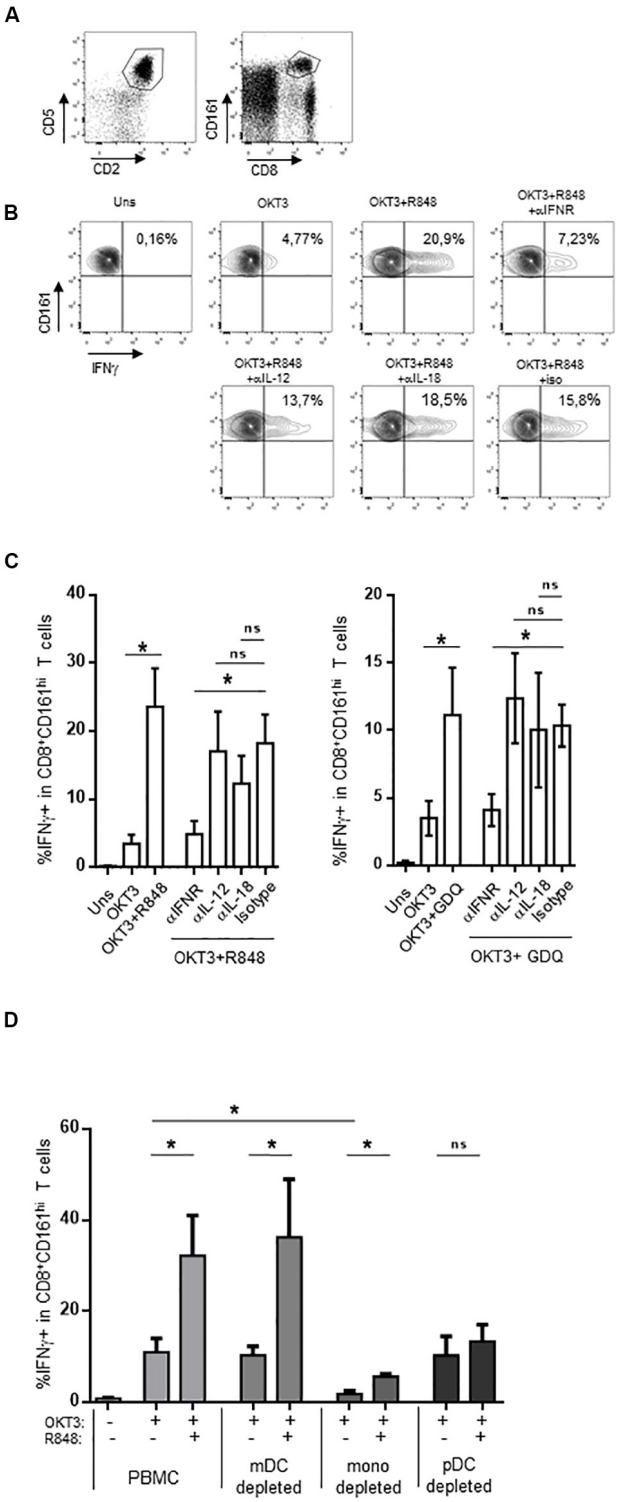

FIGURE 1 | $CD161^{hi}CD8^{+}$ T cell activation by TLR7/8 ligands. **(A)** Gating strategy, based on T cell gating as CD5 + CD2 + lymphocytes (left panel), followed by identification as $CD161^{hi}CD8^{+}$ cells within the CD5 + CD2 + subset. **(B,C)** PBMC were rested or incubated for 16 h with the indicated reagents, in the presence of brefeldin A, before cell surface staining, fixation, permeabilization, and intracellular staining with anti-IFNγ antibody.

(Continued)

FIGURE 1 | Continued

$CD161^{hi}CD8^{+}$ T cells were identified as described in **(A)**. **(B)** A representative example obtained with R848 in one donor. **(C)** The cumulative frequencies (mean ± SEM) of IFNγ-producing CD161hi CD8 + T cells when R848 (left panel) or GDQ (right panel) was used as a co-stimulator, $N = 4$. **(D)** PBMC were used unseparated or after depletion of CD11c-, CD14-, or CD123-expressing cells, and stimulated with OKT3 in the presence or absence of R848. After staining, CD161hi CD8 + T cells were analyzed for intracellular IFNγ production. Cumulative frequencies (mean ± SEM) of IFNγ + MAIT cells are shown, $N = 4$. Mann–Whitney test was used to compare frequencies. $^{*}p < 0.05$; ns, not significant.

RESULTS

Role of Type 1 IFN in the Adjuvant Effect of TLR Agonists on $CD161^{hi}CD8^{+}$ T Cells

Compared to conventional memory T cells, MAIT cells are hypofunctional to TCR-dependent stimulation, resulting in a blunted functional response to purified antigens or anti-CD3 stimulation (37, 38). A strong response can be obtained by the use of adjuvants such as TLR ligands. In particular, the TLR7/8 ligand R848 strongly activates monocytes, which in turn secrete IL-12 and IL-18; these two cytokines strongly potentiate TCR-dependent MAIT cells response. We reasoned that other signals could be involved in this indirect effect of R848; in particular, R848 binds TLR8 but also TLR7, which is strongly expressed by IFNα-secreting plasmacytoid dendritic cells (51–54). We thought that type 1 interferons could also be involved in the potentiation of MAIT cell activation, and tested this hypothesis in whole PBMC. We stimulated PBMC with a low dose of soluble OKT3 (sOKT3), with or without a previous incubation with R848, and quantified the frequency of MAIT cells expressing intracellular IFNγ. Anti-CD3 stimulation induces CD3 downregulation and may lead to experimental artifacts (55), precluding the use of both anti-CD3 and anti-TCR mAbs. It is well known that MAIT cells comprise >90% of $CD8^{+}CD161^{hi}$ T cells (56). Thus, we used the $CD5^{+}CD2^{+}CD8^{+}CD161^{hi}$ phenotype to study a $CD8^{+}$ MAIT cell-enriched subset (**Figure 1A**). OKT3 stimulation alone resulted in a low proportion of IFNγ+$CD161^{hi}CD8^{+}$ T cells (**Figures 1B,C**), whereas the presence of R848 dramatically increased this functional response, both with respect to the frequency of IFNγ-producing cells and the amount of IFNγ/per cell (assessed by the MFI of IFNγ staining). We then tested the role of IL-12, IL-18, and type 1 IFN by testing the ability of blocking antibodies against these cytokines or their receptors to inhibit the action of R848. IL-12 and IL-18 blockade showed a minor effect on R848-induced potentiation of MAIT cell response (**Figures 1B,C**, left panel). In contrast, blocking the common IFNα/β receptor (IFNAR) dramatically inhibited the effect of R848 on IFNγ production in anti-CD3-stimulated $CD161^{hi}CD8^{+}$ T cells, suggesting a major role for type I IFN in this adjuvant effect. To confirm these data, we made use of another TLR agonist, gardiquimod (GDQ). GDQ is a specific TLR7 agonist and potent IFNα inducer. As shown in **Figure 1C** (right panel), GDQ significantly potentiated the $CD161^{hi}CD8^{+}$ T cell response to sOKT3, and this effect was reversed by blocking

the IFNAR. These two pieces of data confirm that type 1 IFN is strongly involved in the potentiation of CD161^{hi}CD8^{+} T cell response upon TLR7 (GDQ) or TLR7+8 (R848) ligation. pDC are the major IFNα-producing cells (on a per cell basis) upon TLR7 activation, but TLR8 agonists may also induce a strong production of IFNβ by monocytes or mDC (57). To identify the main cellular source(s) of type 1 IFN in our settings, we performed cell-depletion experiments. Thus, we separately depleted mDC, monocytes, and pDC from the PBMC of 3 individuals and assessed the effect of R848 on CD161^{hi}CD8^{+} T cell response to anti-CD3 stimulation (**Figure 1D**). The depletion of mDC had no impact on CD161^{hi}CD8^{+} T cell response to sOKT3 alone or with R848, suggesting that R848 can mediate its effects in the absence of mDC. Depleting monocytes resulted in a very low response to sOKT3, probably because this accessory cell type is necessary to the stimulatory effect of sOKT3 on T cells in the absence of exogenous co-stimulation. Nevertheless, the addition of R848 increased the CD161^{hi}CD8^{+} T cell response to the same extent as in whole PBMC, compared with OKT3 alone. Finally, depleting pDC completely abolished the adjuvant effect of R848, strongly pointing to this cell type as the main mediator of R848 action on CD161^{hi}CD8^{+} T cells. Altogether, these data show that R848 potentiates CD3-mediated CD161^{hi}CD8^{+} T cell response in a pDC- and type 1 IFN-dependent manner. Since GDQ, a TLR7 agonist, shows similar adjuvant effects on CD161^{hi}CD8^{+} T cells, it seems likely that R848 activates pDC through TLR7 as well in this setting. Therefore, we decided to analyze in depth the co-stimulatory action of type 1 IFN on MAIT cells response, selecting recombinant IFNα2b as a model type 1 IFN.

IFNα Strongly Potentiates CD161^{hi}CD8^{+} T Cells Effector Functions

The addition of IFNα alone induced CD69 expression on PBMC CD161^{hi}CD8^{+} cells in a dose-dependent manner (**Figure 2A**); however, the production of intracellular IFNγ was minimal (**Figures 2B,C**). In contrast, IFNα very strongly potentiated the production of IFNγ and TNFα, but also of CCL4, by CD161^{hi}CD8^{+} T cells in response to anti-CD3 (**Figures 2B,C**). Type 1 IFN have pleiotropic effects, as most lymphocytes express the IFNAR. Therefore, the observed action of IFNα on CD161^{hi}CD8^{+} T cells could only be representative of its broad action on memory CD8 T cells in general. To test this, we analyzed within the same PBMC the effect of IFNα on CD161^{hi}CD8^{+} T cells and conventional CD8 memory T cells (as defined by the CD5^{+}CD8^{+}CD45RA^{-}CD161^{-} phenotype). This adjuvant effect of IFNα on cytokine production was dramatically higher in CD161^{hi}CD8^{+} T cells than in conventional memory CD8 T cells (**Figure 2D**). Thus, although IFNα acts on conventional memory CD8 T cells, CD161^{hi}CD8^{+} T cells, including MAIT cells, display a higher sensitivity to this cytokine.

As our experiments were performed on whole PBMC, IFNα could act either directly on CD161^{hi}CD8^{+} T cells or indirectly, by inducing in accessory cells other soluble or membrane factors that would co-stimulate this subset. To address this question, we purified MAIT cells (this time using anti-TCRVα7.2 antibody, see section "Materials and Methods") and stimulated them with tetrameric CD2/CD3/CD28 antibodies alone or in the presence of IFNα. As shown in **Figure 2E**, purified MAIT cells responded directly to IFNα co-stimulation, in the absence of accessory cells (data not shown). Similar results were obtained with purified CD8 + T cells. We conclude that MAIT cells respond directly to type 1 IFN co-stimulation which synergizes with TCR stimulation for IFNγ production.

Besides cytokine production, MAIT cells display cytotoxic functions; however, resting MAIT cells express low levels of cytolytic molecules and require some level of priming to display their full effector capacities (12, 13). We then asked whether type 1 IFN may prime CD161^{hi}CD8^{+} T cells for cytotoxicity. IFNα alone was able to increase both perforin and granzyme B expression in CD161^{hi}CD8^{+} T cells (**Figures 3A,B**), whereas OKT3 had no effect on these 2 molecules. When looking at the combined effects of these 2 stimuli, the level of perforin expression remained unchanged as it reached the same level than IFNα alone, but there was a very strong potentiation of granzyme B expression. We also tested the capacity of IFNα-co-stimulated CD161^{hi}CD8^{+} T cells to degranulate, as assessed by the surface expression of the endosomal marker CD107a. IFNα alone did not induce CD107a expression, but it dramatically increased its expression upon sOKT3 stimulation (**Figures 3C,D**). Altogether, IFNα very strongly potentiates CD161^{hi}CD8^{+} T cell effector functions, in terms of cytokine production, degranulation, and expression of cytotoxic molecules. It is noteworthy that the effect of IFNα was seen only when it was added before sOKT3, but not at the same time (**Figure 3E**). This suggests that IFNα acts by priming MAIT cells for full response to TCR-dependent stimulation.

We reproduced these data by using a different, more specific stimulus for MAIT cells. We used the *E. coli* supernatant, which contains bioactive MR1 ligands (58). This supernatant is able to induce both intracellular IFNγ production and surface expression of CD107a in PBMC CD161^{hi}CD8^{+} T cells (**Figures 4A,B**), in an MR1-dependent manner (**Figure 4B**). In contrast, the frequency of responding CD161^{-}CD8^{+} T cells was very low. It is noteworthy that IFNα pretreatment especially increased the frequency of polyfunctional CD107a^{+}/IFNγ^{+} MAIT cells (**Figures 4A,B**, right panel).

Preferential Use of STAT4 in IFNα-Stimulated MAIT Cells

We then wanted to investigate what appears to be a specific sensitivity of MAIT cells to IFNα. Type 1 IFN have pleiotropic effects on different cell types; moreover, depending on the context, it can even show opposite effects on the same cell types. For example, in CD8 T cells, type 1 IFN can mediate either anti-proliferative effects or promote IFNγ production, akin to IL-12 (59–61). The mechanisms behind these differential effects have been partially unraveled in mice. It was shown in the murine LCMV model that the balanced expression of STAT4 and STAT1 modulates the response of CD8 T cells and NK cells to type 1 IFN. Indeed, although type 1 IFN predominantly uses the STAT1 pathway, leading to anti-proliferative effects, viral infection

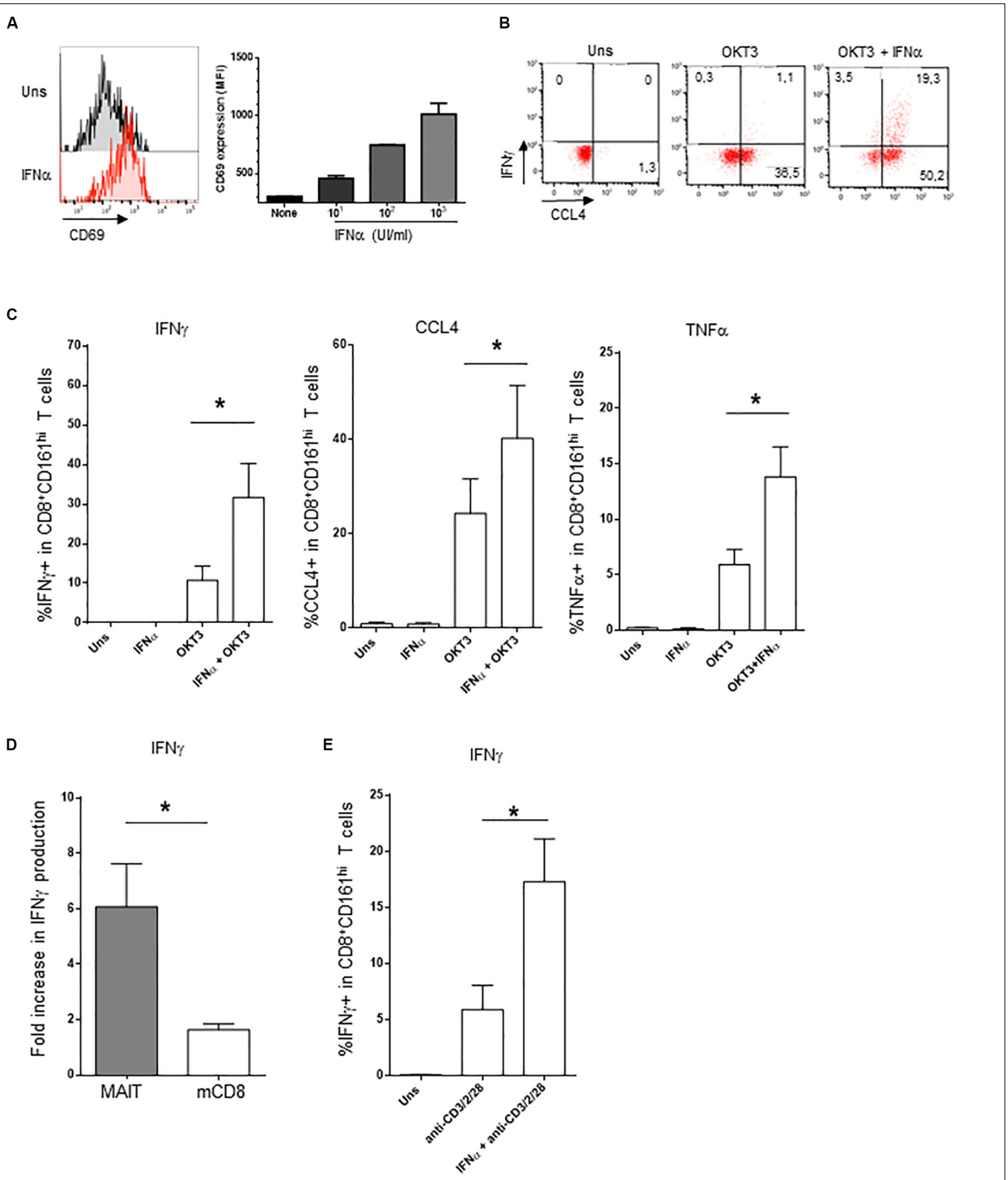

FIGURE 2 | Co-stimulation of CD161^{hi}CD8^{+} T cells activation by IFNα2b. **(A)** Left panel: representative example of membrane CD69 expression by CD161^{hi}CD8^{+} T cells left unstimulated (upper panel) or stimulated for 20 h with 1000 IU/ml of rIFNα2b (lower). Right panel: dose-response of CD69 expression by CD161^{hi}CD8^{+} T cells with increasing doses of rIFNα2b. **(B)** Representative example of intracellular IFNγ and CCL4 production by CD161^{hi}CD8^{+} T cells left unstimulated or after stimulation with the indicated stimuli. **(C)** Cumulative frequencies (mean ± SEM) of intracellular IFNγ + (left), CCL4+ (middle), and TNFα + (right) CD161^{hi}CD8^{+} T cells treated as in **(B)**, $n = 6$. **(D)** Ratio (mean ± SEM) of IFNγ + cells after OKT3 + IFNα2b stimulation to OKT3 stimulation alone, in CD161^{hi}CD8^{+} T and conventional memory CD8 T cells. **(E)** Intracellular IFNγ production (mean ± SEM) by purified MAIT cells left unstimulated or stimulated with anti-CD3/CD2/CD28 tetrameric antibodies alone or in combination with IFNα2b, $n = 4$. Mann–Whitney test was used to compare frequencies. *$p < 0.05$; ns, not significant.

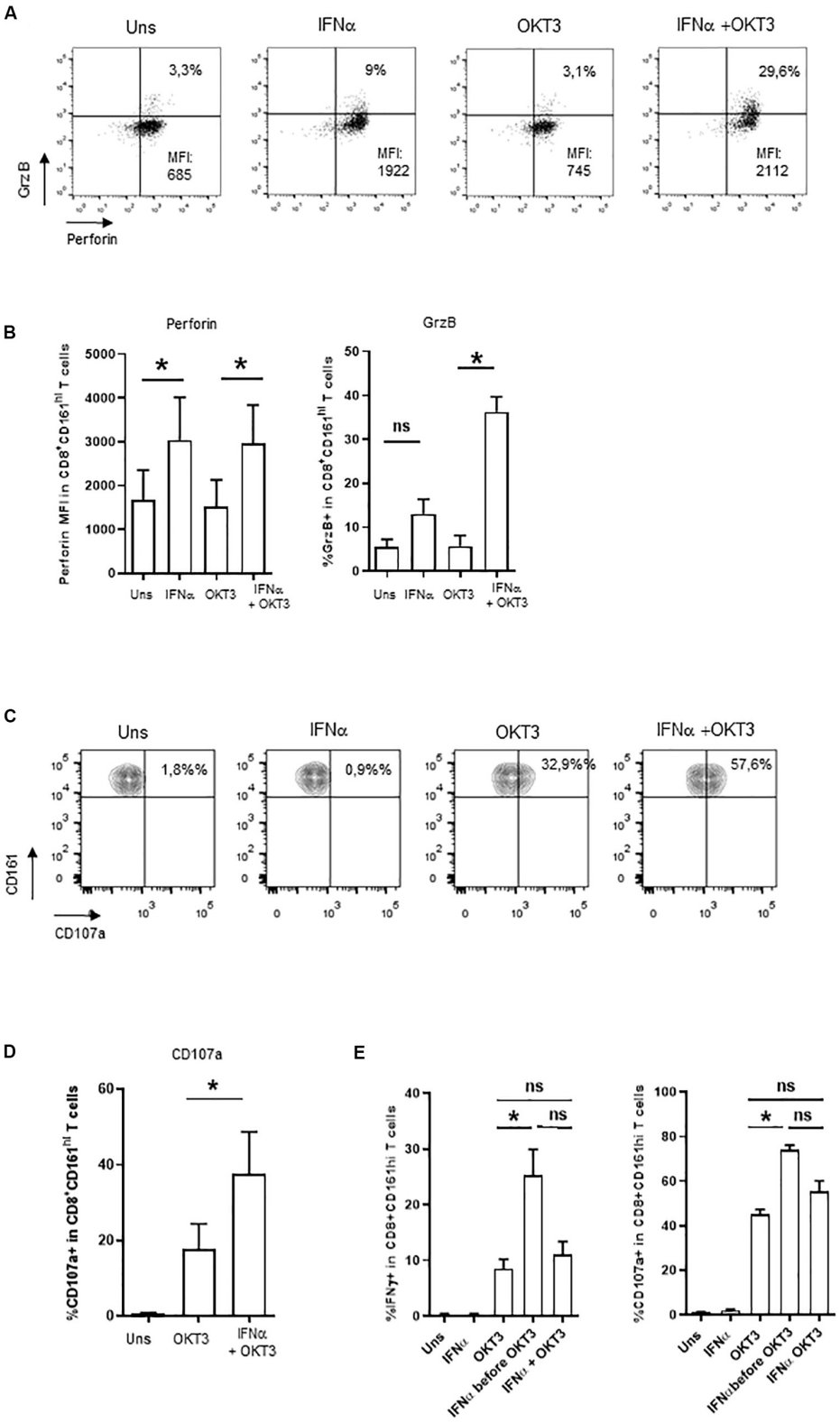

FIGURE 3 | Induction of cytotoxicity in IFNα2b-co-stimulated CD161^{hi}CD8^{+} T cells. **(A)** Representative example of intracellular perforin and granzyme B expression by CD161^{hi}CD8^{+} T cells left unstimulated or stimulated under the specified conditions. **(B)** Cumulative data (mean ± SEM) for the mean fluorescence intensities of perforin staining (left panel) and frequencies of granzyme B + (right panel) CD161^{hi}CD8^{+} T cells, stimulated in the indicated conditions. **(C)** Representative example

(Continued)

FIGURE 3 | Continued
of CD107a staining by CD161^{hi}CD8^{+} T cells. Dot plots are gated on CD8^{+} T cells. **(D)** Cumulative frequencies (mean ± SEM) of CD107a^{+}CD161^{hi}CD8^{+} T cells after stimulation with OKT3 alone or in combination with IFNα2b. Mann–Whitney test was used to compare frequencies. $n = 4$, $^{*}p < 0.05$; ns, not significant. **(E)** PBMC were either left unstimulated, or stimulated in different conditions: IFNα or OKT3 for 20 h, IFNα for 3 h before adding OKT3 for the next 17 h, or IFNα and OKT3 for 20 h. Brefeldin A was added 4 h after the beginning of the culture. At the end of the incubation period, PBMC were collected and stained for extracellular and intracellular markers as stated in the section "Materials and Methods." For CD107a expression, the anti-CD107a mAb was incubated with cells during the whole culture period. Figures show cumulative data ($n = 4$) for IFNγ production (left panel) and CD107a expression (right panel) by CD161^{hi}CD8^{+} T cells under the specified conditions. Statistical comparison was performed with a Friedman test with Dunn's correction for multiple comparisons. $^{*}p < 0.05$; ns, not significant.

induces predominant STAT4 expression and promotes IFNγ (62). From these data, we hypothesized that the response of MAIT cells to IFNα could be at least partially explained by the differential use of STAT4 and STAT1-mediated pathways downstream of the IFNAR. To test this hypothesis, we first quantified STAT1 and STAT4 gene expression by QRT-PCR in purified MAIT (defined with the anti-TCR Vα7.2 mAb, see section "Materials and Methods") versus TCRVα7.2-CD161- memory CD8 T cells. As shown in **Figure 5A**, the STAT4/STAT1 ratio in MAIT cells was >6 fold higher than in TCRVα7.2^{-}CD161^{-}CD8^{+} T cells, showing that resting MAIT cells chiefly express STAT4 over STAT1. To verify that STAT4 is indeed phosphorylated upon IFNα incubation, we used phosphoflow cytometry to quantify p-STAT4. STAT4 was phosphorylated upon 15 min of incubation with IFNα in both MAIT and conventional memory CD8 T cells (**Figure 5B**); however, the MFI of pSTAT4 expression was significantly greater in the former than in the latter (**Figure 5C**). Finally, we checked the nuclear translocation of p-STAT4 in MAIT cells by use of imaging flow cytometry. Resting, inactivated MAIT cells (defined as CD161^{+}CD8^{+} cells) expressed no p-STAT4; upon 15 min of incubation with IFNα, we could detect a significant expression of pSTAT4, which entirely co-localized with DAPI in the nucleus of MAIT cells (**Figure 5D**). We conclude from all this set of experiments that resting MAIT cells express an elevated level of STAT4, which is preferentially phosphorylated and translocated in the nucleus upon IFNα incubation, compared to conventional memory CD8 T cells.

IFNα Elicits a Specific Transcriptional Program in MAIT Cells

Our data strongly suggest that IFNα selectively acts on MAIT cells to dramatically increase their responsiveness to TCR-dependent activation. To more formally address this hypothesis, we performed a microarray analysis of gene expression regulated by IFNα in MAIT versus conventional memory CD8 T cells. Of note, the isolation procedure excluded TCRVα7.2^{-}CD161^{-} cells from this latter subset. MAIT and conventional memory CD8 T cells were purified from the blood of 4 healthy donors, and whole-genome microarrays were performed on resting and IFNα-stimulated samples. We deliberately chose an early time-point (90 min after stimulation) to investigate the immediate effect of type 1 IFN on gene expression. PCA analysis of the 4 types of samples confirmed that resting MAIT cells express a specific set of genes (**Figure 6A**). We first looked at the expression of genes that have been previously described as MAIT-enriched. Indeed, we found a high expression (compared to conventional memory CD8) of *KLRB1* (encoding CD161), *ZBTB16* (encoding PLZF), *CCR6*, *RORC*, *IL-23R*, *CXCR6*, *DPP4* (encoding CD26), and *IL18R1* (**Figure 6B**). These data confirmed the quality of our protocol and encouraged us to analyze further comparisons. A total of 610 and 928 genes were found to be significantly regulated by IFNα in conventional memory CD8 and MAIT cells, respectively (**Figure 6C**). Interestingly, 464 genes were found in common between both cell types, including multiple interferon-specific genes (ISG) as expected (**Supplementary Table S1**). Half (464) of the genes regulated in MAIT were specific and not found in conventional memory T cells. We took a closer look and found that IFNα downregulated 189 genes in conventional CD8 and 430 genes in MAIT, 70% of the latter being specific (**Figure 6C**, right panel, middle). In contrast, IFNα upregulated 421 genes in conventional CD8 and 498 genes in MAIT cells, with only 33% of the latter being specific to this subset (**Figure 6C**, left panel). In other words, IFNα down- and upregulates approximately the same number of genes in MAIT cells, whereas more genes are upregulated than downregulated in conventional CD8 T cells. Interestingly, *furin*, encoding a proprotein convertase shown to be involved in IFNγ production (63), showed an increased transcription in treated MAIT cells only, providing a possible mechanism for the selective action of type 1 IFN. We then used the Ingenuity Pathway Analysis software to analyze canonical pathways regulated by IFNα in MAIT cells. As expected, we obtained very low *p*-values for pathways directly associated with IFN signaling, interferon-regulatory factors, or pattern-recognition receptors (**Figure 6D**). Interestingly, we also detected modules associated with cytotoxic lymphocytes and natural killer cell signaling. We next analyzed side-by-side the disease and function pathways regulated by IFNα in conventional CD8m and MAIT cells. This revealed common and specific pathways for both subsets. Interestingly, MAIT cells specifically upregulated pathways associated with cytotoxicity of lymphocytes, and T cell response, compared with conventional CD8m T cells (**Figure 6E**). Altogether, these experiments confirm that the MAIT cell response to IFNα partially induces a specific set of genes, not found in conventional memory CD8 T cells, and therefore that MAIT cells display a specific responsiveness to this cytokine.

DISCUSSION

How pathogen-derived signals activate protective antimicrobial functions in MAIT cells remains incompletely solved. We provide here strong evidences that IFNα2b (and most likely other type 1 IFN) are important actors in this process, confirming a very recent report (64). We also provide compelling evidences that

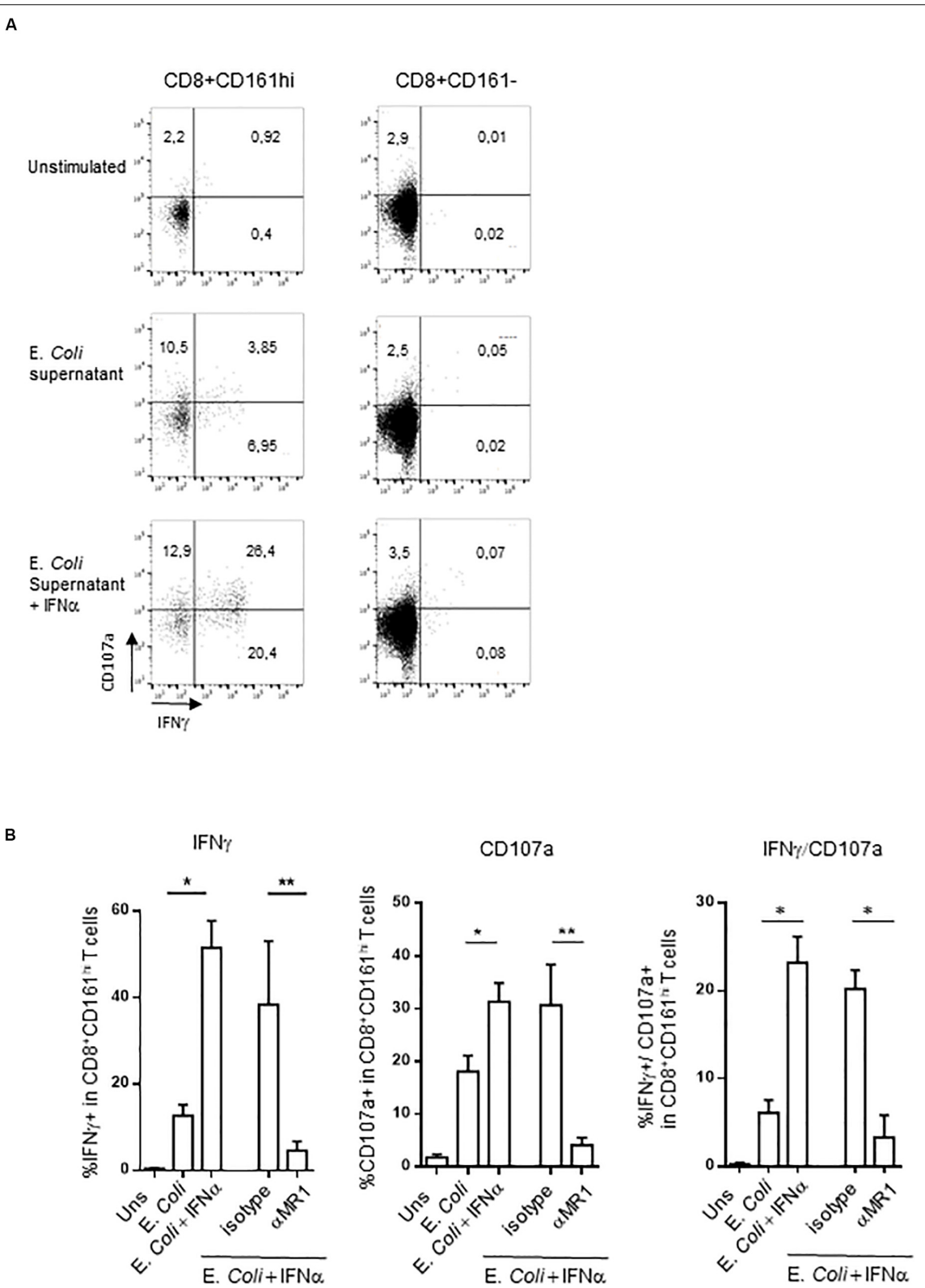

FIGURE 4 | (A) Representative flow cytometry dot plots showing membrane CD107a and intracellular IFNγ expression by $CD161^{hi}CD8^{+}$ T cells (left) or conventional CD8 T cells (right) left unstimulated, or after stimulation with *E. coli* supernatant alone or in combination with IFNα2b. **(B)** Cumulative frequencies (mean ± SEM) of IFNγ+ (upper) and CD107a+ (lower panel) $CD161^{hi}CD8^{+}$ T cells stimulated under the indicated conditions, $N = 5$. Mann–Whitney test was used to compare frequencies. $^{**}p < 0.01$, $^{*}p < 0.05$; ns, not significant.

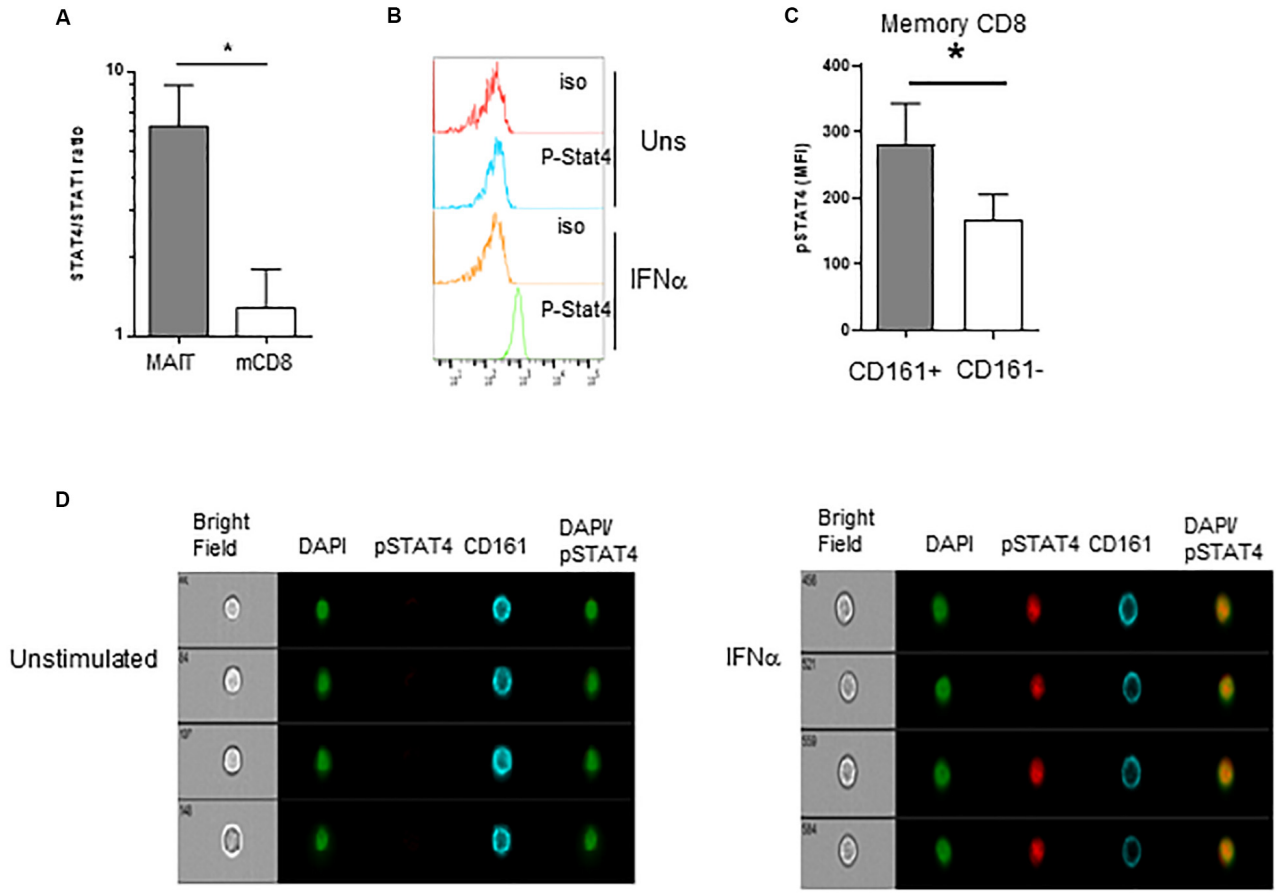

FIGURE 5 | Preferential usage of the STAT4 pathway by IFNα-stimulated MAIT cells. **(A)** Ratio of STAT4 to STAT1 mRNA expression in purified MAIT cells and conventional memory CD8 T cells, determined by quantitative RT-PCR, $N = 4$. **(B)** Representative flow cytometry histograms of phospho-stat4 expression by MAIT cells left unstimulated or after IFNα2b stimulation. **(C)** Cumulative data (mean ± SEM) of MFI of pSTAT4 staining in MAIT and conventional memory CD8 T cells, after IFNα2b stimulation. Mann–Whitney test was used to compare frequencies. $n = 6$; $^*p < 0.05$. **(D)** Representative example of flow imaging data, showing pSTAT4 and CD161 expression in $CD161^+CD8^+$ MAIT cells, left unstimulated (left panels) or after IFNα2b stimulation (right panels).

IFNα induce a specific signaling and transcriptional program in MAIT cells, which could be harnessed for future intervention.

Type 1 IFN have broad actions on the immune system, and one could argue that their stimulatory effects on MAIT cells only reflect their general action on CD8 T cells. However, this is actually not the case (65). In *in vivo* murine models, IFNα mostly stimulates naïve CD8 T cells and fosters their differentiation into memory cells (66, 67). On the contrary, its effect on memory CD8 T cells is mostly anti-proliferative. Similarly, IFNα promotes memory differentiation from human naïve CD8 T cells *in vitro* (68) and only modestly increases cytokine production by CD8m T cells (69). In contrast, we showed that MAIT cells responded promptly to IFNα incubation by directly upregulating cytotoxic molecules and dramatically increasing cytokine response to TCR triggering. Although we did not directly address mechanistic issues, we provided circumstantial evidences that a preferential usage of the Stat4 pathways by the IFNAR signaling is occurring in MAIT cells. It is well known that besides the canonical Stat1/Stat2 pathway, IFNAR signaling may involve any other Stats, depending on the cell type and probably other factors. Seminal work from the Biron laboratory has shown in the murine LCMV infection model that Stat4 is required for innate IFNγ production by natural killer cells but also that viral infection induces a switch in CD8 + T cells from a Stat1-dependent, anti-proliferative effect to a Stat4-dependent, IFNγ production in response to IFNα (62, 70, 71). Our data suggest that mature MAIT cells are equipped with a Stat4-dependent signaling module that drives their response to IFNα. If so, it remains to be determined when, how, and where this specific signaling pathway is plugged into the IFNAR receptor in MAIT cells. Thus, it is possible that thymic positive selection drives this process, perhaps upon PLZF expression, implying that MAIT cells would be developmentally programmed to provide this kind of response. Interestingly, type 1 IFN drives the development of innate-like CD8 T cells in mice (72). More studies are needed to address this question.

We found that IFNα has a potent effect when pre-incubated with PBMC before OKT3 stimulation. This is at odds with the report from Lamichhane et al. (64), where simultaneous

treatment with IFNα and 5-A-RU/MG (a potent MAIT cell-stimulating ligand) showed a synergistic effect. The reason for this discrepancy is not clear but may involve the different setup between our two studies. Indeed, the use of 1 nM of 5-A-RU/MG is known to strongly activate MAIT cells through MR1 presentation by APC, whereas we deliberately chose to use a low dose of soluble OKT3. Our gene expression analysis confirmed that IFNα induces a partially specific transcriptional program, in line with MAIT cells' specific sensitivity to IFNα. We chose a timeframe of 90 min to minimize positive and

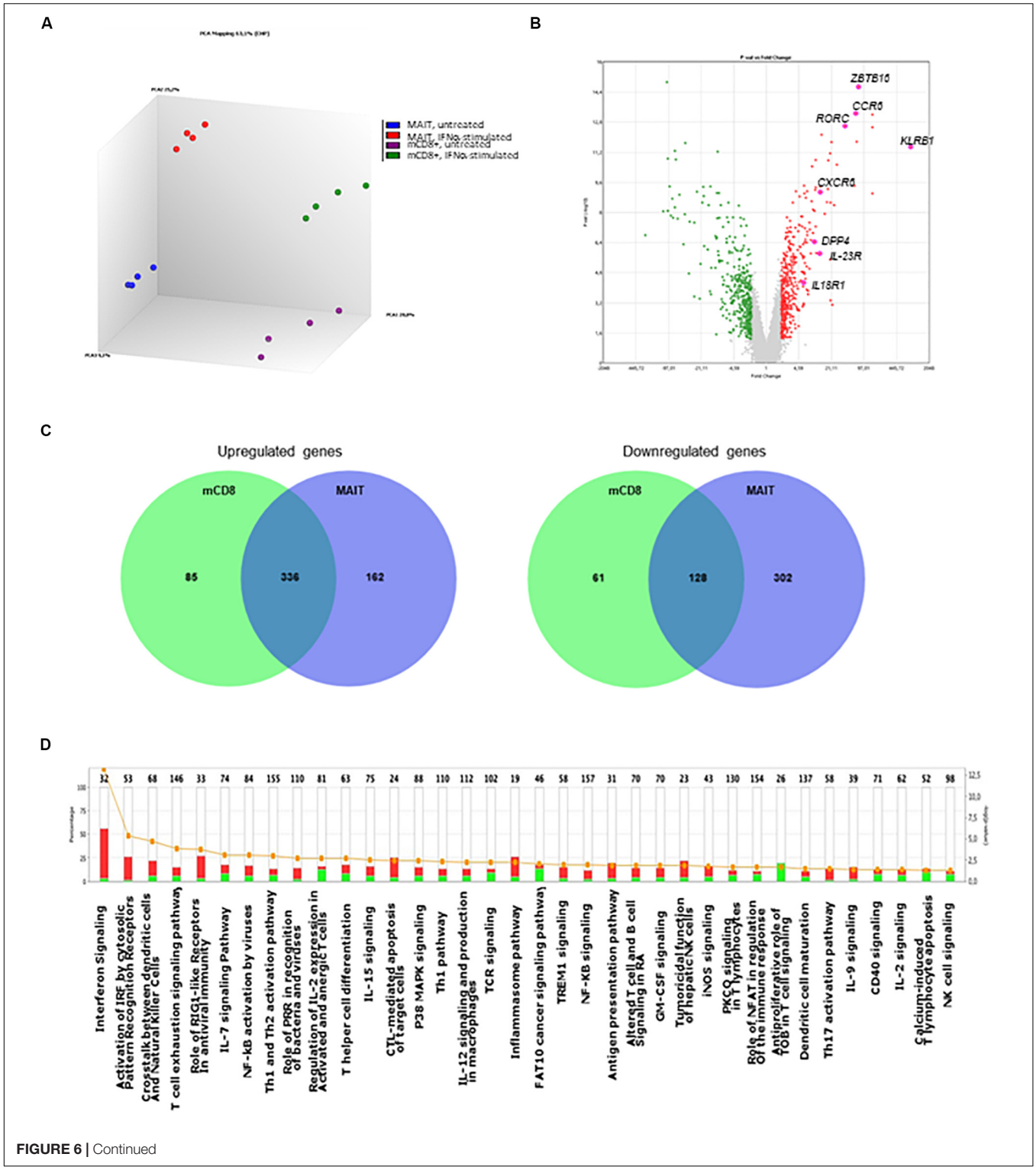

FIGURE 6 | Continued

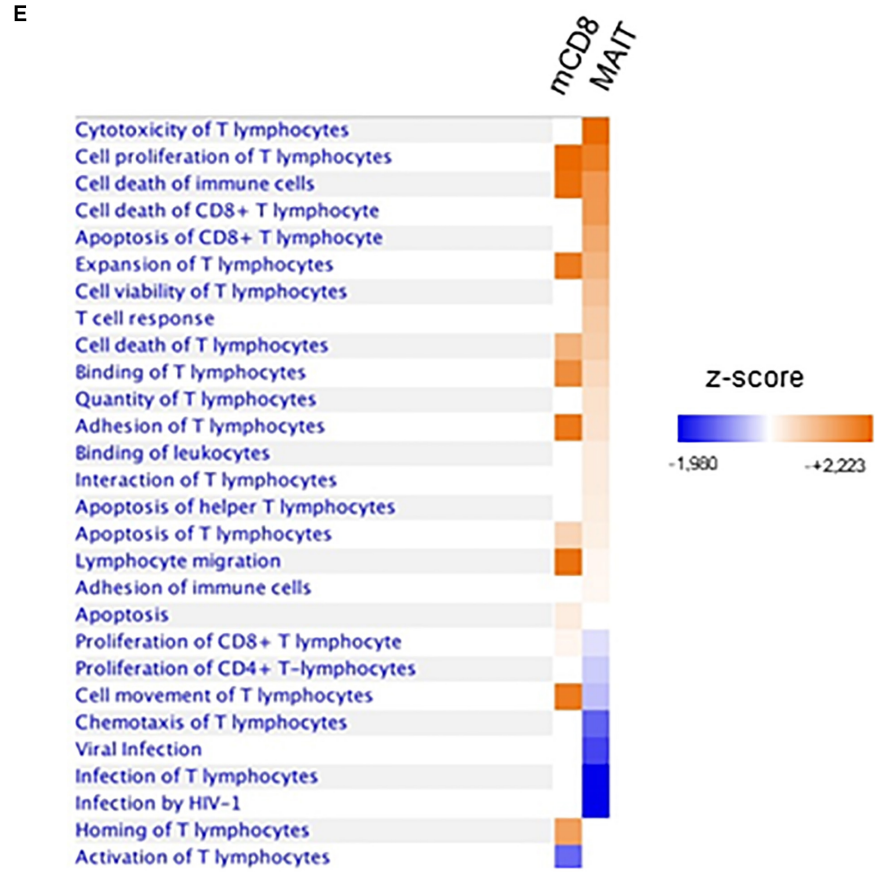

FIGURE 6 | IFNα2b induces a specific set of genes in MAIT cells. **(A)** Principal component analysis of gene expression by untreated MAIT cells (blue), IFNα-stimulated MAIT cells (red), conventional memory $CD8^+CD161^-$ T cells (purple), and IFNα-stimulated conventional memory $CD8^+CD161^-$ T cells (green). Analysis was performed with the Transcriptome Analysis Console (TAC, version 4.0) software. **(B)** Volcano plot of differentially expressed genes (DEG) between MAIT and conventional memory CD8 T cells. Colored dots represent genes with at least a 2-fold difference. The main MAIT-associated genes are labeled. **(C)** Venn diagrams of the number of DEG induced by IFNα2b stimulation in conventional CD8 memory and MAIT cells. **(D)** Pathway analysis of the genes differentially expressed in IFNα2b-stimulated versus unstimulated MAIT cells. The left axis represents the % of genes within each pathway, and the right axis shows the –log (*p*-value). Red bars represent upregulated, and green bars downregulated genes. Orange dots and line show the –log (*p*-value) for each pathway. **(E)** Comparison of the main pathways regulated by IFNα2b stimulation in conventional memory CD8 versus MAIT cells. Four different donors were analyzed in these experiments. Pathway analyses were performed with the Ingenuity Pathway Analysis software.

negative feedback loops that may occur in response to IFN; further, it is close to the peak of the IFN response as shown recently in human and mouse cells (73). We made the surprising finding that IFNα downregulates many more genes in MAIT cells than in conventional mCD8 T cells; indeed, it has been shown recently that in most leucocytes, more genes are upregulated than downregulated in response to IFNα signaling (73). Among these downregulated genes, we found genes involved in the regulation of TCR and NF-κB signaling (**Supplementary Table S1**). These data will require experimental validation. However, we speculate that the hypo-responsiveness of MAIT cells might be the consequence of the wiring of negative regulators of TCR signaling, which would then be downregulated by IFNα (and possible other signals), allowing full responsiveness to antigenic stimulation. If so, it will also raise the issue of the developmental regulation of this hypo-responsive phenotype in MAIT cells, from the thymus to the periphery.

Although the STAT4 pathway did not come out as strongly induced in MAIT cells in the microarray analysis, we found a selective induction of transcripts for *furin* in MAIT cells only. Furin is a ubiquitously expressed proprotein convertase with many substrates, involved in various biological processes. Interestingly, furin is induced by IL-12 in a STAT4-dependent manner and preferentially expressed in human Th1 cells (63). Another publication confirmed that *furin* is a target of STAT4 binding in Th1 cells (74). The absence of *furin* leads to a decreased production of IFNγ in Th1 cells, suggesting an IFNγ-enhancing role for this protease (63). It is therefore possible that furin expression is upregulated by IFNα in a STAT4-dependent manner in MAIT cells and could be important for the enhanced IFNγ production. Future studies will address this possibility.

Our work focused on $CD8^+$ MAIT cells, which represent the majority of MR1-restricted T cells. The reason behind this strategy was our goal to select a homogeneous population to

compare with their mainstream counterparts, i.e., conventional $CD161^-$ memory $CD161^{hi}CD8^+$ T cells. However, this excluded $CD8^-$ MAIT cells, composed of a very small fraction of $CD4^+$ and a significant population of $CD4^-CD8^-$ (DN) MAIT cells (7). Furthermore, DN and $CD8^+$ MAIT cells are developmentally related but display distinct transcriptional and functional profiles, with the $CD8^+$ subset expressing elevated levels of NK-related markers (NKG2A, NKG2D, and others), cytotoxic molecules (GrzB), and type 1 cytokines (IFNγ, TNFα), compared to DN MAIT cells (75). Thus, it is possible that DN MAIT cells behave differently than their $CD8^+$ counterparts with respect to their response to IFNα. Future studies will undoubtedly address this question.

We must stress that comparisons between MAIT cells and conventional memory CD8 + T cells involved slightly different definitions of this latter subset, including total $CD8^+CD161^-$ (functional studies), $CD8^+TCRV\alpha7.2^+CD161^-$ (QRT-PCT experiments) or $CD8^+TCRV\alpha7.2^-CD161^-$ (microarray analysis). Published data showed several differences between $TCRV\alpha7.2^+$ and $TCRV\alpha7.2^-$ cells within the $CD8^+CD161^-$ subset, although this analysis was performed without selecting specifically for memory cells (76). Nevertheless, it is conceivable that some of these comparisons between MAIT cells and conventional $CD8^+$ cells are biased because of subset definitions. On the other hand, the $TCRV\alpha7.2^-$ represent a very minor subset of $CD161^-CD8^+$ T cells, which should not behave so much differently than other $CD161^-$ cells. Although this represents an objective limitation, we believe this does not impede conclusions drawn from our experiments.

Previous work has already shown how inflammatory signals derived from TLR-stimulated APC synergize with TCR signaling to induce full effector functions in MAIT cells. Monocytes have been the main focus of these studies and are shown to be strong MAIT cells stimulators upon TLR8 or TLR4 stimulation (77). Of note, the specific soluble factors produced by monocytes and responsible for MAIT cells co-stimulation were not identified (39). In mice, *in vivo* activation of MAIT cells was observed only when TLR agonists (of TLR2, TLR3, or TLR9) were co-administered with the purified MR1 ligand 5-OP-RU. The specific APC(s) and cytokines involved in this effect were not investigated (37). We show here that pDC can be the source of a strong MAIT cell co-stimulation, at least upon TLR7 stimulation. pDC express MR1 transcripts and could be potent APC for MAIT cells. It remains to be investigated whether pDC are mostly (or solely) cytokine producers in this setting or are also able to potently present MR1 ligands to MAIT cells.

With regard to the prospect of harnessing MAIT cells' functions in a therapeutic or prophylactic manner, a number of reports have demonstrated the importance of inflammatory cytokines in MAIT cell co-stimulation. Several authors have shown the potency of the IL-12 + IL-18 combination, where the role of IL-12 is probably to increase IL-18 responsiveness; on the other hand, IL-7 is also a potent MAIT cell co-stimulator but mostly for cytotoxicity more than for IFNγ production. Recent reports have provided insights into the effects of cytokine stimulation on MAIT cells at the transcriptional and functional levels, mostly focusing on IL-12 + IL-18 (41, 42, 44). These cytokines, along with others such as IL-15 and TL1-A, strongly synergize with TCR stimulation to induce full antimicrobial effector functions, including IFNγ production and cytotoxicity, akin to our observations with IFNα. In one paper, IL-12 + IL-18 stimulation alone was sufficient to induce the expression of IFNγ and cytotoxic effectors at the mRNA and protein levels. Further, most DEGs were upregulated in these studies, whereas we found a lot of downregulated genes in response to IFNα stimulation. It must be noted that these responses were described as slow, and analyzed at late time points (after 24 h of stimulation), suggesting that it could involve feed-forward loops that we tried to avoid in our study of the immediate response to IFNα. As we and others did not compare all these cytokines in their ability to influence MAIT cell activation, it would be of great interest to analyze the transcriptional response of MAIT cells to these stimuli, as a way to stratify their interest in the context of immune intervention but also to analyze in deeper molecular details the response of MAIT cells.

Besides these important issues, our work raises hypotheses and possibilities. For instance, it is well known that viral infections are strong inducers of type 1 IFN. A recent report very elegantly showed that a localized antiviral vaccination induces an IFN response that rapidly spreads to the entire organism, endowing distant cells from prophylactic antiviral mechanisms (78). It is tempting to suggest that this wave of IFN signaling would also provide a priming signal to circulating MAIT cells, which would then be ready for response. Viral infections often dampen inflammatory neutrophilic response, with increased susceptibility to bacterial infections. Priming this specific, highly potent antibacterial subset of T cells would make high evolutionary sense to avoid major damages due to secondary infections. More generally, the type 1 IFN response in antibacterial and fungal immunity is often assumed to be detrimental (79), but this is still debated. Our data emphasize, at least from a MAIT-centered prospective, that IFNα can be beneficial for antibacterial mechanisms and may foster new endeavors to analyze the therapeutic and prophylactic consequences.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the Local Legislation and Institutional Requirements. Written informed consent for participation was

not required for this study in accordance with the National Legislation and the Institutional Requirements.

AUTHOR CONTRIBUTIONS

MP, CG, and CC performed the experiments and analyzed the data. TS helped with preparation of *E. coli*. ET designed the study, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.02097/full#supplementary-material

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REVIEW
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MAIT Cells in Barrier Tissues: Lessons from Immediate Neighbors

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Mucosal-associated invariant T (MAIT) cells are innate-like T cells present at considerable frequencies in human blood and barrier tissues, armed with an expanding array of effector functions in response to homeostatic perturbations. Analogous to other barrier immune cells, their phenotype and function is driven by crosstalk with host and dynamic environmental factors, most pertinently the microbiome. Given their distribution, they must function in diverse extracellular milieus. Tissue-specific and adapted functions of barrier immune cells are shaped by transcriptional programs and regulated through a blend of local cellular, inflammatory, physiological, and metabolic mediators unique to each microenvironment. This review compares the phenotype and function of MAIT cells with other barrier immune cells, highlighting potential areas for future exploration. Appreciation of MAIT cell biology within tissues is crucial to understanding their niche in health and disease.

Keywords: mucosal-associated invariant T cells, microenvironment, microbiome, metabolism, tissue resident cells, mucosal immunology, diet

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INTRODUCTION

Mucosal-associated invariant T (MAIT) cells have been intertwined with barrier immunity ever since they were coined (1, 2). Abundant in human blood, making up to 10% of peripheral T cells, they are also enriched in tissues (2–4). MAIT cells are defined as *TRAV1-2*$^+$ (TCR Vα7.2$^+$) T cells restricted by the MHC class I-related molecule (MR1), which recognizes non-peptide riboflavin biosynthesis intermediates conserved among bacteria and fungi (5–10). All mammalian barrier surfaces are colonized by riboflavin-synthesizing commensals, and MR1 has co-evolved with MAIT cells through most mammalian evolution (11), thus being an example of barrier surfaces imprinting human immunity.

Barrier surfaces are sites of cross-talk between the host and diverse external environments. MAIT cells are found in the intestine, skin, respiratory, oral and female genital mucosa, which all house microbial communities adapted to the local environment and in symbiosis with the host (12) (**Table 1**). MAIT cells are dependent on this microbiome and are part of a community of tissue immune cells anatomically close to epithelial surfaces (20, 35), all poised for rapid effector functions to maintain tissue homeostasis (36, 37).

The range of MAIT cell effector functions is only just being explored. It is increasingly appreciated that resident macrophages and lymphocytes are in constant cross-talk with tissues, integrating cues from the local microbiome, cellular, environmental and metabolic milieus for their development and function (38–44). In this review, we show that MAIT cells occupy a similar niche, engage in similar cross-talk and could sense similar factors (**Figure 1**,

TABLE 1 | MAIT cells in healthy human barrier tissues.

Barrier tissue MAIT location	Frequency	Phenotype and activation	Ref
Oral			
Buccal mucosa Close to basement membrane, both within the epithelial layer and connective tissue.	No enrichment compared to blood (50% of oral CD8αα T cells)	↑CD103$^+$ (20-80%) – most are CD8$^+$ ↑HLA-DR, CD69, PD-1, CTLA-4 ↓CD38, perforin PMA/ionomycin stim: ↑IL-17A, GzmB (esp. CD103$^+$MAIT) ↓TNF, IFN-γ	(13, 14)
Gut			
Gastric mucosa Lamina propria	LPMC 2% (0-12%) Gastric 1% (0.4-2.39)	↑CD103$^+$CD69$^+$ (80%)	(15) *(16)
Duodenum Lamina propria and epithelium	1.7% IEL 3% (0.1-5%) LPL 2% (0.5-4%)	↓IL-18Rα IL-12+IL-18 stim: ↓IFN-γ	(17, 18)
Jejunum Lamina propria and epithelium	IEL *60% (n = 1)*: *(*Vα7.2$^+$ T cells)		(6)
Colon Lamina propria and epithelium	No data in healthy Cancer: IEL 1%; LPL 2% Inactive UC 12% (30% of CD8$^+$ are MDR1+)	Most CD8$^+$ CD103$^+$ *(n = 1)* ↑CD69 (>90%), HLA-DR, CD25, TIGIT, PD-1, CTLA-4, LAG-3 ↑GzmB (baseline) ↑Tbet, RORγt PMA/ionomycin stim: ↑TNF, IFN-γ, IL-17A, IL-22 E coli stim: ↓IFN-γ	*(19) (20–22)
Rectum	2% (0.5-8%)	CD8$^+$ > DN MAIT ↑IL-23R, CSF1, TNF, CD40LG, CRTAM ↓GzmK	(23, 24)
Small intestine *Fetal 2nd trimester*	0.5% (0.2-1%)	CD8α (30%), Ki67 (20%) PMA/ionomycin stim: ↑IL-22 E coli stim: ↑IL-22, ↓IFN-γ	(25)
Lung			
Bronchial tree Epithelium > lamina propria	5% (Endobronchial biopsy) *TRAV1-2$^+$%CD8*: Trachea (42%*)* > proximal (35%*)*: > distal bronchus (22%) (*n = 1)*		(26) (27)
Lung parenchyma	6% [TRAV1-2$^+$%CD8]		(27)
Lung parenchyma Fetal 2nd trimester	0.8% (range 0.6-2)	CD127$^+$IL-18Rα$^+$ (>90%) CD8α (20%), Ki67 (15%) E coli stim: ↑IL-22, ↓IFN-γ	(25)
Sputum	2%		(26)
BAL adult	2% *4% [TRAV1-2$^+$%CD8]*	↑CD103$^+$ (75%), PMA/ionomycin stim: ↑IL-17A (esp. CD103$^+$)	(26, 28) (27)
BAL children	1% 3% (in CAP)	Most CD103$^-$ – in CAP >50% CD103$^+$ Plasma: ↑IL-12p70 ↓IFN-γ, IL-22, IL-23, MIP-1α, MIP-1β CAP: ↑IL-17A, IL-22, IL-23, IL-1β, IL-6, IL-12p70, MCP-1, MIP-1α, MIP-1β ↑ IL-17A: IFN-γ ratio ↑ *HIF1A, AHR, BATF, PLZF* ↓*TCF7*	*(29)
Skin			
Epidermis and dermis, especially papillary dermis and adjacent to the superior vascular plexus	3.8+/-0.32% (by IF) Epidermis: 1.5±0.5%	↑CLA$^+$ (80%) ↑CD103$^+$(80% epidermis, 40% dermis)	(30) *(31)

(Continued)

TABLE 1 | Continued

Barrier tissue MAIT location	Frequency	Phenotype and activation	Ref
	(11.6±11.0% of CD8$^+$) Dermis: 0.5±0.1% (4.6±4.0% of CD8$^+$)		
Female Genital Tract			
Endometrium Lamina propria close to and within glandular epithelium	1% (range 0-3%)	↓PLZF (DN vs CD8$^+$ MAIT ↓ Tbet; ↑PLZF, RORγt, Helios) E coli stim: ↑polyfunctional, IL17-A, IL-22 ↓IFN-γ, TNF, GzmB	(32, 33)
Cervix Endocervix, adjacent to simple columnar epithelium; Transformation zone lamina propria; Ectocervix on both side of basement membrane, predominantly in clusters within epithelium	2% (range 0-6%)	↓Eomes	(32)
Placenta Decidua parietalis	2% (IVB 4%)	↑CD69 (80%), CD25 (25%), HLA-DR (35%), PD-1 (70%), Ki67 (15%) ↓CD127 (50%) E coli stim: ↑GzmB, Perforin	(34)

Location, frequency, phenotype, and function of MAIT cells in healthy human tissue compiled from studies to date. Frequency is expressed as a % of total CD3$^+$ unless otherwise specified. Studies highlighted (*) defined MAIT cells using MR1-tetramer. All other studies used proxy measures of variable stringency to identify MAIT cells, predominantly CD161$^+$Va7.2$^+$. Enrichment and comparisons of phenotype or function are compared to blood. BAL, bronchoalveolar lavage; Ca, cancer; CAP, community acquired pneumonia; DN, double negative; GzmB, granzyme B; DP, decidua parietalis; DN, double negative; IEL, intraepithelial lymphocytes; IF, immunofluorescence; IVB, intervillous blood; LPL, lamina propria lymphocytes.

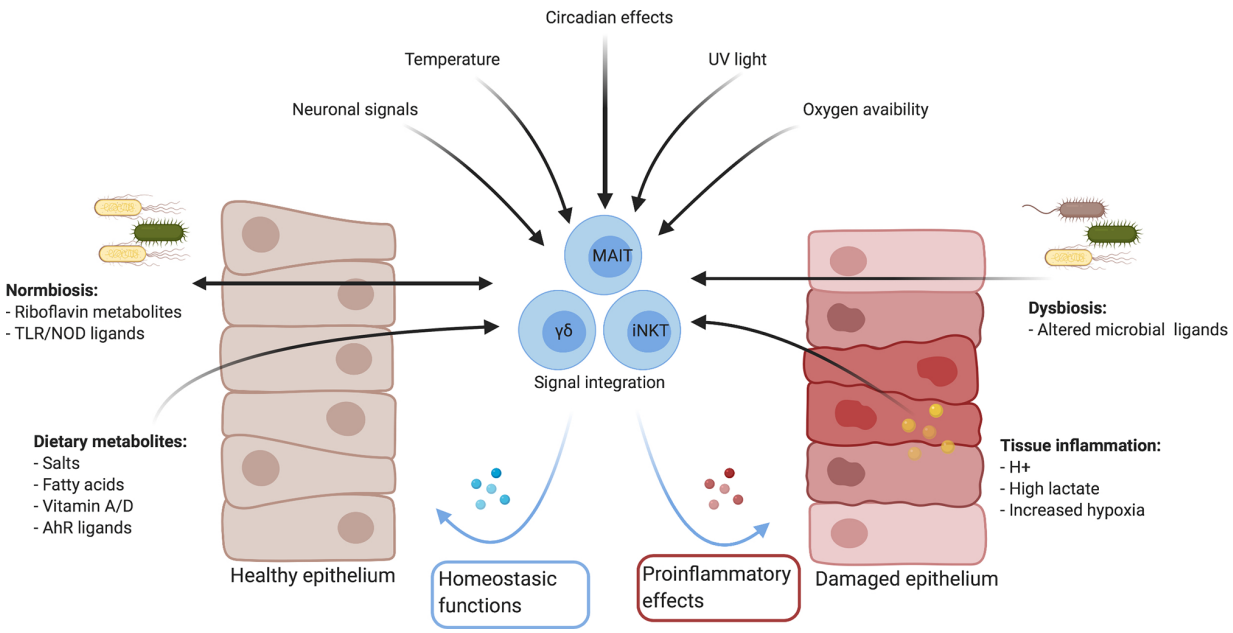

FIGURE 1 | Summary of mucosal environmental factors which could influence immune responses directly and indirectly. Created with Biorender.com.

Table 2). Furthermore, we draw parallels with other barrier lymphocytes to explore tissue-specific factors which could modulate their function at mucosal surfaces, with the hypothesis that there are unexplored functional and metabolic adaptions for their survival and execution of tissue-specific effector functions. Understanding these factors is important to expand our knowledge of their role in shaping tissue function in health and disease.

TABLE 2 | Environmental factors and sensors.

		Protein Atlas	Fergusson (45)	Park (46)	Salou (47)	Hinks (48)	Hinks TCR (48)	Lamichhane E.coli (49)	Leng TCR (20)	Sharma anti-CD3 (50)	Sharma BCG (50)	Lu CAP (29)
Physical factors	**Sensor***											
Hypoxia	HIF1A (effector)						↑	↑		↑	↑	BAL > Blood MAIT17
Acidosis	GPR65		↑	↑	↑	↑				↑	↑	MAIT17
Osmolarity	NFAT5						↑		↑	↑	↑	
Mechanical	PIEZO1					↑						
Metabolite												
Vitamin A	RARG	↑		↑	↑	↑				↑		
Vitamin D	VDR						↑	↑	↑		↑	
Lactate	SLC16A1 (*transporter*)						↑	↑	↑			
Tryptophan metabolites	AHR									↑		BAL > Blood
	GPR35				↑	↑						
Oxysterols	GPR183							↑				MAIT1
Purines	P2RX7									↓		
	P2RY14				↑	↑			↑			
Neuropeptide												
Noradrenaline	ADRB2		↑	↑	↑	↑	↓		↓			
Neuromedin U	NMUR1	↑			↑		↓					
BigLEN	GPR171	↑			↑	↑	↑	↑		↑	↑	

Examples of tissue factors which could modify human MAIT cells in an analogous manner to other resident immune cells. Transcriptional expression of purported sensors, in some cases including relevant non-sensor genes. All transcriptional datasets are from human blood MAIT cells, except (29) which is from matched BAL and blood MAIT cells. Protein atlas, genes enriched in MAIT cells compared to other blood immune cells; Fergusson (45), genes enriched in CD161^{+} T cells; Park (46), genes enriched in CD161^{+} Va7.2^{+} compared to conventional T cells; Salou (47), genes enriched in MAIT cells compared to conventional CD8^{+} T cells; Hinks (48), genes enriched in MR1-tetramer^{+} MAIT cells compared to CD8^{+} T cells; Hinks TCR (48), genes upregulated on 5-OP-RU activation of MAIT cells; Lamichhane E coli (49), genes upregulated on E coli activation of MAIT cells; Leng (20), genes enriched in MAIT cells stimulated with TCR-dynabeads; Sharma (50), genes upregulated with anti-CD3 or BCG stimulation; Lu (29), genes enriched in BAL MAIT cells from children with community acquired pneumonia (CAP). MAIT17 = type-17 MAIT cells; MAIT1 = type-1 MAIT cells.*

BARRIER TISSUES

Mammalian barrier surfaces are diverse physiological, chemical and cellular niches. The skin is dry, lipid-rich and acidic, primarily due to epidermal fatty acids, with unique exposures to high-salt content, UV-radiation and large fluctuations in temperature (51, 52). The female genital tract is even more stressed, driven by *Lactobacillus sp.*-derived lactic acid and hydrogen peroxide, with additional structural and immunological changes with the menstrual cycle, pregnancy and age (12, 53). The digestive system varies along its length, both in terms of physical factors and the unique composition of the microbiome: the oral mucosa is subject to occasional physical trauma from mastication, while the gut has the largest microbial biomass in the body in addition to a nutrient rich environment consisting of bile acids and diet-derived metabolites. Finally, the lungs are subject to fluctuating mechanical shear stress from ventilation and gravitational posture dependent changes, and also differ in structure and microbial sterility from higher to lower order bronchi and alveoli (54).

Most sites have underlying dense neural networks and varying concentrations of physiological parameters such as oxygen tension, lactate, glucose and amino acids depending on the specific location and inflammatory context; relative hypoxia is physiological in healthy skin hair follicles and the intestinal lumen, and induced in the face of increasing tissue demand during inflammation in all tissues. The inflammatory tissue microenvironment is further dysregulated by the catabolic processes necessary for generating an immune response, through excess nutrient consumption and generation of potentially toxic metabolic by-products. Moreover, these physical and biochemical changes interact with and shape the microbiome, therefore directly and indirectly interacting with resident immune cells, including MAIT cells.

BARRIER MAIT CELLS

Barrier tissue lymphocytes include innate, innate-like and adaptive immune cells (37). Activation is antigen-independent in natural killer (NK) and innate lymphoid cells (ILCs), and predominantly mediated through cytokines. Antigen-dependent mucosal protection is provided by $CD4^+$ T helper 17 (T_H17), regulatory T (T_{reg}), and $CD8^+$ tissue-resident memory (T_{RM}) and Tc17 cells. MAIT cells and other unconventional T cells, such as invariant natural killer T (iNKT) and gamma-delta T (γδT) cells, share features with both innate and adaptive cells. They can be activated in a TCR-dependent or independent manner (20, 48, 49, 55), and are important for preserving tissue integrity and function in homeostasis.

Ontogeny and Tissue Residency

Human MAIT cells in blood have a tissue-homing effector-memory phenotype (3), but the ontogeny of cells in barrier surfaces is less clear. Murine tissue MAIT cells and iNKT are resident populations in homeostasis and following infection, expressing the transcription factor retinoic acid-related orphan receptor (ROR) γt (RORγt). After parabiosis, almost all thymic, and the majority of splenic, hepatic, lymph node and $ROR\gamma t^+$ lung MAIT cells are host-derived, with some recirculation of $ROR\gamma t^-$ lung MAIT cells (47, 56). Following *Staphylococcus epidermidis* (*S. epidermidis*) challenge similar frequencies of murine skin MAIT, γδT, and iNKT cells are host-derived and thus tissue-resident (35).

In humans however, it is unclear to what extent tissue MAIT cells are permanently resident. Expression of αE-integrin (CD103) associated with $CD8^+$ T_{RM} cells is rare in blood MAIT cells (<3%) and common, but not universal, among MAIT cells within the oral and gastric mucosa (13, 15), skin (31, 57), and lungs (27–29) (**Table 1**). Thus tissues in health may represent a mixture of resident and recirculating MAIT cells, which could vary micro-anatomically: although most epidermal MAIT cells express CD103 and CLA (cutaneous lymphocyte antigen), only half of dermal MAIT cells express CD103 (31). Resident populations may behave differentially in disease – for example, among bronchoalveolar $CD8^+CD161^{++}V\alpha7.2^+$ cells, which are mostly MAIT cells, only the CD103+ fraction is depleted in HIV infection (28).

Studies on the formation and longevity of tissue MAIT cells in humans are challenging. Remarkably, some MAIT cells do migrate into fetal small intestine, liver and lung as early as the 2nd trimester - before exposure to the conventional commensal flora (25). Fetal tissue MAIT cells, unlike circulating MAIT cells in adults, are cycling in the steady state with appreciable Ki67 expression (25). It is unclear if these early tissue resident MAIT cells could persist into adult life, similar to other fetal tissue-resident cells (58). One study using HLA allele mismatching to discriminate between donor- and recipient-derived T cells following small bowel transplantation showed that although the majority of tissue MAIT ($CD161^+V\alpha7.2^+$) cells are derived from the host long term, donor-derived cells can be found over a year after transplantation (59); this suggests that adult mucosal MAIT cells can persist for prolonged periods. The dynamics may of course vary in tissues with different cellular turnover, and analogous to the differential proliferative capacity and function of resident and monocyte-derived macrophages in tissues (60), the function of long-lived and newly formed MAIT cells may be distinct.

It is also unclear if MAIT cells can leave tissues. Thoracic duct lymph-derived and blood MAIT cells are $CCR7^-$ and present at comparable frequencies with overlapping TCR clonotypes (61), which suggests that MAIT cells in lymph directly derive from blood. This could imply either migration after transit through tissues, or direct CCR7-independent migration from blood through high endothelial venules. Further work is needed to understand the drivers for tissue migration, residency and persistence, including potential functional differences between long lived and nascent tissue MAIT cells.

Tissue Phenotype and Cytokine Production

MAIT cell expression of the NK-cell marker CD161 and the transcription factor RORγt (*RORC*) are features shared with other barrier tissue-resident cells, including T_H17, peripherally derived T_{reg} (pT_{reg}), ILC3, iNKT and γδT cells (62–67). $CD161^+$ T cells share a transcriptional program for innate-like cytokine-responsiveness in the absence of TCR triggering, through high

expression of cytokine receptors (e.g., IL-12R, IL-18R) driven by the transcription factor promyelocytic leukemia zinc finger protein (PLZF) (45, 55). RORγt is crucial for barrier protective IL-17 production common to many mucosal lymphocytes (43, 68). Unusually, in homeostasis many human MAIT cells co-express the type-1 transcription factor T-bet (*TBX21*) and RORγt, whereas naïve SPF mice have two almost mutually exclusive populations (69, 70): nearly all murine tissue resident MAIT cells are RORγt^{+}T-bet^{-} IL-17A producing MAIT17, with less numerous T-bet^{+}RORγt^{-} IFN-γ producing MAIT1 found in the circulation (47). Following infection with intranasal Salmonella or Legionella, an increase in RORγt^{+}T-bet^{+} lung MAIT cells is observed, suggesting that the diverse stimuli that human MAIT cells receive may explain part of the differences between species (71).

Functionally, human tissue MAIT cells are usually more activated than their circulating counterparts in homeostasis and capable of increased cytokine production (19, 23, 72) (**Table 1**). Additionally, barrier MAIT cells in the female genital tract and oral mucosa are predominantly CD8^{+} cells biased towards type-17 function (13, 32). Another study found that CD4^{-}CD8^{-} MAIT cells in healthy endometrium have a more mature phenotype with higher RORγt and lower T-bet expression (33). This skewed tissue biased phenotype is present from early development as fetal small intestine MAIT cells produce more cytokines (IFNγ, IL-22) than their circulating counterparts in response to *Escherichia coli* (*E. coli*) (25). It is unclear however if this phenotype varies between barrier tissues in adults. Barrier specific heterogeneity is observed in mice: murine skin MAIT cells display a transcriptional profile distinct from lung or liver (35), and both colonic and lung MAIT cells produce higher IL-17 than non-barrier tissues, such as the liver and spleen (73). Ultimately, as IL-17A and IL-22 promote barrier integrity, it will be important to understand the relative contributions of pre-programmed transcriptional and environmental cues to MAIT cell heterogeneity observed in human tissues.

Tissue Homeostasis and Tissue Repair

Given their anatomical location and similarity to other tissue-resident lymphocytes, it is perhaps unsurprising that MAIT cell effector function is important in tissue-homeostasis. This parallels tissue homeostatic roles for H2-M3 restricted CD8^{+} T cells (and T_{regs}) in mouse skin (74, 75), and γδT cell populations in the lung and gut (76–78). In NOD mice, *Mr1*-deficient animals have impaired intestinal barrier integrity, implicating MAIT cells in maintaining surface homeostasis (79). This concept was further supported in a model of colonic graft versus host disease (GvHD); *Mr1*-deficient animals had increased proinflammatory donor-derived CD4^{+} T cell expansion and reduced tight junction expression (73). GvHD in human bone marrow transplant recipients is also associated with lower MAIT cell frequency (80, 81), again potentially implicating a role in maintaining mucosal health.

Further recent data from both mouse and human studies has identified an important role for MAIT cells in tissue repair. Constantinides et al. found that murine skin resident MAIT cells are enriched for a distinct tissue repair transcriptional signature, similar to that observed in the previously described H2-M3 restricted CD8^{+} T cells responsive to *S. epidermidis*-derived N-formylated peptides (35). To assess MAIT cell specific tissue repair *in vivo* without confounding skin γδT and H2-M3 restricted CD8^{+} cells which can perform analogous functions, *Tcrd*$^{-/-}$ mice were infected with a strain of *S. epidermidis* that fails to induce CD8^{+} H2-M3-recognizing T cells. Tissue repair in response to *S. epidermidis*, measured by epidermal tongue length growth after skin punch biopsy, was higher in *Tcrd*$^{-/-}$ compared to *Mr1*$^{-/-}$*Tcrd*$^{-/-}$ mice, implicating the additional MAIT cell deficiency.

How do these data relate to human MAIT cells? Recently, murine and human MAIT cells were also found to have a shared tissue repair transcriptional profile (as seen with the H2-M3 restricted CD8^{+} T cells) on resolution of infection from *Legionella longbeachae* and with re-infection, suggesting significant functional parallels (48). Two additional studies in human MAIT cells showed activation of such tissue repair gene expression patterns predominantly following TCR-mediated triggering (20, 49). *In vitro* assays of wound healing also revealed a functional repair role for MAIT cell derived soluble factors, which could be blocked using anti-MR1 antibodies (20). Taken together all these studies suggest that a local repair program analogous to other tissue resident cell types is active in MAIT cells and likely triggered *in vitro* through encounter with microbiota. This is supported by the remarkable observation *in vivo* that direct topical application of the MAIT cell TCR ligand 5-OP-RU alone prior to skin injury, in the absence of additional cytokines, is sufficient to selectively induce cutaneous MAIT cell expansion and expedite tissue repair (35). Much more work is needed to define the importance of this in human disease and also the exact mechanisms through which this large panel of soluble mediators exert their impact.

In addition to tissue-repair functions in response to commensals in the absence of inflammation, there is evidence that innate-like T cells can regulate barrier surface homeostasis by shaping the microbial landscape. CD1d and intestinal iNKT cells influence murine intestinal homeostasis and microbial colonization, with reduced *Bacteriodales* colonization in iNKT-deficient mice (82). *Mr1*-deficient animals, which lack MAIT cells, have reduced intestinal microbial diversity, similar to that found in IL-17A deficient animals (73). Conversely, in obese mice MAIT cells seem to promote microbial dysbiosis and ileal barrier dysfunction. Fecal transplantation from obese *Mr1*-deficient animals reduces barrier permeability in mice fed a high-fat diet, and the microbiome of obese Vα19$^{+/-}$ mice with a high MAIT cell frequency has lower *Bifidobacteriaceae* and *Lactobacillaceae* species (83). Given the importance of a diverse microbiome to human health, further work is needed on the interactions of MAIT cells and a healthy microbiome in maintaining tissue homeostasis.

These expanding tissue-specific functions raise the tantalizing possibility that similar to other innate-like lymphocytes, MAIT cell barrier functions may be more diverse than initially appreciated (84, 85). We know that γδT cells can remarkably

promote stem-cell remodelling (86), adaptive thermoregulation in response to cold stress (87), and sympathetic nervous innervation (88). Furthermore, cytokine activated ILC3 can promote antigen specific $CD4^+$ T cells responses directly in vitro through cell surface MHCII and co-stimulatory molecule expression (89). Indeed MAIT cells have the capacity to indirectly manipulate tissue adaptive responses, through dendritic cell maturation (90), and could potentially act as a sink for IL-7 similar to IL-$7R^+$ ILC to limit homeostatic proliferation and preserve TCR diversity in neighboring tissue T cells (91).

In summary, MAIT cells in tissues are distinct from the population most frequently studied thus far in blood. The array of effector functions is expanding beyond the traditional cytotoxicity and cytokine production first described, and further understanding of the context in which these effector programs are engaged together with knowledge of how to modulate them are key to enable translation of MAIT cell biology to effective human therapeutics.

BARRIER ADAPTATION

Metabolism

T cell development, differentiation, activation, function and survival are regulated by the cell intrinsic metabolism of glucose, amino acids and lipids (92–94). Nutrient availability differs between T cell compartments: circulating lymphocytes function in secondary lymphoid organs with high glucose and amino acid concentrations, whereas barrier tissues are more restrictive anatomically with variable nutrient composition. Permanent tissue-resident cells must therefore adapt to these niches for continued survival and proliferation which are also required for their tissue-specific effector functions.

Activated peripheral blood MAIT cells upregulate glucose uptake as glycolysis is required for their cytotoxicity and IFN-γ production (95, 96), and human circulating MAIT cells have reduced mitochondrial activity compared to $CD161^-CD8^+$ T cells (95). Amino acid metabolism is also crucial - peripheral MAIT cells express high levels of the L-type amino acid transporter, SLC7A5, and L-amino acid oxidase (96, 97). The metabolism of tissue MAIT cells has however not been explored.

Tissue MAIT cells may rely on OXPHOS, which correlates with TNF and IL-17 production in their circulating counterparts (96, 98). Given low tissue glucose concentrations, many resident immune cells are adapted to oxidative phosphorylation of fatty acids abundant in the skin and intestine (99). Tissue-resident but not circulating memory $CD8^+$ T cells require these exogenous fatty acids for their survival, and upregulate transporters and fatty acid binding proteins (FABP) necessary for long term maintenance (100, 101). FABP isoform expression is tissue-specific, shared among resident T_{RM}, IEL, ILC and γδT cells, with T_{RM} able to modulate isotype specific expression on relocation to a new environment (102); *Fabp4* and *Fabp5* expression are enriched among skin resident cells, whereas *Fabp1* is enriched among liver resident cells, including invariant NKT cells. Endogenous fatty acid metabolism is also important in IL-17 producing T_H17 (103), with murine Tc17 cells and human IL-17 producing bronchoalveolar MAIT cells also enriched for genes in fatty acid and lipid metabolism (29, 104). It would therefore be logical to explore tissue MAIT cell mitochondrial and lipid metabolism in understanding their barrier specific effector functions.

Tissue Stress

Tissue stress includes homeostatic perturbations in metabolic or environmental factors. Examples include insufficient nutrients or accumulations of potentially toxic byproducts, including oxidative stress from excessive free radicals. Barrier tissues with an active immune response frequently have minor homeostatic perturbations tolerated by resident immune cells.

Autophagy is a metabolic stress response that recycles intracellular proteins and provides an additional nutrient source advantageous in tissues and activating environments (93, 105). MAIT cells in the liver have higher basal autophagy compared to their circulating counterparts, which may be required given the higher mitochondrial depolarization observed in stressed tissue-resident cells (106). *In vitro*, inhibition of autophagy reduces acquisition of a tissue-resident phenotype in circulating $CD8^+$ T cells (106), thus enhanced autophagy may be a requirement for MAIT cell tissue survival.

Tissue oxidative stress can also be mitigated in barrier tissues by xenobiotic transporters, such as multidrug resistance protein 1 (MDR1). MDR1 (ABCB1) is an ATP-binding cassette B1 drug resistance transporter expressed on IL-17 producing $CD4^+$ T cells in the ileum, which protects against bile acid induced oxidative stress to maintain intestinal homeostasis (107); a subset of patients with ileal Crohn's have loss of function in MDR1, highlighting an important role in controlling tissue inflammation. MAIT cells and other $CD161^+$ T cells also express high levels of MDR1 (3, 45, 108), and it would be interesting to evaluate in future studies whether this has similar implications for their survival and function in toxin rich niches.

Tissue oxidative stress also produces free radicals and hydrogen peroxide (H_2O_2). H_2O_2 can be transported by the plasma membrane water channel, aquaporin 3 (*AQP3*), which is part of a core transcriptional signature shared among type-17 secreting NKT17, ILC3, γδT, and T_H17 cells in mice (109). *Aqp3*-deficient T cells have impaired chemokine mediated trafficking to the skin (110), and *AQP3* expression is higher in human $CD161^+V\alpha7.2^+$ MAIT cells compared to circulating $CD161^-$ cells expressing the same TCRα (46). It is therefore possible that tissue stress regulates MAIT cell migration and survival in inflammatory tissues such as the skin.

Finally, given the often overlapping functions, some adaptations may serve to prune the tissue response to only the most appropriate cells. Murine T_H17, iNKT and T_{RM} are enriched for the purinergic receptor, P2RX7, which recognizes extracellular purines (ATP, NAD^+) released after cell lysis and has cell type specific effects (111–113). Purines released from high microbial turnover can regulate barrier specific immune cell function, promoting murine T_H17 differentiation (111) but

inhibiting human ILC3 IL-22 production (114). P2RX7 expression on tissue iNKT and T_{RM} however induces pyroptosis and limits immunopathology from bystander activation (113, 115). As purinergic receptor expression is downregulated on TCR engagement, release of purines from tissue damage preferentially depletes bystander activated T_{RM} (115). Over time this could serve to shape the barrier immune response by conserving predominantly T cells specific to regularly encountered antigens. Given their presence in tissues and potential for bystander activation in response to cytokines, MAIT cell function may also be shaped by variable adaptations to tissue damage. Indeed, one could imagine that as a mammal ages, MAIT cell TCR-dependent responses could be superseded by antigen-specific T_{RM} populations.

BARRIER SENSING

Microbiome

Barrier surveillance of the commensal microbiome is essential for tissue immunity in homeostasis through shaping the composition and phenotype of innate and adaptive cells (116–118). Numerous mucosal cell types are perturbed in germ-free (GF) and antibiotic treated mice, including RORγt-expressing tissue T_{reg}, T_H17 and innate-like IL-17 producing γδT cells (119). Conversely cohousing laboratory mice with wild mice to induce a more diverse microbiome promotes a human adult-like immune composition, with increased tissue innate and differentiated memory $CD8^+$ T cell populations (120).

We have known MAIT cells are also reliant on the microbiome since Treiner *et al.* discovered that they were absent in the lamina propria of GF mice (2). It was subsequently found that metabolites from riboflavin-synthesizing commensals, which engage the MAIT cell TCR, are necessary for most stages of MAIT cell intra-thymic development and subsequent peripheral expansion (56, 121). Accordingly, the dominant murine population of $ROR\gamma t^+$ MAIT17 dependent on TCR-triggering for proliferation and function are depleted in the thymus and tissues of GF-mice, skewing the response to IFN-γ production (56). Recolonization of GF mice with microbes can rapidly restore this $ROR\gamma t^+$ MAIT population. Remarkably, metabolites from skin riboflavin-synthesizing commensals, even in the absence of bacteria, can drive intra-thymic MAIT cell development remotely (56), in addition to sustaining the development and function of local skin-resident MAIT cells (35). There is however a narrow neonatal window until 3 weeks of age where recolonization of GF mice can restore MAIT cell development; recolonization of adult mice with bacteria after 7 weeks does not increase MAIT cell frequencies in the skin. Although they have complementary functions, this may be due to a finite niche for competing innate-like T cells shaped by the early microbiome; *Tcrd*-deficient animals have increased tissue-resident iNKT and MAIT cell populations (35), *Cd1d*-deficient animals have increased splenic and thymic MAIT cells (121), while GF mice have increased iNKT cells (122). A competing or compensatory interaction is also supported by the massive expansion of $V\delta2^+$ T cells in a patient with a homozygous point mutation in MR1 and MAIT cell deficiency (123). Supporting complementary functions, human blood MAIT cell and iNKT cell frequencies positively correlate (124, 125). Additional work is needed to clarify the relationship between innate-like T cells, and whether this is influenced by age, disease and ligand abundance.

In humans, with a much lower frequency of tissue iNKT and γδT cells, it remains to be seen if this window period for reconstitution and niche exist in adulthood. BAL MAIT cells depleted in HIV are increased with ART; as ART partially restores a dysregulated lung microbiome (126), it is tempting to speculate this contributes to MAIT cell reconstitution. Peripheral MAIT cells however are not reconstituted by ART (21, 24). Long term reconstitution is also seen after allogeneic hematopoietic stem cell transplantation (81), dependent on the microbiome and potentially continued thymic output given the negative correlation with age.

Microbial Diversity and Pathogenicity

The mammalian microbiome is diverse and heterogeneous, varying between sites with different microenvironments. Skin hair follicles, sweat glands and sebum promote distinct commensals and immune responses compared to the intestine (52, 127, 128); diet, age and antimicrobials all shape the gut microbial landscape (129). Furthermore, dysbiosis can result from changes in the tissue microenvironment which drives the expansion of more suitably adapted commensals.

The appreciation of different microbial phyla to MAIT cell expansion and function is expanding. A screen of bacterial species *in vitro* found that the capacity to stimulate MAIT cells correlated with riboflavin secretion (130). Colonization of GF mice with *Proteus mirabilis* alone in the neonatal period is sufficient for MAIT cell expansion in the skin and lungs (35). In reality we have communities of microbes and there is evidence that increased microbial diversity is associated with improved MAIT cell reconstitution after allogeneic hematopoietic stem cell transplantation (81). This could partly be through a reduction in their activation induced cell death as in *vitro* microbial diversity has been shown to reduce MAIT cell activation (131). Testing common intestinal commensals *in vitro* has demonstrated that MAIT cell activation correlates with net riboflavin secretion, with higher diversity resulting in predominant riboflavin uptake and thus lower presentation to MAIT cells (131). This is supported by observations in apical periodontitis oral mucosa, where prominent riboflavin-expressing taxa correlate negatively with Vα7.2-Jα33 and IL17A transcripts (132). Furthermore, *Il17a*-deficient mice, which have microbial dysbiosis and reduced barrier protection, actually have increased MAIT cell frequencies in the lung and colon. As *IL17a*-deficient mice have increased *Candidatus Homeothermaceae* and *Bacteriodaceae*, it is tempting to speculate that the composition of the microbiome is crucial for MAIT cell expansion (73). As microbial diversity varies between tissues in health and disease, normally high in healthy colon and reduced in dysbiosis and metabolic diseases, this could be a mechanism to manipulate MAIT cell function and through which they may contribute to pathology.

In addition to diversity, healthy tissues are characterized by an intact barrier, disruption of which induces inflammation and could impact MAIT cell function. Most microbes are commensals living in symbiosis with the host; pathogens induce barrier disruption, inflammation and cytokine production. For example, colonization with commensal *S. epidermidis* does not induce inflammation and is important for tissue homeostasis and a MAIT cell tissue repair signature (35). However, stimulation of human MAIT cells *in vitro* with cytokines in addition to TCR engagement promotes an antimicrobial program, including the cytokine IL-26, capable of directly killing extracellular bacteria (133), and effector recruiting chemokines CXCL9 and CXCL10 (20, 49). Thus, the pathogenicity of the human microbiome could tune the MAIT cell response, and further studies should assess the various pathogen-specific factors which induce antimicrobial rather than more tolerant repair effector programs.

Microbial Metabolism and Environment

Microbial metabolism is also shaped by the tissue microenvironment and could tune MAIT cell activation. One mechanism is through availability of TCR ligands derived from riboflavin synthesis: heat stress in *Streptococcus pneumoniae* induces expression of the riboflavin operon (134); and acid stress increases purine and folate metabolism, as well riboflavin uptake (19, 131). Bacterial co-culture conditions influence their capacity to activate human MAIT cells *in vitro* (19); hypoxia, simulating the low oxygen tension in colonic crypts, stationary growth phase and hypercarbia increase MAIT cell activation, whereas hypoglycemia, acetate and pyruvate inhibit bacterial control (19). Even chemicals and pesticides, which cause gut dysbiosis on ingestion of food, have been found to increase *E. coli*-induced MAIT activation (135). MAIT cells could therefore survey the nature and state of the microbiome as a proxy measure of tissue health.

Non-riboflavin microbial metabolites could also modulate tissue MAIT cells, contributing to tissue homeostasis or pathogenic inflammation. *Lactobacilli*, enriched in the female genital tract, produce high levels of L(+)-lactate; and both lactate and *Lactobacilli*-derived factors dampen *Staphylococcus aureus* (*S. aureus*)-induced MAIT cell activation in whole PBMC (136). Given that the antibacterial response of MAIT cells resident in the female genital tract is biased towards IL-17 and IL-22 production compared to their circulating counterparts (32), it is tempting to speculate that microbial derived factors at barrier surfaces might directly skew MAIT cell responses.

There is evidence that other products of bacterial metabolism, short chain fatty acids (SCFA), can directly modulate barrier RORγt^+ immune cell responses (137). Acetate, propionate and butyrate are products of dietary fiber fermentation which signal via G-protein coupled receptors (GPCR) to inhibit histone deacetylases (HDACs) (137). These SCFA directly promote barrier preservation and can reverse some of the immune dysregulation in GF-mice; SCFA rescue the colonic T_{reg} depletion seen in GF mice (138) and are capable of augmenting RORγt^+T_{reg} expansion (139–141) and ILC3 IL-22 production (142). Barrier cells such as ILC3 can directly sense acetate through *Ffar2 (*free fatty acid receptor 2) (142), which interestingly is also enriched transcriptionally in murine skin MAIT cells compared to $CD4^+$ T cells (35). As SCFA reduce MAIT cell antimicrobial function *in vitro* (19), it would be of interest to determine if microbial metabolites could be sensed and tune MAIT cell responses in a TCR-independent manner.

Dietary Factors

In addition to the microbiome, mucosal immune cells are capable of directly sensing chemicals, including nutrients in the mammalian diet (39). Dietary-derived metabolites, particularly lipophilic compounds, rapidly diffuse and bind to intracellular ligand-dependent transcription factors and can regulate tissue resident cells (143); these include receptors for vitamin A (retinoic acid receptor, RAR), vitamin D (vitamin D receptor, VDR), and tryptophan metabolites (aryl-hydrocarbon receptor, AhR). Given their often-shared function and location, MAIT cells may also be regulated by these dietary factors.

Vitamin A

The fat soluble Vitamin A is enriched in human intestine and is important for mucosal health (39). Dietary vitamin A, as all-trans-retinol, retinyl esters, or β-carotene, is metabolized to bioactive *all-trans*-retinoic acid (ATRA) and *9-cis*-retinoic acid (144), which bind to nuclear retinoic acid receptors RARα (*RARA*), RARβ (*RARB*), and RARγ (*RARG*) (39). In mice RA maintains barrier homeostasis by directly modulating T_{reg}, T_H17 and ILC3 function. RORγt^+pT_{reg} development, homing and differentiation are RA dependent (145–148), with dietary deficiency promoting T_H17 mediated tissue pathology (141). Similarly ILC3 gut homing (149), plasticity (150), and IL-22 mediated protection in DSS colitis are RA dependent (151). In humans, ATRA directly increases CD161 and gut homing CCR9 expression in a population of $RORC^+CD161^+$ colonic T_{reg} associated with tissue repair (64). Vitamin A deficiency is also strongly associated epidemiologically with severe mucosal infections, which may partly be through direct effects on barrier protective immune cells.

The role of RA in innate-like T cells is less clear. RA reduces invariant NKT induced-sterile tissue damage by inducing P2RX7 expression, rendering bystander but not TCR-activated cells more susceptible to extracellular ATP-induced pyroptosis (152). γδT cell function is also reduced: $CD27^-$γδT cell IL-17 production is inhibited by RA through reduction in IL-1R, IL-23R, and pSTAT3 expression (153). In IBD tissue however, RA levels correlate with increased γδT and MAIT cell function (IL-17, IFN-γ) (154). As *RARG* is upregulated in blood MAIT cells relative to conventional T cells (46), vitamin A could conceivably modulate intestinal MAIT cell migration and function to ultimately maintain mucosal integrity in an analogous manner to neighboring $CD161^+$ T cells.

Vitamin D and Cholesterol Metabolites

The lipophilic oxysterol derivate **Vitamin D** can be derived from the diet or photochemically synthesized in the skin (39), and binds to its heterodimeric receptor, composed of VDR and the retinoid X receptor (RXR). Immune cells, particularly those in

the intestine and skin, are enriched for expression of the nuclear VDR which is reduced in inflammatory bowel disease and implicated in the moderation of mucosal inflammation (39, 155). VDR expression is upregulated on TCR signaling and decreases type 17 associated immunopathology in humans and mice, increasing the ratio of T_{reg}: T_H17 (156, 157), inhibiting ILC3 IL-23R expression (158), and directly competing with NFATc1 for binding to the *IL17A* promoter (159). *Vdr*$^{-/-}$ mice also have impaired iNKT and CD8$\alpha\alpha^+$ IEL development, suggesting a broad role in tissue immunity (159–161).

MAIT cell frequency and function may also be subject to regulation by vitamin D. MAIT cells triggered through their TCR upregulate *VDR*, either *in vitro* in humans or during acute *Legionella longbeachae* infection in mice (48). In asthmatic patients, seasonal fluctuations in peripheral MAIT cell frequency correlate with serum phytochemically derived vitamin D_3 levels and peak in August (162). In cystic fibrosis patients however, although baseline serum vitamin D3 correlates with peripheral MAIT cell CD38 expression, there was a trend for reduced MAIT cell frequency in those receiving oral vitamin D supplementation (163). Dietary and photochemically-derived vitamin D may differentially regulate MAIT cells in different compartments and activation states – whether responding to commensals in homeostasis or during active inflammation.

Other cholesterol derivatives of host or microbial metabolism, including **oxysterols**, are abundant in the intestine and can act as RORγt ligands to promote development and function of RORγt$^+$ intestinal cells (164). Stromal cells produce 7-α,25-hydrocycholesterol, which binds to GPR183 expressing ILC3 to promote their migration in homeostasis (165). As tissue IFN-γ producing MAIT cells transcriptionally express the oxysterol receptor GPR183 (29), oxysterol sensing may also functionally regulate MAIT cells.

Bile acids are cholesterol-derived surfactants crucial for fat digestion that bathe the ileum as part of the enterohepatic circulation and regulate both the microbiota and mucosal immunity (166). Secondary bile acids derived from microbial metabolism, including deoxycholic acid (DCA) and lithocholic acid (LCA), can directly promote mucosal homeostasis by increasing colonic FOXP3$^+$ RORγt$^+$ T_{reg} (167). A screen of secondary bile acids also found that LCA derivatives can reduce the T_H17:T_{reg} balance in the intestinal lamina propria, by directly blocking RORγt-induced T_H17 differentiation and promoting T_{reg} *Foxp3* expression and differentiation in a mitochondrial ROS-dependent manner (168). MAIT cell activation and PLZF expression negatively correlate with serum concentrations of conjugated bile acids in teenage children, and *in vitro* bile acids inhibit MAIT cell activation in response to *E. coli* (169), so it would be important to explore whether intestinal bile acids promote homeostatic MAIT cell responses against commensals within a healthy functioning symbiotic intestinal environment.

Aryl Hydrocarbon Receptor

Aryl hydrocarbon receptor (AhR) is a conserved ligand activated transcription factor highly expressed by cell types at barrier surfaces, in keeping with its role as an environmental sensor promoting mucosal integrity. Physiological AhR ligands include: indole-derived ligands from dietary cruciferous vegetables; host and microbe-derived tryptophan-metabolites (e.g., kynurenine); and exogenous chemicals (40). Initially discovered and enriched in intestinal T_H17 and T_{reg} (41, 170), AhR signaling is also important for the function of mucosal IL-17 producing γδT, iNKT and ILC3 (66, 171, 172). Sensing of diverse environmental signals promotes T_{reg} differentiation, IL-22 production, ILC3 survival and IEL homeostasis, thus promoting barrier integrity (173). *Ahr*-deficient mice have dysfunctional skin and intestinal γδT cells and absent IEL (172, 174), with reduced capacity for T_H17 differentiation and IL-22 secretion. AhR expression in CD8$^+$ T cells is crucial for T_{RM} and IEL persistence in tissues (175, 176), while cytokines upregulate AhR expression in NK cells and iNKT to promote cytotoxicity and IL-22 production respectively (66, 177).

The role of AhR in MAIT cells has only been tentatively explored. *Ahr* is dispensable for MAIT cell thymic development in mice (56). In HIV patients on ART, increased tryptophan catabolism and generation of the AhR ligand, kynurenine correlates with lower peripheral blood MAIT cell frequency and higher frequency of T_{reg} (178). As *AHR* expression is higher in bronchoalveolar MAIT cells compared to matched circulating cells in children with pneumonia (29), further work should explore specifically whether tissue MAIT cells are selectively regulated by AhR in a similar manner to other IL-22 producing cells in particular.

Lipids

The predominant calorie source of diet can influence barrier immunity in mice. A high glucose diet exacerbates colitis by increasing mitochondrial metabolism to drive T_H17 differentiation (179). Mice fed a high fat diet also have increased T_H17 differentiation through induction of the lipid sensitive kinase, acetyl co-A carboxylase 1, crucial for de novo FA synthesis and oxidative phosphorylation (103, 180). A ketogenic high fat diet however protects against influenza challenge and promotes improved lung barrier integrity associated with early lung γδT cell recruitment, expansion and barrier type-17 function (181). In addition to diet, tissue free fatty acids have also been shown to induce a regulatory phenotype in iNKT (182). Lung type-17 MAIT cells in the context of pneumonia are enriched in genes for OXPHOS, glycolysis, lipid efflux and translocation, while other MAIT cells are enriched in genes for steroid metabolism, fatty acid synthesis and lipid uptake (29). Further studies should investigate the regulation of MAIT cell function by lipids and metabolism.

Tissue Environment

Tissue immune cellular and soluble mediators, particularly cytokines, manipulate the function of MAIT cells and other resident populations (4). This is further nuanced by the confined shared niche occupied by resident cells which compete for space and local survival signals (175). Tissue inflammation and infiltration of metabolically active, cytotoxic cells into this niche can disrupt homeostatic regulation by depleting nutrients (glucose, amino acids) and oxygen, producing waste

products such as reaction oxygen species and lactate which contributes to tissue acidosis (183, 184). Resident immune cells are themselves in turn tuned by these non-immune tissue parameters.

Oxygen Sensing

Oxygen tension and regulation varies *in vivo*: blood and primary lymphoid organs have tightly regulated levels, whereas physiological hypoxia is observed in tissues such as the skin and intestine (38, 52, 185). Microbes can also indirectly induce colonic oxygen consumption through SCFA (186). Hypoxia regulates immune cells directly by preventing cytosolic degradation of the oxygen sensing transcription factor, hypoxia-inducible factor (HIF1A). T cell upregulation of *HIF1A* expression is STAT3-dependent and promotes $CD8^+$ T cell effector functions (187), T_{reg} plasticity (188, 189), and T_H17 differentiation through induction of glycolysis, *Ror*γt expression and *Foxp3* proteasomal degradation (190, 191).

In vitro sorted human MAIT cells co-cultured with proximal tubular epithelial cells are more activated in hypoxic conditions, with increased cytotoxicity albeit no difference in cytokine production (192). In children with pneumonia, bronchoalveolar MAIT cells have higher *HIF1A* expression compared to their blood counterparts which correlates with their capacity for increased IL-17 production (29). Furthermore, the tissue repair signature enriched in MAIT cells engaged through their TCR includes upregulation of *HIF1A* in addition to factors associated with angiogenesis (*VEGFA, PDGF2, CSF2*) (48). It would seem plausible that tissue MAIT cells, likely to experience local hypoxia during the course of an immune response, could tune their effector functions accordingly to ultimately induce tissue repair and improve oxygenation.

pH

Although circulating pH is homeostatically maintained around pH 7.4, deviations are seen in tissues: healthy skin is acidic due to a high free-fatty acid content; and inflammation drives tissue acidosis through glycolytic products (52). Many immune cells possess mechanisms for proton sensing, including acid-sensing ion channels (ASIC), transient receptor potential (TRP) channels, and GPCRs (193). Among T cells, human MAIT cells and other $CD161^+$ T cells share functionality and a conserved transcriptional signature by bulk microarray, which includes enrichment for two candidate GPCR proton sensors, *GPR65* and *GPR68* (45, 194, 195). GPR65 may play an important role in *ROR*γt^+ T cells; the *Gpr65* promoter has a *ROR*γt binding site (196), and Gpr65 expressing T cells regulate the development of EAE in mice which is driven by type 17 inflammation (197). Naïve $Gpr65^{-/-}$ $CD4^+$ T cells differentiated under T_H17 conditions, or memory $Gpr65^{-/-}$ $CD4^+$ T cells reactivated with IL-23 produce less IL-17A *in vitro*, and the adoptive transfer of $Gpr65^{-/-}$ $CD4^+$ T cells into $Rag1^{-/-}$ recipients prior to EAE induction markedly delays and reduces disease (197). Another study however found that *Gpr65*-deficient mice develop exacerbated EAE, which was lost in the absence of iNKT (198); functionally deficient $Gpr65^{gfp/gfp}$ but not $Cd1d^{-/-}$ $Gpr65^{gfp/gfp}$ mice develop more severe disease compared to wild-type. It is particularly interesting to note that murine *Gpr65* expression is important for $CD4^+$ T survival in culture and highest in iNKT, followed by γδT and NK cells, suggesting a homeostatic role for acid sensing in innate-lymphoid cells. MAIT cells were not assessed in this study, but in humans share similarities with and are a hundred times more common than iNKT (67), thus may represent the most prominent GPR65 expressing cell. Indeed type 17 bronchoalveolar MAIT cells in children with pneumonia are enriched for *GPR65* expression so future studies should explore whether acid-sensing can promote MAIT cell mucosal function (29).

Lactate

Increased lactate is concomitantly seen with acidosis in inflammation, and can be directly sensed by $CD4^+$ T and $CD8^+$ T cells through SLC5A12 and SLC16A1 transporters respectively; these function to inhibit T cell migration and potentially promote tissue retention (199–201). However in mice, cells with low glycolytic capability, including murine iNKT, show reduced survival under high lactate conditions *in vitro* (202). MAIT cells and other IL-$23R^+$ lymphocytes could also be indirectly regulated by lactic acid augmentation of TLR-induced IL-23p19 production (203), which would skew towards type 17 responses in tissues (32, 204).

Temperature

Fever is a conserved response to infection and autoinflammatory disease across species. Although core temperature is rigorously regulated, peripheral tissues such as the skin where MAIT cells reside, are prone to deviations (205). High temperatures have long been known to enhance human lymphocyte proliferation and cytotoxicity *in vitro* (206), as well as $CD8^+$ T cell differentiation and $CD4^+$ T cell activation through increased membrane fluidity and reduced co-stimulation thresholds (207, 208). In mice, antipyretics (aspirin, ibuprofen) inhibit T_H17 differentiation, with high temperatures selectively promoting inflammatory T_H17 differentiation and increased lung neutrophil recruitment (209). Pulmonary MAIT cells and IL-17A producing innate-like T cells clearly protect against bacterial and viral infections in mice, which become pyrexial during the course of a normal immune response (210–212). It is unclear if pharmacological or pathological alteration of this normal febrile response, or significant exposure to cold environments, could modulate the response of tissue MAIT cells.

Electrolytes and Osmotic Stress

Similar to pH, electrolytes such as sodium, potassium, and chloride are normally tightly regulated in blood. Elevated extracellular potassium is, however, found in necrotic tissues and tumors, which paralyses human cytotoxic T cell responses (213). It is also appreciated that salt (NaCl) concentration can be enriched in barrier tissues such as the skin, particularly during inflammation (44, 52, 214). High salt diet increases EAE severity in mice due to increased T_H17 differentiation from naïve precursors; direct salt-sensing ultimately induces T_H17 IL-23R expression (215, 216) and inhibits T_{reg} differentiation (217, 218).

In humans, high salt *in vitro* augments both naïve $CD4^+$ T_H17 polarization and memory $CD8^+$ T cell IL-17A production in response to TCR-activation in the absence of polarizing cytokines (219). Interestingly, the skin of patients with atopic dermatitis has higher salt concentrations, and salt promotes both T_H2 and T_H17 cytokine production and skin-homing CCR8 expression by TCR-activated memory $CD4^+$ T cells, thus potentially linking the environment with pathogenic mucosal T cell responses (219). Salt also indirectly regulates mucosal T cell function through differential production of polarizing cytokines: osmotic stress increases macrophage IL-1β production and Th17 generation in mice (220); and humans with a fixed high salt diet have increased plasma IL-23 (221). As MAIT cells are $IL\text{-}23^+$ T cells in the skin, it would be interesting to determine whether their responses are also skewed in a similar way by the tissue electrolyte composition and if this contributes to disease.

Neuroendocrine System

The dense peripheral neuronal network underlying barrier surfaces co-ordinate rapid often reflex responses to external insults, such as itch, pain or cough reflex. Remarkably peripheral nerves directly regulate tissue immunity through soluble factors, including neuropeptides: neuromedin U (NMU) modulates ILC2-mediated tissue protection (222, 223); vasoactive intestinal peptide (VIP) increases ILC3-mediated epithelial barrier protection (224, 225); and catecholamines have inhibitory and stimulatory effects on ILC2 and NK cells

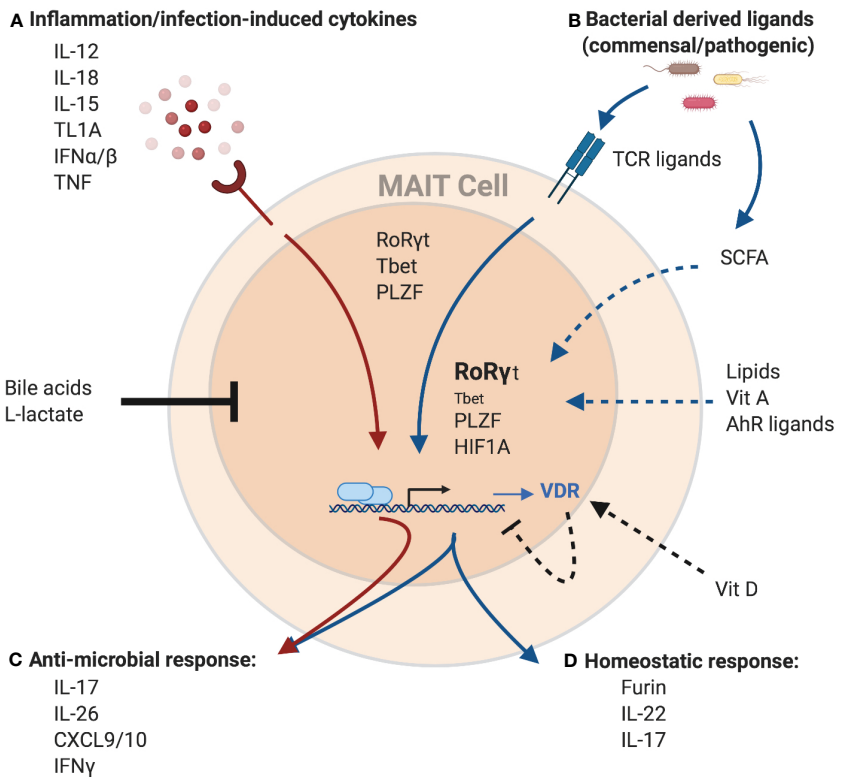

FIGURE 2 | Potential regulation of the MAIT cell transcriptome and effector function by environmental cues. MAIT cells can be activated **(A)** independent of TCR-ligands by cytokines, or **(B)** through TCR-mediated recognition of microbial-derived riboflavin derivates presented by MR1. These signals can work both independently and synergistically to induce a spectrum of different effector programs. Cytokine-mediated MAIT activation results in the induction of a strong anti-microbial program **(C),** including the production of cytokines like IFNγ, IL-26 and members of the IL-17 family as well as pro-inflammatory chemokines like CXCL9 and CXCL10. These antimicrobial functions are further amplified with concurrent TCR signaling. TCR signals result in the induction of a homeostatic response **(D)**, including cytokines associated with barrier maintenance (IL-22, IL-17), and proteins associated with tissue repair, such as the endoprotease furin. MAIT cells effector functions are controlled by the transcription factors PLZF, RoRγt, and Tbet. Importantly, while PLZF expression within MAITs is stable, expression of the homeostatic effector program is associated with increased expression of RoRγt and decreased expression of Tbet. Finally, TCR-mediated MAIT cell activation also leads to expression of HIF1A, another transcription factor associated with tissue repair. In addition to TCR-ligands and cytokines, several other factors have the potential to modulate MAIT cell activation. Bile acids and L-lactate were shown to generally reduce MAIT cell responses, while binding of Vitamin D to its receptor (VDR), the expression of which is upregulated in MAIT cells in response to TCR-signaling, has the potential to specifically inhibit the homeostatic response. In contrast, recognition of several other metabolites including AhR ligands, Vitamin A and lipids was associated with the expression of homeostatic effector molecules in other T cell populations and hence, could positively influence the expression of these molecules in MAIT cells as well. Similarly, short-chain fatty acids (SCFA), a product of bacterial metabolism, were shown to stimulate production of IL-22 and expansion of RORγt-expression lymphocytes in other immune cells, while reducing the antimicrobial function of MAIT cells, which could overall present a mechanism to preserve tissue homeostasis. Created with Biorender.com.

respectively via adrenoceptor beta-2 (ADRB2) (226). The enteric nervous system can also indirectly control resident lymphocytes, through nociceptor induced IL-18 and IL-23 expression (227, 228). Given their location within this neuroimmune network, and expression of relevant receptors for cytokines and neuropeptides transcriptionally, MAIT cells could be subject to rapid manipulation by the nervous system.

Growth factors also regulate tissues and could influence MAIT cells. One example is insulin-like growth factor 1 (IGF-1) signaling, which promotes STAT3 signaling and aerobic glycolysis to increase type-17 effector functionality of T_H17 and ILC3 (229). IGFbp4, an important modulator of IGF1 signaling, is enriched in murine RORγt$^+$ T_H17, T_{reg}, and ILC3. In humans, *IGFBP4* is enriched in CD161$^+$ T cells, so may be an important regulator of MAIT cell type-17 functionality (45). Indeed insulin resistance and fasting insulin levels in obese children correlate with circulating IL-17A producing MAIT cells (230), which may imply that feedback circuits regulating tissue glucose metabolism play an important role in skewing MAIT cell function.

External Environment

UV-light can regulate the immune system (231), partly through photochemical synthesis of vitamin D. Additionally, UV light dampens inflammatory pathology in psoriasis and has been shown to degrade numerous photosensitive MAIT cell ligands, including folic-acid derived 6-FP (6-formyl-pterin) (232). Given the unstable nature of MAIT cell ligands, the impact of light on skin MAIT cell responses to commensals in particular deserves further attention.

Finally, the **circadian rhythm** has a role in entraining barrier RORγt$^+$ cells (233). Clock genes regulate the *RORC* promoter to dictate T_H17 differentiation (234) and ILC3 function (235–237); and disruption of the light-dark cycle in mice exacerbates T_H17 IL-17A-dependent DSS colitis. Pathway enrichment for innate-like T cells suggests that circadian clock regulation is a shared feature among human innate-like T cells, with transcription factors *ARNTL* (encoding BMAL), *RORA*, *PER1*, and *CRY1* enriched among MAIT, iNKT, and Vδ2$^+$ $\gamma\delta$T cells (238). As circadian biology regulates mammalian behavior and exposure to environmental factors, including food, this could be particularly relevant to mucosal MAIT cell function.

CONCLUSION

MAIT cells serve an increasingly appreciated role in barrier tissues, yet the full range of effector functions remain to be determined. Similar to other mucosal lymphocytes, they are engaged in cross-talk with the tissues and microbiome via their TCR and through cytokine receptors (**Figure 2**). The context of this cross-talk in tissues, in addition to the array of increasingly recognized signals sensed by resident lymphocytes, suggest that other factors may influence MAIT cell activation, function, and plasticity. Indeed, these environmental factors were identified with mouse models that may have missed the impact on MAIT cell biology as these cells are infrequent in murine mucosal tissues. Humans, however, have an abundance of MAIT cells and in contrast to laboratory mice, are exposed to phenomenally diverse environmental factors unique to each individual. Exploring local environmental factors in addition to fixed pre-programmed factors in the investigation of MAIT cell tissue biology will be crucial to understanding the variability in humans and could pave the way for personalized therapies in the context of disease.

AUTHOR CONTRIBUTIONS

AA conceived the review, conducted the literature review, and wrote the bulk of the manuscript. DP contributed to writing of the manuscript. C-PH edited and revised the manuscript, and created the figures. PK contributed to the planning, editing, and scope of the review. All authors contributed to the article and approved the submitted version.

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