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**Review**

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Review

# Classic costimulatory interactions in MAIT cell responses: from gene expression to immune regulation

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## Summary

Mucosa-associated invariant T (MAIT) cells are evolutionarily conserved, innate-like T lymphocytes with enormous immunomodulatory potentials. Due to their strategic localization, their invariant T cell receptor (*i*TCR) specificity for major histocompatibility complex-related protein 1 (MR1) ligands of commensal and pathogenic bacterial origin, and their sensitivity to infection-elicited cytokines, MAIT cells are best known for their antimicrobial characteristics. However, they are thought to also play important parts in the contexts of cancer, autoimmunity, vaccine-induced immunity, and tissue repair. While cognate MR1 ligands and cytokine cues govern MAIT cell maturation, polarization, and peripheral activation, other signal transduction pathways, including those mediated by costimulatory interactions, regulate MAIT cell responses. Activated MAIT cells exhibit cytolytic activities and secrete potent inflammatory cytokines of their own, thus transregulating the biological behaviors of several other cell types, including dendritic cells, macrophages, natural killer cells, conventional T cells, and B cells, with significant implications in health and disease. Therefore, an in-depth understanding of how costimulatory pathways control MAIT cell responses may introduce new targets for optimized MR1/MAIT cell-based interventions. Herein, we compare and contrast MAIT cells and mainstream T cells for their expression of classic costimulatory molecules belonging to the immunoglobulin superfamily and the tumor necrosis factor (TNF)/TNF receptor superfamily, based not only on the available literature but also on our transcriptomic analyses. We discuss how these molecules participate in MAIT cells' development and activities. Finally, we introduce several pressing questions vis-à-vis MAIT cell costimulation and offer new directions for future research in this area.

**Keywords:** MAIT cells, costimulation, CD28, ICOS, CD40 ligand, OX40, 4-1BB, CD27

**Abbreviations:** Ag(s): antigen(s); APC(s): Ag-presenting cell(s); 5-A-RU: 5-amino-6-D-ribitylaminouracil; B6: C57BL/6 [mouse strain]; 4-1BBL: 4-1BB ligand; CAR: chimeric Ag receptor; CD: cluster of differentiation; CD40L: CD40 ligand; CTLA-4: cytotoxic T-lymphocyte-associated antigen-4; DC(s): dendritic cell(s); dsDNA: double-stranded deoxyribonucleic acid; *E. coli*: *Escherichia coli*; HIV: human immunodeficiency virus; HOMA-β: homeostatic model assessment of β cell function [index]; HPV: human papillomavirus; *H. pylori*: *Helicobacter pylori*; IAV(s): influenza A virus(es); ICOS: inducible costimulator; ICOSL: ICOS ligand; IFN: interferon; IL: interleukin; ILC2: type-2 innate lymphoid cell(s); *i*NKT: invariant natural killer T [cell(s)]; *i*TCR: invariant T cell receptor; KDM6B: histone lysine demethylase 6B; LAG-3: lymphocyte-activation gene 3; LPS: lipopolysaccharide; mAb: monoclonal antibody; MAIT: mucosa-associated invariant T [cell(s)]; MAITfh: T follicular helper-like MAIT [cell(s)]; MG: methylglyoxal; MHC: major histocompatibility complex; MR1: MHC-related protein 1; mRNA: messenger ribonucleic acid; *Mtb*: *Mycobacterium tuberculosis*; mTOR: mammalian target of rapamycin; mTORC: mTOR complex; NK: natural killer [cell]; NKG2D: NK group 2, member D; 5-OP-RU: 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil; OX40L: OX40 ligand; PB: peripheral blood; PBMC(s): peripheral blood mononuclear cell(s); PD-1: programmed death-1; PLZF: promyelocytic leukaemia zinc finger [transcription factor]; PMA: phorbol myristate acetate; RIPK3: receptor-interacting protein kinase 3; RORγt: retinoic acid receptor-related orphan receptor γt; SAP: signaling lymphocytic activation molecule-associated protein; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; scRNA-seq: single-cell RNA-sequencing; SHP-2: Src homology region 2 domain-containing protein tyrosine phosphatase 2; SLE: systemic lupus erythematosus; T-bet: T-box expressed in T cells; TB: tuberculosis; $T_{conv}$: conventional T [cell(s)]; T1D: type 1 diabetes; T2D: type 2 diabetes; Tfh: T follicular helper [cell(s)]; TIGIT: T cell immunoreceptor with immunoglobulin and ITIM domains; T1-IFN(s): type 1 IFN(s); TIM-3: T cell immunoglobulin and mucin-3; TL1A: TLR(s): tumour necrosis factor-like protein 1A; TLR: Toll-like receptor(s); TNFR: TNF receptor; ZAP-70: zeta chain-associated protein kinase 70.

## MAIT cells: a brief introduction

### Definition and homing characteristics

Mucosa-associated invariant T (MAIT) cells are innate-like T lymphocytes with a unique gene rearrangement pattern in their invariant T cell receptor (*i*TCR) α chain, typically *TRAV1-2-TRAJ33/12/20* (Vα7.2-Jα33/12/20) in humans and *Trav1-Traj33* (Vα19-Jα33) in mice [1–3]. The TCR Vβ repertoires of human and mouse MAIT cells are also limited in diversity, with the predominant usage of Vβ2/13 and Vβ6/8, respectively [3, 4].

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Unlike conventional T ($T_{conv}$) cells that see antigenic peptides presented by the highly polymorphic major histocompatibility complex (MHC) molecules, MAIT cells are restricted by the monomorphic MHC-related protein 1 (MR1) [5]. MR1 can display vitamin B metabolites synthesized by commensal and pathogenic bacteria and fungi, typified by 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) [6–8], among other potential ligands. MR1 appeared early in mammals, co-evolved with TRAV1, and remained conserved ever since [9]. The cross-reactivity between mouse and human MAIT cells points to the similarity of MR1 ligands recognized by these cells [10].

MAIT cells are scarce in standard laboratory mouse strains [11] in which they primarily show a $CD4^-CD8^-$ phenotype [12]. Therefore, *in vivo* studies on MAIT cells have often relied on experimental pre-enrichment [13], or on genetically altered strains with an adequately large MAIT cell compartment, such as Vα19*i* TCR transgenic [14] and C57BL/6 (B6)-$MAIT^{CAST}$ mice [11]. In contrast, human MAIT cells, which are predominantly $CD4^-CD8^+$, are abundant in the peripheral blood (PB), in the liver, and in barrier tissues, including in the skin and in gastrointestinal, respiratory, and urogenital tracts, where they help maintain homeostasis and combat pathogens at their entry sites [15, 16]. Human MAIT cells express high levels of CD161, which has enabled their identification as $CD3^+Vα7.2^+CD161^+$ cells in many investigations. The advent of MR1 tetramer reagents has allowed for the accurate detection of mouse and human MAIT cells and their comparative analyses [12, 17].

## Intrathymic and extrathymic development

MAIT cell maturation in the thymus occurs in three stages. In mice, $CD24^+CD44^-$, $CD24^-CD44^-$ and $CD24^-CD44^+$ cells define stages 1, 2, and 3, respectively [18]. In humans, the $CD161^-CD27^-$ stage is followed by the appearance of $CD161^-CD27^+$ cells before $CD161^+CD27^{-/+}$ cells emerge [18]. While early thymic MAIT cell precursors are $CD4^+CD8^+$, their differentiation into more mature cells is accompanied by a gradual loss of CD4 expression. In both mice and humans, the transition from stage 2 to stage 3 is dictated by the transcription factor promyelocytic leukemia zinc finger (PLZF) and interleukin (IL)-18 [18, 19].

Preserved *i*TCR signaling events and intermediates, including ZAP-70 (zeta chain-associated protein kinase 70) and diacylglycerol, are essential for mouse MAIT cell development [19, 20]. In addition, mice lacking miR-181a/b-1, a microRNA pair serving as a rheostat in TCR signal strength regulation, exhibit early MAIT cell maturation arrest [18, 21]. The potential roles of microRNAs during human MAIT cell education are currently unclear. Normal mouse MAIT cell development appears to depend on SAP (signaling lymphocytic activation molecule-associated protein) [19, 22], RIPK3 (receptor-interacting protein kinase 3), [23] and CXCR6 [19], among other signaling entities.

Unlike $T_{conv}$ cells whose positive selection is executed by cortical thymic epithelial cells displaying self-peptide:MHC complexes, MAIT cells are positively selected by MR1-expressing $CD4^+CD8^+$ thymocytes [24]. Another major difference between $T_{conv}$ and MAIT cells is the location in which functional commitments are made. $T_{conv}$ cells leave the thymus as mature but naïve cells before they encounter cognate antigens (Ags) in secondary lymphoid tissues, proliferate, and differentiate into various effector cell subsets. By contrast, mouse MAIT cells develop in the thymus as transcriptionally and functionally distinct subpopulations, primarily as MAIT1 and MAIT17 lineages that express T-bet (T-box expressed in T cells) and RORγt (retinoic acid receptor-related orphan receptor γt), respectively [18–20, 22]. These transcription factors are not mutually exclusive in the case of human MAIT cells. In their stage 3, thymic human MAIT cells are $RORγt^+T\text{-}bet^+$ and capable of producing interferon (IFN)-γ and tumor necrosis factor (TNF)-α [18], likely among other cytokines.

The thymic differentiation of mouse MAIT1 cells and their accumulation in the thymus and spleen depend on IL-2 and IL-15, whereas signaling through inducible costimulator (ICOS) promotes MAIT17 cell development [20]. Furthermore, the presence of commensal microbes and stronger TCR signals, as judged by Nur77 expression, seems to favor the MAIT17 program in the thymus [25].

Mouse MAIT cells complete their maturation in the periphery after birth and in the presence of B lymphocytes and commensal microbes [26, 27]. In humans, MAIT cell development occurs primarily *in utero* before commensal microbial communities are established [28]. During the second trimester, functional human MAIT cells are detectable within the fetal small intestine, liver, and lungs [28]. It is possible that microbial MR1 ligands present in maternal circulation and mucosal tissues might cross the placenta to access the foetal thymus and mediate positive MAIT cell selection [28]. This is not far-fetched since 5-OP-RU has been detected in the mouse thymus shortly after its cutaneous application [25].

## Activation pathways and consequences

MAIT cell activation occurs through *i*TCR-dependent and -independent mechanisms. Exposure to microbes that metabolize riboflavin or to 5-OP-RU, a riboflavin derivative of bacterial and fungal origin, primes mouse and human MAIT cells in an MR1/*i*TCR-dependent manner [6–8]. A photodegradation product of folic acid called 6-formylpterin [29], certain drugs and drug-like compounds, such as diclofenac and methotrexate [30], and the dietary molecules vanillin and ethylvanillin [31], can also serve as MR1 ligands with various functional outcomes as far as MAIT cell responses are concerned.

Unlike mature $T_{conv}$ cells that are either $CD4^+$ or $CD8^+$ and that rely on their respective co-receptor for stable TCR–MHC interactions, the vast majority of mature MAIT cells in mice are double-negative but still responsive to MR1 ligands or *i*TCR cross-linking. Therefore, co-receptor expression is not required for MR1/*i*TCR-driven MAIT cell activation in mice. By comparison, CD8 expression on human MAIT cells enhances MR1 binding and cytokine production [32].

In addition to or in the absence of *i*TCR signaling, MAIT cells can be activated by cytokines released after infection or Toll-like receptor (TLR) engagement. This pathway is particularly critical in antiviral host defence since viruses lack the riboflavin biosynthesis machinery [33]. IL-7, IL-12, IL-15, IL-18, IL-33, type I IFNs (T1-IFNs), and TNF-like protein 1A (TL1A) are among cytokines to which MAIT cells react in the face of infection, sterile inflammation, tissue damage and other microenvironmental changes [34–38].

Once activated, MAIT cells swiftly release potent immunomodulatory mediators, including IFN-γ, TNF-α, IL-4, IL-5, IL-13, IL-17, and IL-22, in various combinations. This is likely influenced by the strength and/or duration of cognate, cytokine and costimulatory signals MAIT cells receive,

their functional pre-programming, their tissue imprinting, and their epigenetic regulation [39], among other factors. Owed to their remarkable cytokine production capacity, and also through contact-dependent mechanisms, activated MAIT cells modulate the physiological or pathological behaviors of many cell types, including myeloid and plasmacytoid dendritic cells (DCs) [40], monocytes and macrophages [40–43], natural killer (NK) cells [40, 44–46], type-2 innate lymphoid cells (ILC2) [47], NKT cells [44], $T_{conv}$ cells, [44, 48, 49] and B cells [50, 51].

MAIT cells express NK group 2, member D (NKG2D), a C-type lectin-like receptor that enables *i*TCR-independent cytotoxicity against infected, stressed, damaged, or transformed cells [52, 53]. They also harbor a powerful arsenal of cytolytic effector molecules, including perforin, granzymes, and granulysin, which can be upregulated and mobilized toward $MR1^+$ target cells, attack extracellular bacteria, and help overcome resistance to carbapenems, a class of last-resort antibiotics [53, 54].

Steady-state and activated MAIT cells demonstrate transcriptomic signatures suggesting potential functions in tissue repair and remodeling mechanisms [26, 38, 55–57]. For instance, following skin incision, dermal mouse MAIT cells secrete amphiregulin to promote wound healing [57].

For all of the above reasons, one could envisage protective, pathogenic, or sometimes seemingly paradoxical roles for MAIT cells in a wide variety of conditions, including infections and superinfections [58, 59], autoimmunity [44, 60–62], malignancy, [63–65] and fibrotic processes [46, 66]. Therefore, understanding signaling cascades that control MAIT cell responses, including those mediated by costimulatory molecules, is key to successful MR1/MAIT cell-based interventions.

## MAIT cell costimulation: basic principles

MAIT cell activation can be tuned by several molecules not falling under the "classic" costimulatory families of receptors. For instance, signaling through IFN-α/β receptor and several TLRs may potentiate, augment, or regulate MAIT cell responses to 5-OP-RU [40, 49, 67–69]. This mode of action is often indirect and reliant on accessory cells, such as Ag-presenting cells (APCs), in co-culture systems or in complex *in vivo* settings. Mouse MAIT cell responses to certain bacterial infections also depend on concurrent stimulation through IL-12 and IL-23 receptor signaling [70, 71]. As another example, the *i*TCR-driven TNF-α production capacity of MAIT cells was boosted in the presence of α-ketoglutarate, a cofactor for the epigenetic regulator histone lysine demethylase 6B (KDM6B), in human peripheral blood mononuclear cell (PBMC) cultures [39]. In this review, we only cover classic costimulatory molecules belonging to the immunoglobulin, TNF, and TNF receptor (TNFR) superfamilies.

Naive $T_{conv}$ cell priming requires at least two signals. Signal 1 is generated when cognate peptide:MHC complexes are detected by TCRs. This signal, which accounts for the specificity of T cell-mediated immunity, is necessary but insufficient for optimal naïve $T_{conv}$ cell activation, which also depends on costimulatory interactions that supply signal 2. In fact, TCR triggering in the absence of costimulation may lead to T cell anergy or death [72]. Cytokines present in the T cell priming milieu can provide a third signal, which is responsible for or contributes to proliferation, differentiation and functional polarization of the ensuing effector cells and memory cell precursors [73].

Costimulatory molecules work through several mechanisms to enable naïve $T_{conv}$ cell activation. For instance, CD28 engagement makes signaling-rich lipid rafts cluster [74] and triggers actin cytoskeletal remodeling within the immunological synapses formed between $T_{conv}$ cells and APCs [75]. Moreover, CD28 costimulation upregulates the anti-apoptotic protein Bcl-xL to promote T cell survival [76], enhances IL-2 gene transcription [77] and messenger RNA (mRNA) stability [78] to enable T cell growth, and increases glycolytic flux to meet the suddenly raised demand for energy [79]. Effector and memory $T_{conv}$ cells have much less stringent costimulatory requirements compared with their naïve counterparts.

MAIT cells are effector memory-like T lymphocytes, as judged by their $CD45RO^+CD45RA^-CD127^{hi}CD95^{hi}CD62L^{lo}$ phenotype in humans [80], and mount rapid and robust responses to antigenic stimulation. However, within an "innateness gradient," MAIT cells stand closer to $T_{conv}$ cells when transcriptionally compared with *i*NKT and γδ T cells [81]. Accordingly, MAIT cells contain lower levels of pre-formed mRNA encoding cytokines and cytolytic molecules while appearing to exhibit the highest proliferative capacity among innate-like T cells.

Importantly, in response to TCR cross-linking, MAIT cells launch weaker and more transient effector functions than do $CD8^+$ conventional memory T cells [82]. On the contrary, MAIT cells respond more rigorously to inflammatory cytokines. A higher activation threshold for *i*TCRs may serve as a checkpoint to prevent unnecessary MAIT cell activation in mucosal layers where commensal bacterial metabolites are plentiful [82]. However, MAIT cells stay poised and prepared to react to infectious agents and inflammatory stimuli by virtue of the cytokine receptors they express.

We previously compared the costimulatory needs of *i*NKT and $T_{conv}$ cells [83]. In this review, we focus on MAIT cells, which are more frequent than *i*NKT cells in humans. We highlight differences in the expression of classic costimulatory molecules (Table 1) and discuss the significance of these molecules in MAIT cell responses.

## Costimulatory molecules of the immunoglobulin superfamily

### The CD28-CD80/CD86 system

In the mouse thymus, CD28 expression is highest among $CD4^+CD8^+$ thymocytes before its gradual decline as MAIT cell development progresses from stage 1 to stage 3 [19]. Using CD319 and CD138 as surrogate markers for stage-3 MAIT1 and MAIT17 cells, respectively, Koay *et al.* [19] reported a link between higher CD28 expression and the MAIT1 functional program.

Pulmonary mouse MAIT cells constitutively express CD28 at levels comparable to those found on $T_{conv}$ cells [70]. To assess the *in vivo* significance of CD28 costimulation in MAIT cell functions, or lack thereof, Wang *et al.* compared wild-type (WT) and *Cd80/Cd86* double-knockout B6 mice following intravenous administration of a live vaccine strain of *Francisella tularensis*, which elicits a protective MAIT1-skewed response [71]. In this model, defective CD28 signaling reduced non-MAIT αβ T cell, but not MAIT cell, numbers

**Table 1.** Expression of classic costimulatory molecules by conventional T and MAIT cells

| Family | Costimulatory molecule | Cell type | | Expression during MAIT cell development | Ligand(s) | Ligand expression | Reference(s) |
|---|---|---|---|---|---|---|---|
| | | MAIT cells | $T_{conv}$ cells | | | | |
| IgSF | CD28 (Tp44) | + | + | **Stage 1: +++*** | CD80 (B7-1) | APCs | [19, 71, 84–86] |
| | | | | **Stage 2: ++** | | Activated $T_{conv}$ cells | |
| | | | | **Stage 3: +** | CD86 (B7-2) | APCs | |
| | ICOS (CD278, AILIM, CVID1) | + | ↑ | **Stage 3:+++* MAIT17** | ICOSL (LICOS, B7h, B7RP-1, B7-H2, GL50, GL50-B, CD275) | B cells<br>DCs<br>Macrophages<br>Non-lymphoid cells | [20, 51, 70, 71, 87, 88] |
| TNFSF | CD40L (CD154, TNFSF5, gp39, TRAP, TBAM) | *↑** | ↑ | ND | CD40 (TNFRSF5, Bp50, CDW50, p50) | B cells<br>DCs<br>Macrophages<br>Monocytes<br>Platelets<br>Non-hematopoietic cells | [36, 40, 55, 89–91] |
| TNFRSF | OX40 (CD134, TNFRSF4) | *↑** | ↑ | ND | OX40L (CD252, TNFSF4) | B cells<br>DCs<br>Macrophages<br>$T_{conv}$ cells<br>Endothelial cells | [92–97] |
| | 4-1BB (CD137, TNFRSF9, ILA) | ↑ | ↑ | ND | 4-1BBL (CD137L, TNFSF9) | B cells<br>DCs<br>Macrophages<br>Monocytes<br>$T_{conv}$ cells | [40, 55, 70, 98, 99] |
| | CD27 (TNFRSF7, Tp55, S152) | + | + | *Stage 1: -** | CD70 (CD27L, TNFSF7) | Thymic epithelial cells<br>Activated DCs<br>Activated B cells<br>Activated $T_{conv}$ cells | [21, 27, 40, 70, 100] |
| | | | | *Stage 2: +* | | | |
| | | | | *Stage 3: +* | | | |

APC: antigen-presenting cell(s); DC(s): dendritic cell(s); IgSF: immunoglobulin superfamily; $T_{conv}$: conventional T [cell(s)]; TNFRSF: tumor necrosis factor receptor superfamily; TNFSF: tumor necrosis factor superfamily.
+: constitutive expression; ↑: inducible expression; ND: not determined.
*Bold and italics fonts/arrows refer to mouse and human data, respectively. Results applicable to both species are shown using regular fonts.

in the lungs. However, pulmonary MAIT1 cell proportions were slightly increased, rather than decreased, in infected *Cd80/Cd86*$^{-/-}$ animals. These investigators also demonstrated that CD28 signaling was dispensable for pulmonary MAIT cell expansion after intranasal inoculation of *Salmonella typhimurium* or *Legionella longbeachae*, which induces a dominant MAIT17 response [70, 71]. MAIT cell responses and their functional bias in these models depend partially, if not largely, on IL-12 and/or IL-23 [70, 71]. Therefore, it would be interesting to explore the contribution of CD28 costimulation to *i*TCR-driven antibacterial responses when cytokine receptor signaling is experimentally ablated.

In rhesus macaques, PB MAIT cells show a memory phenotype with elevated levels of CD69, CD95, IL-18 receptor and CCR6, along with a larger CD28$^+$ fraction, compared with non-MAIT T cells [84, 85]. In fact, CD28 expression helps distinguish between central memory (CD28$^+$CD95$^+$) and effector memory (CD28$^-$CD95$^+$) MAIT cell subsets in both rhesus macaques and cynomolgus macaques [84, 85]. Moreover, at the peak of simian immunodeficiency virus viremia in the latter species, CD28$^+$ memory MAIT cells transiently increased their expression levels of Ki-67, CD69, CD39, T-bet, and RORγt, whereas their CD28$^-$ counterparts exhibited Ki-67 upregulation only among the tested parameters [84, 85]. Whether CD28 signaling directly alters the activation threshold of nonhuman primate MAIT cells is not known.

In humans, PB MAIT cells express CD28 at their steady state [40, 80]. Using publicly available PBMC [101–104] and CD45$^+$ non-parenchymal hepatic [105] single-cell RNA-sequencing (scRNA-seq) datasets, we found a modest but still detectable presence of *CD28* in PB (Fig. 1A and B and Supplementary Fig. 1) and hepatic (Fig. 2A and B) human MAIT cell-enriched populations. Similar levels of *CD28* transcripts were also detectable in non-MAIT $T_{conv}$ cells in both compartments (Figs. 1B and 2B). For these analyses, MAIT cell-enriched clusters were identified by *CD3D* and *SLC4A10* expression [64], and non-MAIT $T_{conv}$ cell clusters were defined by *CD3D* expression only (Figs. 1A and 2A).

Due to the existence of redundant systems within the costimulatory machinery of human T cells, CD28 appears dispensable for protective immunity against many, if not most, infections. However, intact CD28 signaling controls human papillomavirus (HPV) infection in keratinocytes. This notion is supported by a recent article reporting three patients with inherited CD28 deficiency and cutaneous papillomatosis, including a male with an HPV-2-driven "tree man" phenotype and two relatives with severe, recurrent HPV-4-induced warts [107]. Of note, PB MAIT cells were the only innate-like T lymphocytes with low frequencies in these patients compared with healthy controls. This is especially intriguing in light of another case report describing a person with tattoo-associated persistent HPV$^+$ warts who was found to carry a homozygous point mutation in *MR1* resulting in MAIT cell deficiency [108]. Whether and how CD28 signaling in MAIT cells contributes to anti-HPV immunity warrants mechanistic investigations.

Several studies have reported reduced CD28 expression by human MAIT cells in the contexts of viral infections and cancer, accompanied by increases in co-inhibitory molecules and exhaustion markers. For instance, PB MAIT cells from patients with chronic hepatitis delta virus infection exhibited low CD28 and high programmed death-1 (PD-1) levels compared with healthy donor MAIT cells [109]. Similarly, in patients with hepatocellular carcinoma, intratumoral MAIT cells expressed lower CD28 but higher PD-1, cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), and T cell immunoglobulin and mucin-3 (TIM-3) levels than did matched healthy liver MAIT cells [110]. In both scenarios, MAIT cells were hyporesponsive to *i*TCR stimulation with fixed *Escherichia coli* (*E. coli*) [109, 110].

Using cell-free biochemical assays and PD-1-transduced Jurkat T cells, Hui *et al.* [111] demonstrated that PD-1-recruited Src homology region 2 domain-containing protein tyrosine phosphatase 2 (SHP-2) dephosphorylates CD28, thus terminating its signaling cascade. Using mouse models of chronic viral infection and cancer, Kamphorst *et al.* [112] found CD28 costimulation to be necessary for CD8$^+$ T cell proliferation following PD-1 blockade. In addition, anti-PD-1 therapies in lung cancer patients expanded a population of CD8$^+$ T cells that were predominantly CD28$^+$ [112].

It is tempting to suggest that PD-1 upregulation in MAIT cells may interfere with their CD28 signaling . To test this hypothesis, however, the expression kinetics of these molecules and their relationship need to be explored first. Nonetheless, in *in vitro* culture systems, CD28 triggering augments *i*TCR-based responses. For instance, an anti-CD28 monoclonal antibody (mAb) raised the frequency of CD69$^+$IFN-γ$^+$ cells among purified Vα7.2$^+$ T lymphocytes that were co-cultured with monocytes in the presence of fixed *E. coli* [113]. In a separate study, biotin beads coated with anti-CD3 and anti-CD28, which were used as artificial APCs, were superior to those coated with anti-CD3 alone in inducing IFN-γ and TNF-α production by CD8$^+$Vα7.2$^+$ T cells [114]. This response was further boosted when CD161 was additionally ligated.

## The ICOS-ICOSL system

ICOS-ICOS ligand (ICOSL) interactions play a pivotal role in MAIT cell development, functional fate choice determination, and immunomodulatory responses, at least in mice. Tao *et al.* [20] found no ICOS expression by mouse TCRβ$^-$CD4$^+$CD8$^+$ thymocytes and low ICOS levels in TCRβ$^+$ thymocytes. However, ICOS was readily detectable in developing MAIT cells with the lowest and highest levels present in thymic stages 1 and 3, respectively.

Approximately 70% of thymic MAIT cells are lost in *Icos*$^{-/-}$ mice, which can be attributed to a numerical drop in stage-3 cells [20]. In addition, ICOS deficiency selectively but incompletely impedes MAIT17 cell development/maintenance, which reduces the number of splenic, peripheral lymph node, hepatic and pulmonary MAIT cells as a result.

TCR-, ICOS- and cytokine receptor-coupled signaling pathways are known to activate the mammalian target of rapamycin (mTOR) in $T_{conv}$ cells [115–118]. Using mice with a selective deficiency of *Rictor* in their CD4$^+$ cells and bone marrow chimeras, Tao *et al.* found intrinsic mTOR complex 2 (mTORC2) signaling to be strictly required for stage-3 MAIT cell maturation/homeostasis and for ICOS$^+$RORγt$^+$ MAIT17 cell development [20].

Given the more pronounced impact of mTORC2 deficiency on MAIT17 cell development, compared with ICOS deficiency, it is likely that mTOR integrates cytokine cues with other signals generated by TCR and/or ICOS stimulation, to control functional MAIT cell programs. Accordingly, Tao *et*

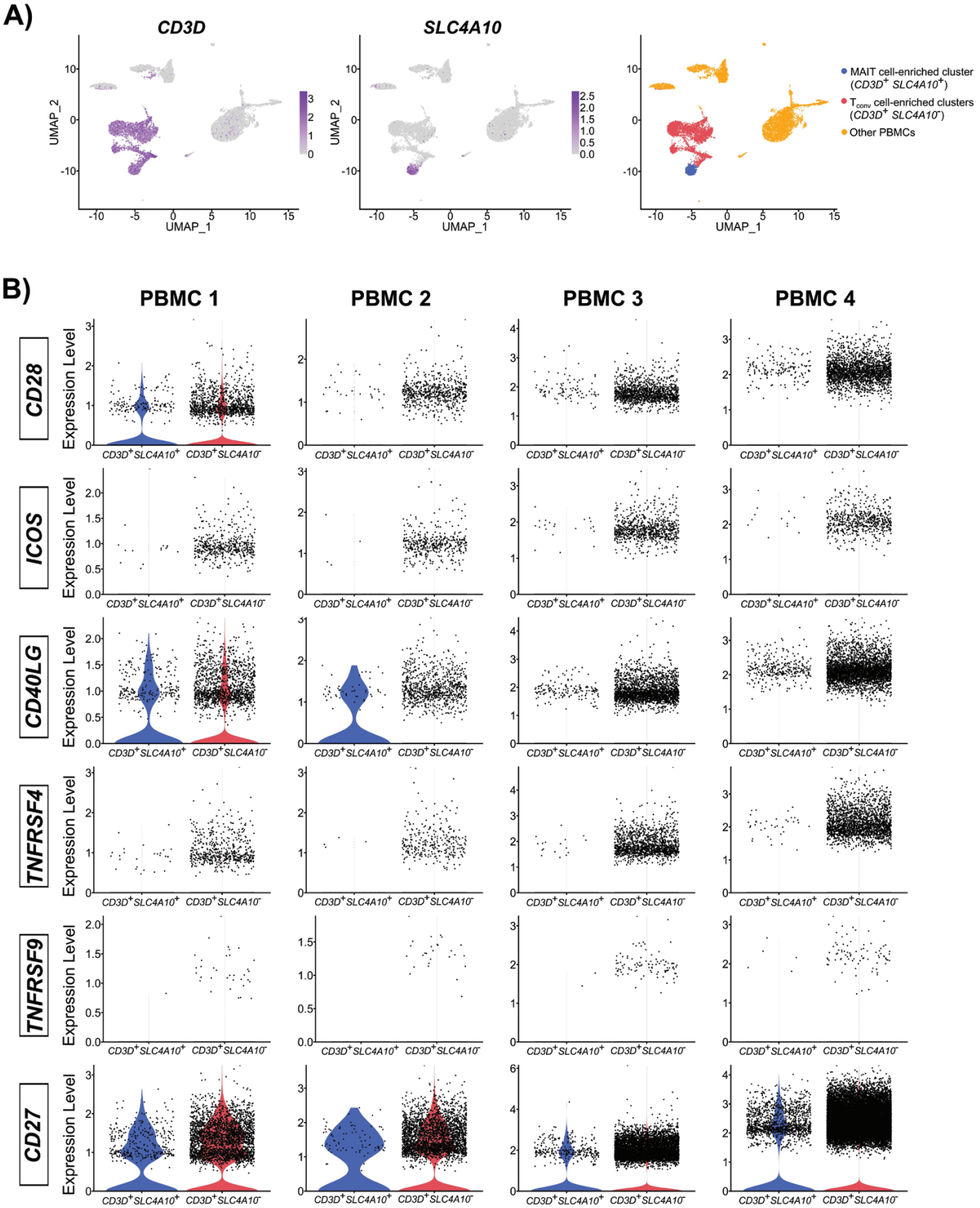


**Figure 1.** Transcriptomic expression of classic costimulatory molecules by MAIT cell-enriched populations identified in human peripheral blood mononuclear cell datasets. Four pre-processed single-cell RNA sequencing datasets [101–104] were accessed on February 22, 2023, from 10x Genomics (https://www.10xgenomics.com/resources/datasets). These include 10k PBMCs from a healthy donor (v3 chemistry), 8k PBMCs from a healthy donor, 33k PBMCs from a healthy donor, and fresh 68k PBMCs (donor A), which are referred to as PBMC 1, PBMC 2, PBMC 3, and PBMC 4, respectively, in this figure. Datasets were analyzed with default parameters using the standard Seurat (4.3.0) workflow [106]. Briefly, matrices were filtered to remove doublets and non-viable cells, transformed by the LogNormalize function, and adjusted with a scale factor of 10 000. The top 2000 variable features were selected and the ScaleData function was applied. Principal component analysis (PCA) was performed, and the top 25 principal components were selected for graph-based clustering and visualization with Uniform Manifold Approximation and Projection (UMAP) plots. Resolution was set to enable MAIT cell-enriched cluster selection for downstream analyses (0.3, 0.7, 0.5, and 1.1 for PBMC 1, PBMC 2, PBMC 3, and PBMC 4,

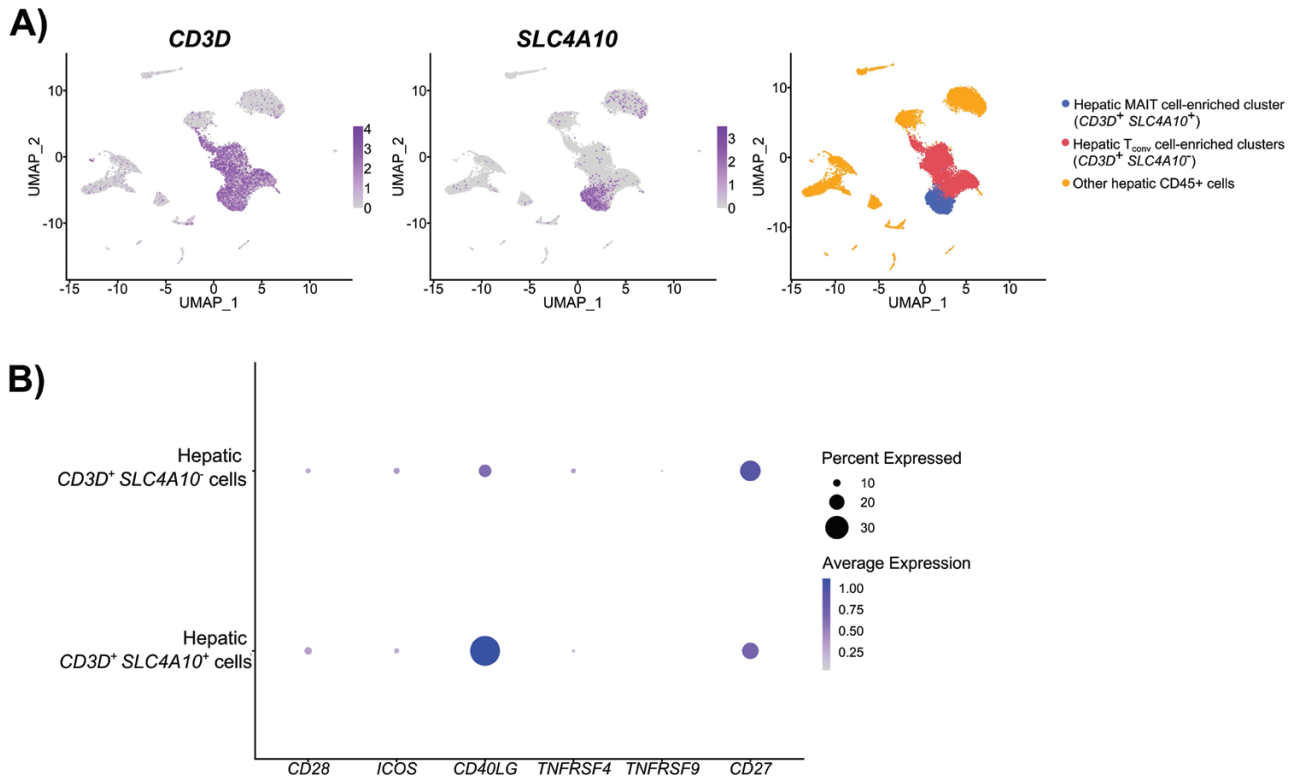


**Figure 2.** Transcriptomic expression of classic costimulatory molecules by hepatic MAIT cells. A pre-processed single-cell RNA sequencing dataset [105] generated using CD45$^+$ cells from five healthy liver samples was obtained from the gene expression omnibus (GEO) public repository (accession number GSE48452). Samples used to create the above dataset were from four males and one female (57.4 ± 7.9 years of age). Standard Seurat (4.3.0) workflow [106] was used for dataset analysis with default parameters. Briefly, the count matrix was filtered to remove doublets and non-viable cells, transformed by the LogNormalize function, and adjusted with a scale factor of 10 000. The top 2000 variable features were selected, and the ScaleData function was then applied. Principal component analysis (PCA) was performed, and the top 25 principal components were selected for graph-based clustering and visualization with uniform manifold approximation and projection (UMAP) plots. Resolution was set to 0.5 to enable MAIT cell-enriched cluster selection for downstream analyses. (A) UMAP plots of *CD3D* and *SLC4A10* illustrate T cells and the MAIT cell-enriched population, respectively. The latter cluster was defined by concomitant *CD3D* and *SLC4A10* expression while *CD3D* expression in the absence of *SLC4A10* was used to identify non-MAIT T cells. Clusters containing other CD45$^+$ hepatic mononuclear cells that expressed neither *CD3D* nor *SLC4A10* are also depicted. (B) A DotPlot for *CD28, ICOS, CD40LG, TNFRSF4, TNFRSF9,* and *CD27* expression in MAIT and non-MAIT T cells was generated.

*al.* proposed a model in which IL-1β works with IL-23 and ICOS to signal through mTORC2 and promote MAIT17 cell polarization. On the other hand, IL-2 and IL-15 involve mTORC1 signaling to enable MAIT1 cell differentiation [20].

It is noteworthy that using ICOS-sufficient mice, Tao *et al.* found CD122$^+$ICOS$^{lo}$ and CD122$^-$ICOS$^+$ phenotypes to be prevalent among T-bet$^+$ and RORγt$^+$ MAIT cells in the thymus and peripheral tissues, which were potent IFN-γ and IL-17A producers, respectively, upon *ex vivo* stimulation with a combination of phorbol myristate acetate (PMA) and ionomycin [20]. Therefore, surface CD122 expression is indicative of the MAIT1 lineage while ICOS can serve as a surrogate marker for MAIT17 cells. In a separate study, transcriptomic and cytofluorimetric analyses of mouse MAIT cells also strongly correlated ICOS expression with RORγt, while the expression of the chemokine receptor CXCR3 was associated with T-bet [119].

Wang *et al.* [70] demonstrated a much higher basal expression level of ICOS in naïve B6 pulmonary MAIT cells than in non-MAIT T cells [70]. This was in contrast with 4-1BB that was absent in MAIT cells, and unlike CD27, CD28 (as noted before) and CD40 ligand whose expression levels were comparable between MAIT and non-MAIT T cells. The authors found lower MAIT17 cell numbers in the lungs of *Icos*$^{-/-}$ mice following infection with *Salmonella typhimurium* or *Legionella longbeachae* [70]. However, this is not surprising since ICOS is necessary for the thymic development of MAIT17 cells before antibacterial responses are initiated in the periphery.

Jensen *et al.* [51] reported negligible expression levels of ICOS by unstimulated pediatric PB MAIT cells, a finding that was corroborated in our bioinformatic analyses of adult human PB (Fig. 1A and B and Supplementary Fig. S1) and hepatic (Fig. 2B) *CD3D*$^+$*SLC4A10*$^+$ cells. However, a large fraction of "T follicular helper (Tfh)-like MAIT (MAITfh) cells", which were discovered in the tonsils of children who had undergone tonsillectomy, expressed ICOS. These MAITfh cells were CXCR5$^+$ MAIT cells that co-expressed the

respectively). (A) Representative UMAP plots from the PBMC 1 dataset. UMAP plots of *CD3D* and *SLC4A10* depict T cells and the MAIT cell-enriched population, respectively. The latter population was defined by concomitant *CD3D* and *SLC4A10* expression while *CD3D* expression in the absence of *SLC4A10* was used to identify non-MAIT T cells. Clusters containing other PBMCs expressing neither *CD3D* nor *SLC4A10* are also illustrated. (B) Violin plots for *CD28, ICOS, CD40LG, TNFRSF4* (OX40), *TNFRSF9* (4-1BB), and *CD27* expression in MAIT and non-MAIT T cells were generated.

Tfh lineage-defining transcription factor *BCL6*, preferentially homed to germinal centers, and produced IL-21 in response to PMA and ionomycin. Together, these features point to a potential role for MAIT cells in providing help to B lymphocytes in mucosal lymphoid tissues. Importantly, MAIT cell transfer into TCR$\alpha^{-/-}$ mice, which are devoid of conventional Tfh cells, gave rise to MAITfh-like cells that supported B cell differentiation into plasmablasts/plasma cells or memory B cells and restored *Vibrio cholerae*-specific IgA production after an intranasal challenge with this pathogen. Future investigations will need to address the significance of ICOS in MAITfh cell activities.

## Costimulatory molecules of the TNFR/TNF superfamily

### The CD40L-CD40 system

The interaction between CD40 ligand (CD40L, CD154) and CD40 results in bidirectional signaling in T cells and their engagement partners, such as DCs and B cells, with clearly important outcomes in adaptive immunity.

In the mouse lung, a substantial proportion of MAIT cells are CD40L$^{+}$ [70]. In humans, unlike unstimulated PB MAIT cells, a fraction of hepatic MAIT cells display CD40L on their surface [55, 89]. We similarly detected *CD40LG* transcripts in hepatic *CD3D*$^{+}$*SLC4A10*$^{+}$ cells at levels that far exceeded those found in *CD3D*$^{+}$*SLC4A10*$^{-}$ cells (Fig. 2B). In addition, varying levels of *CD40LG* were found in PB MAIT cell-enriched populations from healthy donors (Fig. 1B). Steady-state *CD40LG* expression is reportedly enriched among human MAIT cells isolated from the rectal mucosae [82]. However, the expression of CD40L by intestinal MAIT cells at the protein level, or lack thereof, and its potential significance remain to be defined.

As with $T_{conv}$ cells, CD40L expression is inducible upon MAIT cell activation [36, 55, 89, 90]. Lamichhane *et al.* [55] found CD40L upregulation at both mRNA and protein levels in PB human MAIT cells following *E. coli* stimulation. This was phenocopied in cultures containing recombinant IL-12 and IL-18, albeit to a lesser extent. Jeffery *et al.* similarly detected CD40L on human PB MAIT cells in a co-culture system in which T cells were exposed to *E. coli*-primed biliary epithelial cells [89]. This response was partially dependent on MR1, IL-12 and IL-18. Finally, Lepore *et al.* [4] showed that stimulation with *E. coli*-infected THP-1 macrophages prompts human PB MAIT cell clones to secrete soluble CD40L. The *in vivo* relevance of this finding remains unclear.

To study MAIT cells' crosstalk with APCs, Salio *et al.* [40] co-incubated monocyte-derived DCs and MAIT cells in the presence of 5-amino-6-D-ribitylaminouracil (5-A-RU) and methylglyoxal (MG), which together form 5-OP-RU. This resulted in reciprocal activation of DCs and MAIT cells as evidenced by IL-12p40 and IFN-γ production, respectively. Importantly, IFN-γ production was prevented by MR1 blockade and also significantly diminished by CD40L blockade (Fig. 3A). This study yielded two additional findings of interest. First, *i*TCR stimulation of MAIT cells enhanced DC maturation, as judged by the expression of several maturation markers, including B7-1 and B7-2 that bind CD28 to costimulate T cells. Second, CD40L upregulation was evident exclusively among CD161$^{bright}$ IFN-γ-secreting cells that also expressed 4-1BB, another prominent costimulatory molecule expressed by T cells. Interestingly, an MR1-blocking mAb completely abrogated CD40L upregulation but only marginally reduced 4-1BB levels [40]. It is reasonable to assume differences between CD40L and 4-1BB expression patterns in terms of activation thresholds and/or expression kinetics and based also on MAIT cell stimulation means and modes. CD40L upregulation occurs quickly after exposure to 5-A-RU or fixed *E. coli* and reaches its peak within 6 h. By comparison, a combination of IL-12 and IL-18 induces CD40L gradually [55].

A multi-omics approach by Schubert *et al.* [90] identified CD40L as a key driver of transcription in IL-12/18-, but not in anti-CD3/CD28-stimulated human PB MAIT cells. Nonetheless, the strongest MAIT cell responses, either helpful or harmful to the host, are likely to be elicited when both pathways are engaged. In addition, MAIT cell subsets residing in different tissues may behave differently when signaled through *i*TCRs and/or cytokine receptors.

Certain cytokines other than IL-18 may also induce CD40L expression in the absence of *i*TCR stimulation. For example, IL-33, an alarmin implicated in inflammatory responses, homeostasis, and tissue repair [122], synergizes with IL-12 to make *CD40LG* transcripts detectable in human PB MAIT cells [36]. Whether IL-33 alone, or in conjunction with *i*TCR and/or other cytokine signals, promotes or improves the healing capacity of MAIT cells [26, 38, 55–57] and whether the CD40L-CD40 costimulatory axis plays a role in this context are important questions to address.

The significance of CD40L-CD40 interactions after MAIT cell priming has been documented in mice. Pankhurst *et al.* [120] recently reported that CD40L blockade prior to and after intranasal 5-A-RU/MG administration interferes with migratory DC maturation, as judged by CD86 expression in mediastinal lymph nodes. In addition, repeated co-administration of chicken ovalbumin, a model protein Ag, with 5-A-RU/MG increased total and Ag-specific Tfh cell frequencies among activated CD4$^{+}$ $T_{conv}$ cells. These responses could be efficiently ablated by a CD40L-blocking mAb [120]. Therefore, the CD40L-CD40 axis mediates MAIT cell activities in licensing DCs and in optimizing downstream Tfh cell priming (Fig. 3A and 3B). These activities are apart from MAITfh cell roles in humoral immunity [50, 51] whose dependence on CD40L–CD40 interactions remains to be established.

MAIT cells' ability to directly help B cells via CD40L-CD40 signaling has been demonstrated using FcγRIIb$^{-/-}$ *Yaa* mice, which serve as a model for systemic lupus erythematosus (SLE) [121] (Fig. 3C). In these animals, and also in patients with class III or class IV lupus nephritis, activated MAIT cells were detectable in the kidney. Moreover, MR1 deficiency ameliorated the course of lupus in FcγRIIb$^{-/-}$ *Yaa* mice, suggesting an important role for MAIT cells in this model. Finally, lipopolysaccharide (LPS)-stimulated splenic B cells from these animals produced larger quantities of total IgG, total anti-double-stranded DNA (dsDNA) autoantibodies, and anti-dsDNA IgG when MAIT cells were present in cultures. Importantly, these responses were reversed by a CD40L-blocking mAb, but not through ICOS blockade [121]. It will be interesting to adoptively transfer CD40L-sufficient and -deficient MAIT cells into *Mr1*$^{-/-}$FcγRIIb$^{-/-}$ *Yaa* mice to ask whether CD40L signaling in MAIT cells contributes to the SLE-like phenotype *in vivo*.

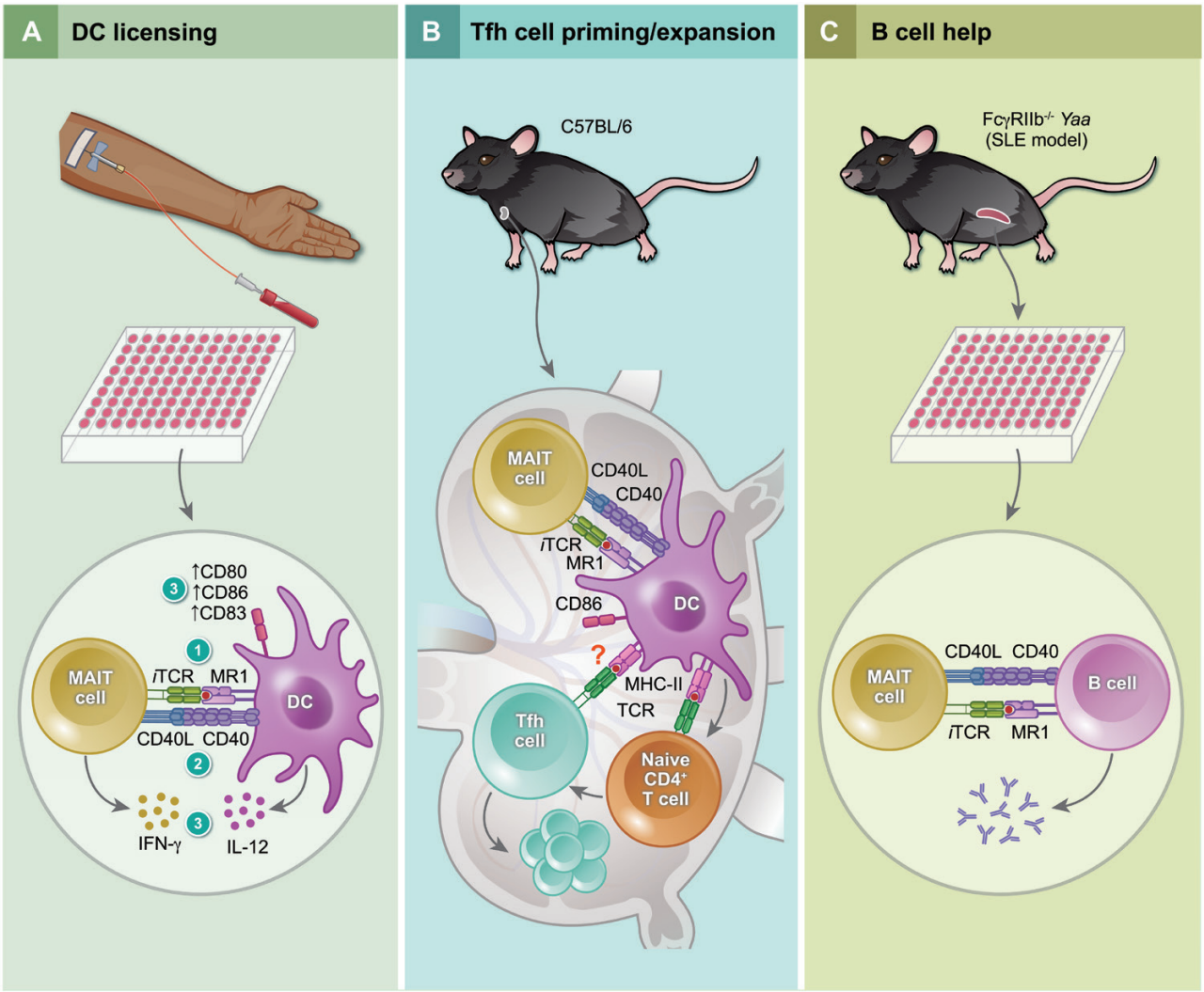


**Figure 3.** CD40L-CD40 interactions mediate MAIT cells' crosstalk with dendritic cells and B cells. (A) *In vitro* riboflavin-derived antigen presentation by MR1 (step 1) licenses dendritic cells (DCs) to activate MAIT cells, which in turn upregulate their CD40L expression to engage CD40 on DCs (step 2). This results in DC maturation, as evidenced by their CD80, CD86 and CD83 upregulation (step 3). At or around the same time, IL-12 and IFN-γ are released by DCs and MAIT cells, respectively (step 3), to promote a type-I response [40]. (B) Intranasal administration of 5-amino-6-D-ribitylaminouracil (5-A-RU)/methylglyoxal to wild-type C57BL/6 mice activates MAIT cells, which in turn promotes conventional DC maturation in mediastinal lymph nodes [120]. This leads to antigen-specific $CD4^+$ T follicular helper (Tfh) cell expansion in the lymph node directly or indirectly [120]. (C) Activated splenic MAIT cells from systemic lupus erythematosus (SLE)-prone $Fc\gamma RIIb^{-/-}$ *Yaa* mice increase autoantibody production upon *in vitro* interaction with lipopolysaccharide (LPS)-activated splenic B cells in a CD40L-CD40-dependent manner [121].

The CD40L-CD40 pathway is known to participate in the pathogenesis of SLE [123], and several clinical trials have tested the efficacy of CD40L antagonists, such as toralizumab and dapirolizumab pegol, in SLE patients [124, 125]. It will be important to determine where human $CD40L^+$ MAIT cells might fit in such clinical pictures.

## The OX40-OX40 ligand (OX40L) system

OX40 serves as a "second-wave" costimulatory molecule whose expression is induced by TCR triggering and then sustained by CD28-B7 interactions to support $T_{conv}$ cell survival, differentiation and memory, especially in the $CD4^+$ compartment [83, 126]. OX40 signaling promotes Bcl-2 and Bcl-xL expression, which bestows upon $T_{conv}$ cells a survival advantage [126]. Conversely, OX40 is considered a death receptor in mouse *i*NKT cells since its engagement activates caspase 1 and induces pyroptotic cell demise [127].

Our bioinformatic analyses of PBMC samples from four healthy donors (Fig. 1B) and hepatic $CD45^+$ cells (Fig. 2B) have demonstrated very low, if any, *TNFRSF4* levels in unfractionated *CD3D*$^+$*SLC4A10*$^+$ cells, which consist primarily of $CD8^+CD4^-$ MAIT cells. Somewhat consistent with our transcriptomic analyses, two independent studies have reported the cell surface expression of OX40 by circulating MAIT cells in some, but certainly not all, healthy individuals [92, 93].

Vorkas *et al.* [92] demonstrated selective OX40 expression by $CD4^+CD8^-$ MAIT cells in a few healthy donor PBMC samples, but not in all samples tested. Interestingly, healthy household contacts of patients with active tuberculosis (TB) from the same community had upregulated levels of OX40 on $CD4^+CD8^-$ MAIT cells, which are known to respond to *Mycobacterium tuberculosis* (*Mtb*) [128]. This is reminiscent of the OX40 expression pattern in $T_{conv}$ cell subsets after TCR stimulation.

Zhang *et al.* [93] reported elevated OX40 levels on PB MAIT cells from type 2 diabetic (T2D) patients compared with healthy controls. Furthermore, OX40$^+$ MAIT cells showed higher CD69 and Fas levels on their surface and increased IFN-γ and IL-17A production capacities when compared with OX40$^-$ cells. Finally, cleaved caspase-3 became detectable within sorted T2D MAIT cells shortly after they were exposed to recombinant OX40L and an anti-CD3 mAb. This stimulation mode also resulted in activation-induced MAIT cell death. Therefore, as with *i*NKT cells [127], OX40 signaling can promote MAIT cell death at least in certain conditions. It will be interesting to examine other T cell subsets, along with MAIT cells, for their OX40 expression and survival *versus* apoptotic potentials not only in T2D but also in other clinical scenarios that have been linked to OX40. These include rheumatoid arthritis, autoimmune colitis, autoimmune encephalomyelitis, asthma, and tissue fibrosis [129].

OX40-OX40L interactions have also been implicated in proinflammatory MAIT cell responses. In patients with *Helicobacter pylori* (*H. pylori*), gastric mucosal MAIT cells reportedly express high levels of surface OX40, with the OX40$^+$ population co-expressing more Ki-67, CD69 and CD25 [94]. This was accompanied by increased intracellular IL-9, a suspected culprit of *H. pylori*-induced gastritis [130], in MAIT cells [94]. In addition, OX40L expression was increased in gastric mucosal DCs at levels that could be positively correlated with IL-9$^+$ MAIT cell proportions. Other positive correlations were found between OX40$^+$ MAIT cell frequencies in gastric biopsies and both IL-9$^+$ MAIT cell percentages and IL-9 serum levels in patients. Finally, IL-9 was readily detectable in MAIT:DC co-culture supernatants after stimulation with recombinant OX40L and/or anti-CD3. By the same token, MAIT cell proliferation in response to *H. pylori*-primed DCs was partially inhibited by OX40- and/or OX40L-blocking mAbs [94].

How OX40-OX40L interactions modulate MAIT cell functions and those of their engagement partners is ill-defined at this point. Certain viral infections enhance the expression of OX40 by *i*NKT cells, which engages OX40L on plasmacytoid DCs to promote T1-IFN production [83, 131]. MAIT cells too respond to a wide variety of viruses and to T1-IFNs [34, 37]. However, the contribution of the OX40-OX40L signaling cascade to antiviral MAIT cell responses remains an open question.

## The 4-1BB-4-1BB ligand (4-1BBL) system

The 4-1BB-coupled signaling cascade appears dispensable in the early phase of $T_{conv}$ cell activation but plays a crucial role subsequently when several other costimulatory molecules are less available. Although 4-1BB can be expressed at comparable levels by CD4$^+$ and CD8$^+$ $T_{conv}$ cells, it preferentially drives or amplifies CD8$^+$ T cell proliferation and cytotoxicity [132].

4-1BB/*TNFRSF9* is not constitutively expressed by mouse pulmonary MAIT cells [70] or by human PB and hepatic *CD3D*$^+$*SLC4A10*$^+$ cells (Figs. 1B and 2B and Supplementary Fig. S1). However, *in vitro i*TCR stimulation with 5-A-RU/MG or fixed *E. coli* induces rapid and robust expression of 4-1BB at both mRNA and protein levels in human MAIT cells [40, 55, 98]. Interestingly, while MR1 blockade in 6-h PBMC cultures with *E. coli* completely abolished 4-1BB upregulation, it was only modestly effective in 24-h cultures [55], suggesting an important role for other signals, such as inflammatory cytokines, in maintaining 4-1BB expression at later time points. Indeed, a combination of exogenous IL-12 and IL-18 can induce 4-1BB expression by MAIT cells [55, 98]. However, this response is much weaker than that generated through *E. coli* stimulation.

The strong linkage between *i*TCR signaling and 4-1BB expression is further supported by functional RNA sequencing using pseudotime analysis that revealed select genes associated with *i*TCR and cytokine stimulation trajectories [133]. Accordingly, while *TNFRSF9* (encoding 4-1BB), *CCL3* and *CCL4* were of great importance for the *i*TCR trajectory, *IFNG* and *IL26* were specifically linked to the IL-12/IL-18 stimulation pathway. This approach provides dynamic information not easily acquired by other methods.

Lamichhane *et al.* sought to determine how viral infection alters MAIT cell responses to 5-A-RU/MG [67]. Interestingly, while adding the influenza A virus (IAV) strain A/Puerto Rico/8/34 or T1-IFNs to human PBMC stimulation cultures enhanced the expression of several activation, effector, or costimulatory molecules by MAIT cells, 4-1BB levels were slightly lowered. Therefore, 4-1BB may need to be tightly regulated during viral infections through a T1-IFN-dependent mechanism(s).

4-1BB also appears to participate in antibacterial MAIT cell responses. Upon *in vitro* stimulation with *Mtb* antigens, 4-1BB was upregulated on PB MAIT cells and more strongly on MAIT cells from the tuberculous pleural effusions of TB patients in an IL-2-dependent fashion [134]. In addition, 4-1BB$^+$ MAIT cells expressed significantly more CD25, Ki-67, IFN-γ, IL-17A and IL-17F compared with 4-1BB$^-$ MAIT cells [134]. These findings partially simulate $T_{conv}$ cell responses to *Mtb* in TB patients in whom 4-1BB expression was associated with IFN-γ production [135].

## The CD27-CD70 system

The CD27 is constitutively expressed on naïve CD4$^+$ and CD8$^+$ $T_{conv}$ cells and upregulated upon T cell activation [136]. After CD27–CD70 interactions, the extracellular domain of CD27 can be cleaved off to form a soluble, active form found in bodily fluids.

Similarly to $T_{conv}$ cells, the majority of MAIT cells express CD27 at steady state [27, 40, 70], a finding that was reproducible in our transcriptomic analyses of PB and hepatic *CD3D*$^+$*SLC4A10*$^+$ cells (Figs. 1B and 2B and Supplementary Fig. S1). Whether MAIT cells release soluble CD27 upon activation is unknown to our knowledge.

Several studies have focused on pathological conditions in which a lack of CD27 on MAIT cells is encountered. For instance, circulating CD27$^-$ MAIT cell frequencies are reportedly increased in newly diagnosed, untreated multiple myeloma [137], human immunodeficiency virus (HIV) infection [138], juvenile type 1 diabetes (T1D) [139, 140], and T2D with obesity [141]. Interestingly, CD27$^-$ PB MAIT cell frequencies were associated with disease progression in T2D since they were positively correlated with hemoglobin $A_{1c}$ levels and also negatively correlated with the homeostatic model assessment of β cell function (HOMA-β) index [141].

In healthy donors and T2D patients, CD27$^-$ MAIT cells were found to produce more IL-17A in response to PMA and ionomycin when compared to CD27$^+$ cells [139, 141]. Consistent with these observations, a higher RORγt:T-bet

ratio was evident in CD27$^-$ MAIT cells from obese/overweight but otherwise healthy adults and also in obese T2D patients [141]. Of note, CD27–CD70 interactions reportedly promote $T_H1$ differentiation and inhibit IL-17 production by CD4$^+$ $T_{conv}$ cells [142–144], and a lack of CD27 expression by CD4$^+$ $T_{conv}$ and γδ T cells confers upon them a T helper 17 ($T_H17$)-like phenotype [142, 145]. Therefore, a role for CD27 signaling in MAIT17 cell differentiation is conceivable.

Examination of the MAIT cell compartment in T2D patients has also revealed a gene signature related to antibacterial responses in circulating CD27$^+$, but not CD27$^-$, MAIT cells [141]. This was accompanied by the overt presence of *Bacteroides ovatus*, a common commensal of the human colon, in circulation, which drove the accumulation of IL-17-producing CD27$^-$ MAIT cells in the PB [141]. *Ex vivo*, T2D PBMC stimulation with heat-killed *B. ovatus* resulted in IL-17A and TNF-α by MAIT cells, which was much more pronounced in the CD27$^-$ subset [141]. Since microbiota dysbiosis can lead to IL-17 production with pathological consequences, a role for IL-17-secreting CD27$^-$ MAIT cells was speculated in T2D exacerbations [141].

## Future directions

Despite recent progress in our understanding of costimulatory pathways that operate in MAIT cells, many questions remain unanswered.

Costimulatory signals that are exchanged between MAIT cells and APCs or target cells to promote or stabilize the corresponding immunological synapses need to be deciphered. Such signals may work bidirectionally to alter the biological behaviors of both parties involved. Costimulatory molecules that regulate MAIT cell priming and effector functions are not always simultaneously available. Therefore, their spatiotemporal expression should be assessed in MAIT cells residing in different microenvironments.

The strength of costimulatory and co-inhibitory signals and their balanced (or imbalanced) expression are likely to determine MAIT cell stimulation outcomes. The classical example is CTLA-4, which is upregulated on $T_{conv}$ cells and outcompetes CD28 for access to B7 molecules [146]. This is due to the much higher avidity of CTLA-4, compared to CD28, for binding to B7, and apart from CTLA-4's inherent negative signaling properties. We have reported the selective upregulation of lymphocyte-activation gene 3 (LAG-3) and T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) on MAIT cells in the contexts of toxic shock [147] and chronic psychological stress [148, 149], respectively, with functional repercussions for antimicrobial immunity. Whether costimulation can be experimentally or therapeutically enforced to help overcome MAIT cell dysfunctions, exhaustion, or even undesired skewedness will be an attractive area of research.

Mouse models will continue to shed mechanistic light on T cell costimulation pathways, especially in clarifying which interactions work in T cell-autonomous and/or -extrinsic manners to shape a given phenotype. MAIT cells, which appear to be the most similar innate-like T lymphocytes to $T_{conv}$ cells [81], are no exception. Importantly, mouse and human MAIT cells are more phenotypically and functionally similar than previously assumed [12]. However, although MAIT cells in these species recognize and respond to similar MR1 ligands, they show drastic differences in terms of tissue abundance, homing properties, and co-receptor usage. Therefore, caution needs to be exercised in interpreting the translatability of findings from mouse models.

*i*NKT cell subsets defined by their co-receptor expression and tissue localization exhibit functional heterogeneity [150–154] and somewhat distinct costimulatory requirements [83, 155]. While CD4$^-$CD8$^-$ cells are the predominant MAIT cell subset in mice, CD4$^+$ and CD8$^+$ MAIT cells are also found in certain locations, for instance in lymph nodes [12]. Whether these subsets fulfil different or complementary functions that may potentially rely on select costimulatory signals remains to be clarified.

In response to PMA and ionomycin, which work together to bypass *i*TCR engagement, CD4$^+$ human MAIT cells were much more potent producers of IL-2 than all other MAIT cell subsets [156]. This stimulation mode also generated more IL-17A-producing MAIT cells within the human CD4$^-$CD8$^-$ subpopulation than in CD8$^+$ cells [157]. Most human PB MAIT cells express CD8αα homodimer or CD8αβ heterodimer as their co-receptors, which enhance MR1 binding and cytokine responses [32]. In addition to the functional MAIT1, MAIT17, and MAIT1/17 subsets that are commonly studied in humans, CD4$^+$ and CD8$^+$ MAIT2-like cells with a delayed capacity to produce IL-13 can be generated in long-term cultures [158] although their significance and signaling requirements need to be validated *in vivo*. Finally, there exist γδ T cells with MR1 reactivity [159, 160], whose degree of innateness and costimulatory requirements warrant future investigation.

Although typical MAIT cells express *TRAV1-2* in their TCRα chain, MR1-restricted T cell repertoires additionally harbor *TRAV1-2*$^-$ cells [161]. Within tissue-resident *TRAV1-2*$^+$ cell populations, *TRAJ33* is enriched in the spleen and liver, and *TRAJ12* and *TRAJ29*/*TRAJ36* are frequently used in the liver and jejunum, respectively [162]. This suggests distinct *TRAV1-2*$^+$ TCR specificities for cognate Ags depending on the metabolomic features of various tissues. It will be interesting to learn whether *TRAV* and *TRAJ* gene usage in MAIT and other MR1-restricted T cells influences not only the range of Ags these cells may recognize but also the costimulatory signals they may require for their activation, antimicrobial functions, and potentially for their tissue repair activities.

The tremendous antimicrobial, cytolytic and immunomodulatory activities of MAIT cells may be harnessed in vaccine design or future immunotherapies. The monomorphic nature of MR1 [5] means its ligands may be used to target MAIT cells in genetically diverse populations [163]. Using mouse models of prime-boost immunization against IAVs and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), we have recently demonstrated that repeated 5-OP-RU administration is possible without inducing MAIT cell anergy [49]. This is in stark contrast with the prototypic CD1d ligand α-galactosylceramide, which induces long-term *i*NKT cell anergy even after a single injection [164].

MR1 ligands and/or certain cytokines may be employed to expand MAIT cells in *in vivo* settings [49, 165] and in *in vitro* protocols generating many "donor-unrestricted" MAIT cells [166]. Future "off-the-shelf" cell therapies of this kind may restore, reinforce, or rejuvenate MAIT cell compartments when they are depleted [167, 168], aged [169], or functionally impaired [147–149, 170]. Using "costimulation-optimized" strategies and protocols will likely yield better outcomes or products.

The cytolytic potentials of MAIT cells can be exploited against cancer, for instance by engineering MAIT cells that express chimeric Ag receptors (CAR-MAIT cells) with a 4-1BB costimulatory domain [47]. Another potential advantage of MAIT cells in cancer immunotherapy is their resistance to certain chemotherapeutic agents by virtue of their unusually high expression of multi-drug resistance protein 1 [80, 171]. This should enable combined interventions with costimulation-optimized, drug-resistant MAIT cells with chemotherapy, when applicable.

We anticipate new and important knowledge arising from future studies in which costimulatory interactions will be interrogated, augmented when MAIT cells are protective, or interfered with when MAIT cells participate in pathological conditions.

## Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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## Ethical Approval

Not applicable.

## Conflicts of Interest

The authors declare no conflicts of interest.

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Conflict of Interest: None declared.

## Data Availability

Publicly available peripheral blood mononuclear cell datasets were obtained from 10x Genomics (https://www.10xgenomics.com/resources/datasets), and transcriptomic data from healthy liver samples were generated using Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo) under the accession code GSE48452.

## Author Contributions

N.I.W. investigation, visualisation, bioinformatic analyses, writing (initial draft). M.N. Investigation, visualisation, writing (initial draft). S.M.M.H. conceptualization, funding acquisition, supervision, investigation, visualization, editing, writing (final manuscript).

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## Clinical Trial Registration

Not Applicable.

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