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# Human liver-derived MAIT cells differ from blood MAIT cells in their metabolism and response to TCR-independent activation

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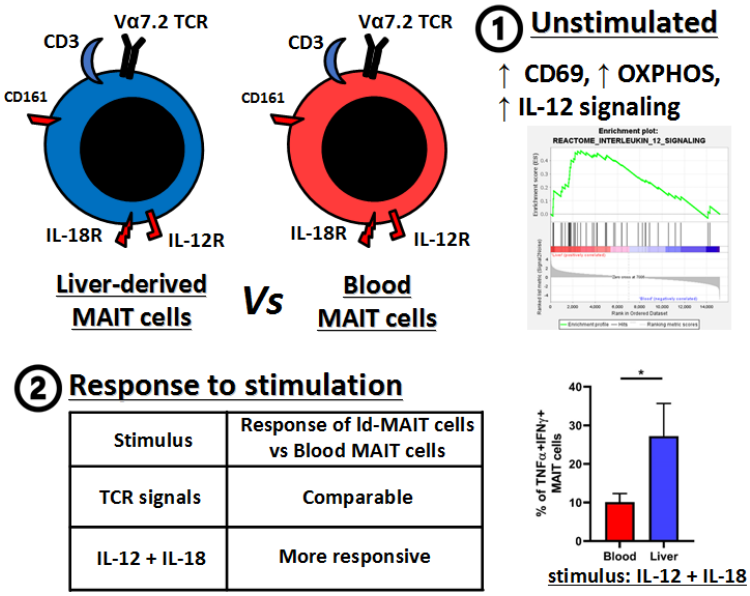
**Abstract**

Mucosal associated invariant T (MAIT) cells are anti-microbial innate-like T cells that are abundant in blood and liver. MAIT cells express a semi invariant T cell receptor (TCR) that recognises a pyrimidine ligand, derived from microbial riboflavin synthesis, bound to MR1.

Both blood and liver derived (ld)-MAIT cells can be robustly stimulated via TCR or by cytokines produced during bacterial or viral infection. In this study, we compared the functional and transcriptomic response of human blood and ld-MAIT cells to TCR signals (E. coli or the pyrimidine ligand) and cytokines (IL-12+IL-18). While the response of blood and ld-MAIT cells to TCR signals were comparable, following cytokine stimulation ld-MAIT cells were more polyfunctional than blood MAIT cells. Transcriptomic analysis demonstrated different effector programmes of ld-MAIT cells with the two modes of activation, including the enrichment of a tissue repair signature in TCR-stimulated MAIT cells. Interestingly, we observed enhancement of IL-12 signalling and fatty acid metabolism in untreated ld-MAIT cells compared with blood MAIT cells. Additionally, MAIT cells from blood and liver were modulated similarly by TCR and cytokine signals. Therefore, we report that blood and ld-MAIT cells are fundamentally different but undergo conserved changes following activation via TCR or by cytokines.

We demonstrate that liver-derived MAIT cells are more responsive to stimulation with proinflammatory cytokines than blood MAIT cells. The transcriptome of unstimulated liver-derived MAIT cells differs from blood MAIT cells, with significant differences in metabolic pathways and cytokine signaling. Together this suggests tissue specific modulation of MAIT cell function.



## Introduction

Mucosal associated invariant T (MAIT) cells are innate-like antibacterial T cells that in humans are abundant in the liver and in mucosal tissues. In contrast to conventional T cells which are activated by peptide epitopes bound to MHC I or II molecules on antigen presenting cells (APCs), MAIT cells are activated by pyrimidine ligands bound to MR1, a non-polymorphic MHC class Ib molecule [1, 2]. The pyrimidine ligands, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), are derived from 5-amino-6-D-ribitylaminouracil (5-A-RU), an intermediate metabolite of microbial riboflavin biosynthesis pathway, by condensation with methylglyoxal and glyoxal respectively [3, 4]. MAIT cells express a semi-invariant T cell receptor (TCR) which consists of Vα7.2 mostly in combination with Jα33, Jα12, or Jα20, and a variety of beta chains, with diverse Vβ usage [1, 5, 6]. Additionally, MAIT cells are activated by proinflammatory cytokines, mainly IL-12 and IL-18, produced during bacterial and viral infections [7-10].

The liver is a major site of immune surveillance against microbes crossing the gut barrier, enabling close contact between lymphocytes and the endothelial cells lining the sinusoids, as well as various antigen presenting cells; approximately 80% of the reticuloendothelial system is present in the liver [11]. In addition to parenchymal hepatocytes (which comprise two thirds of cells in the liver), the liver contains many non-parenchymal cells such as Kupffer cells, biliary and sinusoidal epithelial cells, stellate cells, and intrahepatic lymphocytes, including MAIT cells [11, 12]. MAIT cells are highly enriched in liver compared to any other human tissues, comprising on average 15% of total $CD3^+$ T cells in the liver [12-14]. In the liver, MAIT cells are believed to reside in the sinusoids as well as the peri-biliary areas of portal tracts close to bile ducts [14-16]. Like their peripheral blood counterparts, liver-derived (ld-) MAIT cells can be activated via both their TCR and by cytokines [16-18]. Activation of MAIT cells, including ld-MAIT cells, leads to the production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), as well as cytotoxic molecules [16, 18, 19]. However, the full profile of effector functions of ld-MAIT cells in response to TCR or cytokine signals remains yet to be fully defined.

Given the enrichment of MAIT cells in the liver and the presence of APCs, including Kupffer cells and biliary epithelial cells, capable of presenting pyrimidine ligands on MR1 [16, 20], MAIT cells may have an important role in regulating liver pathophysiology. Recently, we and others showed that human circulating MAIT cells express distinct transcriptional and functional profiles when activated by TCR signals or cytokines [21-23]. However, it not

known whether the responses of ld-MAIT cells are comparable to those of circulating MAIT cells. Therefore, we compared the transcriptional response and effector functions of blood and ld-MAIT cells in response to stimulation via their TCR (*E. coli* or 5-A-RU/MG) or with cytokines (IL-12+IL-18).

## Results

### MAIT cell are enriched in liver

First, we compared the frequency of MAIT cells in patient matched blood and liver; MAIT cells were identified as $CD3^+$ T cells expressing the semi-invariant Vα7.2 TCR and CD161 [12, 24]. As previously reported [12, 14], MAIT cells were more abundant in liver, accounting for 10.8% of total T cells and 16% of $CD8^+$ T cells in liver in comparison to 1.45% and 4.1% of respective cell types in blood (Figure 1A). Consistent with previous reports [16, 25], MAIT cells were predominantly $CD8^+$ followed by $CD8^-CD4^-$ subsets in both blood and liver (Figure 1B).

### IL-12+IL-18-stimulation increases ld-MAIT cell activation more than blood MAIT cells

To test whether ld-MAIT cells can be robustly stimulated by TCR and cytokines *in vitro*, we treated mononuclear cells from patient-matched blood and liver with *E. coli* or 5-A-RU/MG for 6 hours or a combination of IL-12 and IL-18 for 24 hours. Ld-MAIT cells, compared to their blood counterparts, expressed significantly more CD69 on their surface at rest (Figure 1C and D) which is in line with a previous study [14]. MAIT cells from both blood and liver increased CD69 expression on their surface following activation by *E. coli* compared to untreated control; higher CD69 expression was observed with 5-A-RU/MG, albeit at lower levels compared to *E. coli*, however, it was not statistically significant (Figure 1C). Stimulation with IL-12+IL-18 also enhanced CD69 expression on blood and ld-MAIT cells, however, the level of expression was significantly greater on ld-MAIT cells (Figure 1D). Expression of 4-1BB, which was absent on the surface of untreated blood MAIT cells but expressed at low levels by ld-MAIT cells, was significantly upregulated upon activation by TCR signals (Figure 1E). Cytokine treatment also caused 4-1BB expression on blood and ld-MAIT cells, however, significantly more ld-MAITs expressed 4-1BB compared to blood MAIT cells following IL-12+IL-18 activation (Figure 1F). Both TCR and cytokine stimulation resulted in a similar increase in the expression of CD40L on blood and liver MAIT cells (Figure 1G and H).

Next, we compared the ability of blood and ld-MAIT cells to produce cytokines following TCR and cytokine triggering. Interestingly, the percentage of blood and ld-MAIT cells producing

TNFα or IFNγ following activation by *E. coli* or 5-A-RU/MG were comparable (Figure 2A and C); greater production of TNFα and IFNγ was observed in response to 5-A-RU/MG than to *E. coli*. For both blood and ld-MAIT cells, stimulation with either *E. coli* or 5-A-RU/MG resulted in mostly TNFα mono-producers and TNFα-IFNγ double producers, with very few IFNγ mono-producers (Figure 2E). In contrast, most blood MAIT cells activated with IL-12+IL-18 produced only IFNγ, while a significant minority of ld-MAIT cells produced both TNFα and IFNγ (Figure 2B, D, and E and S. Figure 2). Therefore, ld-MAIT cells are more responsive to cytokine stimulation than their blood counterparts, with a polyfunctional response.

Expression of cytokine genes was also assessed in TCR or IL-12+IL-18-stimulated ld-MAIT cells; high expression of MAIT cell TCR (Vα7.2-Jα33/12/20) in the sorted cells confirmed them being MAIT cells (S. Figure 3A). Consistent with the changes seen by flow cytometry, higher expression of *TNF* was seen with early TCR activation, while more *IFNG* expression was seen with IL-12+IL-18 stimulation (S. Figure 3B and C). Increased *IL17A* and *IL22* expression was observed following TCR stimulation whereas a lesser upregulation in *IL22* expression was evident upon cytokine activation (S. Figure 3D and E). Taken together, TCR activation leads to expression of both T1 and T17 cytokines by ld-MAIT cells, while the response to IL-12+IL18 is more T1-focussed.

We further compared the average expression of CD69, 4-1BB and cytokines (TNFα and IFNγ) on unstimulated blood and ld-MAIT cells at 6 and 24 hours to assess if there was any non-specific activation following incubation *in vitro*. No significant increase in expression of these proteins was seen, confirming that MAIT cell activation was in response to the externally added stimuli (S. Figure 4).

### Similar changes in cytotoxic molecule expression in blood and ld-MAIT cells upon activation

Following activation, MAIT cells degranulate and modify their expression of cytotoxic molecules [18, 19, 22]. Next, we assessed the expression of cytotoxic molecules (granzymes A, granzyme B, and perforin) by blood and ld-MAIT cells and the proportion of cells undergoing degranulation (CD107a) following activation with *E. coli,* 5-A-RU/MG, or IL-12+IL-18. In both blood and ld-MAIT cells, stimulation with *E. coli* or 5-A-RU/MG resulted in increased granzyme B expression but reduced granzyme A expression; the extent of modulation of granzyme A was greater with 5-A-RU/MG than with *E. coli* (Figure 3A, B, D, and E). In both blood and ld-MAIT cells, IL-12+IL-18 stimulation significantly increased granzyme B production but did not reduce granzyme A expression (Figure 3A, C, D, and F). A reduction in the proportion of blood and ld-MAIT cells expressing perforin was seen

following stimulation with 5-A-RU/MG but not *E. coli* (Figure 3G and H). In contrast, stimulation with IL-12+IL-18 increased the proportion of both blood and ld-MAIT cells producing perforin (Figure 3G and I). Equivalent CD107a expression was seen on blood and ld-MAIT cells following TCR or cytokine stimulation, suggesting similar levels of degranulation (Figure 3J-L). In summary, blood and ld-MAIT cells rapidly degranulate and express comparable cytotoxic granule contents upon stimulation by TCR or cytokine signals.

### TCR and cytokine stimulated ld-MAIT cells have distinct transcriptional profile

To further characterize the effect of TCR or cytokine stimulation on ld-MAIT cells, we generated transcriptomic profiles of ld-MAIT cells following stimulation with either *E. coli,* 5-A-RU/MG, or IL-12+IL-18. Differentially expressed genes (DEGs) were determined by comparison with untreated ld-MAIT cells, with fold change >2 and adjusted p-value <0.05 used as cut-offs. More DEGs were obtained following TCR triggering (907 for *E. coli* and 561 for 5-A-RU/MG) than with cytokines (262) (Figure 4A and Supplementary Dataset 1). Interestingly, 71 of 80 (89%) DEGs shared among all three treatments were upregulated (Figure 4A). Also, IL-12+IL-18 stimulated ld-MAIT cells shared more DEGs with *E. coli* stimulated ld-MAIT cells than 5-A-RU/MG stimulated ld-MAIT cells. The differences in TCR and cytokine stimulation of ld-MAIT cells were also evident by multidimensional scaling analysis; *E. coli* and 5-A-RU/MG stimulated ld-MAIT cells clustered away from the unstimulated and IL-12+IL-18 activated ld-MAIT cells (Figure 4B). The differences in response to different stimuli were also evident in the heat-map of the normalized expressions of total DEGs (1193) with all three treatments (Figure 4C). Taken together, TCR and cytokine stimulated ld-MAIT cells express distinct transcriptomic profiles.

Next, we compared the expression of genes encoding effector molecules (cytokines, chemokines, and cytotoxic molecules) by ld-MAIT cells following activation (S. Figure 5A). Ld-MAIT cells activated by 5-A-RU/MG or *E. coli* highly expressed *TNF*, *IL17A*, and *IL22*, consistent with our previous observations (Figure 2 and S. Figure 1B-E). Increased expression of multiple other cytokines (including *CSF2*, *IL17F*, and *IL2*) and chemokines (including *CCL3*, *CCL4*, *CCL3L3*, and *CCL20*) was also observed following stimulation with *E. coli* or 5-A-RU/MG, confirming that TCR stimulation leads to a strong inflammatory response by ld-MAIT cells. *IFNG*, *CSF1*, *GZMA*, *IL*32, and *IL26* were markedly upregulated by IL-12+IL-18 triggering. Expression of *IL10* was restricted to *E. coli* and IL-12+IL-18 stimulation whereas increased expression of *CD40LG*, *FASLG*, *GZMH,* and *LTA* was evident in response to all stimuli. Transcription of chemokine receptors also changed following activation: while *CCR2* and *CCR6* expression were reduced in TCR and cytokine

activated ld-MAIT cells, expression of *CXCR6* was increased with IL-12+IL-18 but reduced following TCR stimulation by both *E. coli* and 5-A-RU/MG (S. Figure 5A).

Recent studies on peripheral blood MAIT cells suggest that TCR activation is required for the induction of tissue repair function [21-23]. GSEA analysis confirmed that tissue repair genes were specifically enriched in ld-MAIT cells following TCR mediated activation (Figure 4D). Stronger enrichment was observed with 5-A-RU/MG (normalized enrichment score (NES) 2.13 and nominal p-value <0.001) than with *E. coli* (NES 1.77 and p-value 0.007). Some of the commonly enriched genes from the tissue repair gene list in ld-MAIT cells activated by *E. coli* or 5-A-RU/MG were *CCL3*, *CSF1*, *CSF2*, *TNF*, *EGF2*, *FGF2*, *HBEGF*, *AREG*, *PDGFA* and *VEGFA* (S. Table 2). Interestingly, some of these common tissue repair associated genes enriched with *E. coli* and 5-A-RU/MG stimulation (*HBEGF*, *TNF*, *CSF1*, *CSF2*, *CCL3*, *FGF2* and *VEGFA*) were also upregulated following IL-12+IL-18 stimulation, however, the overall enrichment of the tissue repair signature following IL-12+IL-18 stimulation was not significant (NES 1.21 and p-value 0.101) (Figure 4D and S. Table 2), consistent with our observations with blood MAIT cells [22].

We recently demonstrated that genes involved in type I interferon (IFNs) signaling are enriched in blood MAIT cells following bacterial stimulation and that type I IFNs are important for the activation and effector response of MAIT cells [22, 26]. Therefore, we compared the transcriptome of *E. coli* and 5-A-RU/MG activated ld-MAIT cells. Interestingly, GSEA analysis revealed genes associated with IFN signaling, especially interferon alpha beta signaling, were highly enriched in ld-MAIT cells following *E. coli* activation compared to activation by 5-A-RU/MG (S. Figure 5B). Consistent with blood MAIT cells, ld-MAIT cells also expressed high levels of type I interferon α and β receptor subunit 1 (*IFNAR1*) and subunit 2 (*IFNAR2*), equivalent to the expression of IL-18 receptor (*IL18R1* and *IL18RAP*) (S. Figure 5B).

Expression of certain transcription factors has been associated with specific effector functions following activation [22, 25, 27, 28]. Therefore, to explain the differences in the expression of effector molecules following TCR and cytokine stimulation, we compared gene expression of transcription factors among the DEGs with the different treatments (S. Figure 5C). Expression of *RORC* (encodes RORγt), which controls the expression of *IL17*, was increased in MAIT cells activated via their TCR but was unchanged with IL-12+IL-18 stimulation. Consistent with this, *IL17A* expression was only increased following TCR stimulation but not by cytokine stimulation (S. Figure 3D and S. Figure 5A). Expression of *IKZF2* (Helios) and *TBX21* (T-bet) were also upregulated upon TCR stimulation, whereas

expression of *EOMES* was downregulated. Interestingly, expression of *PRDM1* (Blimp1) was increased with *E. coli* and IL-12+IL-18 whereas 5-A-RU/MG had no effect. This differential expression of transcription factors with the two modes of activation was consistent with our previous study on blood MAIT cells [22]. Also, histone demethylase KDM6B expression, which we recently showed to be important for MAIT cell activation [22] was specifically increased by TCR stimulation. Finally, we confirmed by flow cytometry that ld-MAIT cells express high levels of RORγt and PLZF compared to other T cell subsets which was also observed for blood MAIT cells [22, 29, 30] (S. Figure 5D).

### Comparison of the transcriptome of blood and liver MAIT cells

To assess how different ld-MAIT cells are from the blood MAIT cells, we compared the transcriptome of untreated ld-MAIT cells ($CD3^+$) from this study and untreated blood MAIT cells ($CD3^+CD8^+$) from our recent study [22]. DEGs were determined by comparing ld-MAIT cells with blood MAIT cells, with fold change >2 and adjusted p-value <0.05 used as cut-offs. A total of 1883 genes were differentially expressed, of which 244 were upregulated and 1639 were downregulated (Figure 5A and Supplementary Dataset 2). The list of both upregulated and downregulated DEGs were analysed in Enrichr to get an overview of the differences; the top 10 hits for Reactome pathway analysis are shown in S. Figure 6A. Interestingly, most downregulated pathways were related to signal transduction, especially via G-protein coupled receptors. Some of the top downregulated pathways identified by Enrichr using downregulated DEGs were also identified by GSEA pathway analysis when all gene expression data was considered, with heavy clustering of genes associated with GPCR signaling enriched in blood MAIT cells (S. Figure 6A and B). In contrary, the top 10 upregulated pathways based on upregulated DEGs suggested enhanced cholesterol biosynthesis, and interferon gamma signaling in ld-MAIT cells (S. Figure 6A). Both Reactome and KEGG pathway analysis by GSEA highlighted enrichment of genes associated with biosynthesis of cholesterol, fatty acid β-oxidation/metabolism (including fatty acyl-CoA biosynthesis and synthesis of very long chain fatty acyl CoAs), and oxidative phosphorylation (OXPHOS) in ld-MAIT cells (Figure 5B, S. Figure 6C and data not shown). In contrary, the glycolysis+gluconeogenesis pathway was comparatively but not significantly enriched in blood MAIT cells (Figure 5B and S. Figure 6C). These results suggest fundamental differences between blood and ld-MAIT cells in their signal transducing ability and cellular metabolism. GSEA analysis further revealed upregulation of IL-12 signalling in ld-MAIT cells over blood MAIT cells (Figure 5C). Heatmap analysis of genes in the IL-12 signaling pathway (Reactome) highlighted enhanced receptor expression (*IL12RB1* and *IL12RB2)* and JAK-STAT signaling (*JAK1*, *JAK2*, and *STAT4*) required for signal

transduction [31] (Figure 5D). Additionally, the IFNγ signaling pathway, which has the potential to induce IL-12R expression [32], was also enriched in ld-MAIT cells (Figure 5E) .

Despite these differences, there was no significant changes in their overall circulatory and tissue residency signature of blood and ld-MAIT cells although tissue specific clustering of MAIT cells was seen in the heat-map analysis (S. Figure 7). Furthermore, changes in the transcriptome of blood and ld-MAIT cells following stimulation were similar (S. Figure 8). Overall, a clear separation of MAIT cells based on response to TCR and cytokine stimulation was observed irrespective of the tissue origin, which suggested conserved changes in circulating and ld-MAIT cell transcriptomes following activation. Nevertheless, tissue specific changes were evident in the heatmap, with MAIT cells clustered by tissue of origin within treatments (S. Figure 8).

## Discussion

MAIT cells are highly abundant in liver, reaching up to 45% of the total hepatic lymphocytes [12, 13]. While the detailed transcriptomic response of blood MAIT cells to TCR and cytokine-mediated activation has recently been defined [21-23], the response of ld-MAIT cells to different modes of activation is yet to be examined. In this study, we assessed the functional and transcriptomic response of resting and activated MAIT cells from blood and liver. Ld-MAIT cells were found to be more responsive to stimulation with IL-12+IL-18, which may be relevant in infectious and inflammatory liver diseases. We further demonstrated fundamental differences in the MAIT cell transcriptome from blood and liver, suggesting tissue specific shaping of MAIT cells in humans.

Consistent with previous studies, MAIT cells expressed more CD69 on the surface and were more abundant in liver compared to blood [12, 14, 33]. Interestingly, the response of circulating and liver derived MAIT cells to TCR signals were comparable. One of the reasons could be the comparable TCR diversity of MAIT cells in both compartments [33, 34]. Also, the proportion of antigen presenting cells, mainly monocytes and dendritic cells, present in blood and liver are comparable which may further reflect similarities in the TCR response of blood and ld-MAIT cells [13]. In contrast, the response of ld-MAIT cells to cytokine stimulation (IL-12+IL-18) was more polyfunctional and a greater proportion of ld-MAIT cells produced cytokines compared to blood MAIT cells. A similar trend was seen on cytotoxic granule contents, especially granzyme B and perforin. Transcriptomic analysis demonstrated pre-enhancement of the IL-12 signaling pathway in ld-MAIT cells, including increased expression of IL-12 receptor subunits and the JAK-STAT pathway. Moreover, ld-MAIT cells

showed enhanced IFNγ signaling suggesting heightened sensitivity of ld-MAIT cells to IFNγ, which has the potential to act on T cells in both an autocrine and paracrine fashion and can induce IL-12R expression [32, 35, 36]. Consistently, *IFNG* was found to be one of the highly DEGs in ld-MAIT cells compared to blood MAIT cells; expression of most cytokine receptors on blood and ld-MAIT cells was remarkably similar, however *IL-12R* expression was not assessed [14]. The enhanced cytokine signaling and response could reflect the constant exposure of ld-MAIT cells to bacterial and viral contaminants draining in the portal circulation leading to the arming of ld-MAIT cells, which was also suggested by higher CD69 surface expression than on their blood counterparts [37]. This hyperresponsiveness of ld-MAIT cells to cytokine stimulation suggests a possible role of ld-MAITs in inflammatory liver pathologies.

Our study differs from previous studies in that we assessed TCR-mediated ld-MAIT cell activation early (6 hours) post-stimulation, rather than at 20-24 hours [13, 14]. The early (5-6 hours) response of blood MAIT cells to bacterial stimulation has been shown to be solely via TCR, while later (18-24 hours) they are activated via both TCR and cytokines [9, 22, 38]. The cytokine profile of MAIT cells early and late after bacterial stimulation differs [17, 22]. In most earlier studies on ld-MAIT cells, activation was assessed at 18-24 hours or after overnight incubation post bacterial stimulation [13, 14, 16]. While assessing the late response of MAIT cells to bacterial stimulation has the advantage of robust MAIT cell activation via both TCR dependent and independent signals, it may miss TCR specific features of the MAIT cell response which peak early, such as TNFα production [22]. Focusing on earlier events following activation of ld-MAIT cells by bacteria or 5-A-RU/MG in this study, we showed that MAIT cells attain a strong inflammatory phenotype, with high expression of TNFα (also *TNF*) and *IL17A* and low expression of IFNγ (also *IFNG*), in line with previous studies on blood MAIT cells [22, 39]. TCR stimulated ld-MAIT cells also strongly expressed chemokines such as *CCL3*, *CCL4*, and *CCL20*. In contrast, cytokine stimulation triggered strong production of IFNγ and less TNFα production at a later timepoint (24 hours). Therefore, early TCR activation leads to a polyfunctional inflammatory response by ld-MAIT cells, while cytokine-mediated activation is narrower.

In addition to an effector memory phenotype ($CD45RA^{-}CD45RO^{+}CD95^{hi}CD62L^{lo}$) and elevated CD69 expression, tissue resident MAIT cells display higher expression of certain chemokine receptors, such as CCR6 and CXCR6, that helps them to home to tissues [12, 16]. In our study, expression of both *CCR6* and *CXCR6* by ld-MAIT cells was reduced following TCR activation. Downregulation of *CCR6* but upregulation of *CXCR6* was evident upon cytokine stimulation. Additionally, expression of *CCR2* was found to be reduced in both

TCR and cytokine stimulated ld-MAIT cells. Interestingly, CCR2 and CCR6 were recently demonstrated to be important for different steps (arrest and trans-endothelial migration) of tissue-trafficking of MAIT cells in response to inflammation [40]. While the functional significance of activation induced alterations in chemokine receptor expression is uncertain, it could potentially suggest reduced tissue retention of liver-resident and liver-infiltrated MAIT cells following activation [41, 42]. Lee et al. also demonstrated diminished CCR6 expression on MAIT cells following siRNA mediated known-down of CCAAT/enhancer binding protein delta (CEBPδ) [40]. Consistent with this, we found that the expression of *CEBPD* (which encodes CEBPδ) was downregulated in ld-MAIT cells after TCR stimulation which might have contributed to the reduced expression of *CCR6.*

We demonstrated the enrichment of a tissue repair signature in TCR stimulated ld-MAIT cells. Similar enrichment of tissue repair genes in human blood MAIT cells following TCR stimulation ± cytokine stimulation, and in murine MAIT cells post bacterial challenge has recently been demonstrated [21-23]. Consistent with these reports, the tissue repair signature was not significantly enriched by cytokine stimulation alone [21-23]. Two recent publications strongly suggest the possibility of 5-OP-RU, generated by gut microbes, driving tissue repair function *in vivo* in various different tissues [43, 44]. This suggest that MAIT cells in the liver could be maintaining tissue homeostasis in response to MR1-bound ligand continuously derived from commensal bacteria. Interestingly, the role of MAIT cells during liver inflammation was recently highlighted in $MR1^{-/-}$ mice (lacking MAIT cells) that suffered from severe liver steatosis and inflammation compared to wild type mice [20]. This highlights the importance of MAIT cells in controlling inflammatory liver diseases *in vivo*. In contrary to this hypothesis, Hedge et al and Bottcher et al separately proposed a pathogenic role of MAIT cells which was mediated by the activation and proliferation of hepatic stellate cells (HSCs) and myofibroblasts that are central to hepatic fibrosis [15, 45]. TCR activation of MAIT cells and cell-to-cell contact was shown to be essential to trigger proliferation of HSCs and myofibroblasts, while their activation was dependent on production of TNFα and IL-17 by MAIT cells [15, 45]. In our study, we found that both TNFα and IL-17 were highly expressed by MAIT cells stimulated by *E. coli* or 5-A-RU/MG but not by cytokines. Given that repeated IL-12 stimulation can also trigger IL-17 production by MAIT cells *in vitro*, more *in vivo* studies are warranted to determine the precise role of MAIT cells and their mode of activation in liver pathologies [15].

A striking difference between blood and ld-MAIT cells was observed in their metabolic programming. Normally naïve and memory T cells maintain a low glycolysis rate and utilize the energy efficient oxidative phosphorylation (OXPHOS) pathway (OXPHOS > glycolysis)

whereas a shift to glycolysis is seen in effector T cells (glycolysis > OXPHOS) [46]. The observation of increased fatty acid metabolism and OXPHOS and relatively lower glycolysis in ld-MAIT cells compared to blood MAIT cells is consistent with previous observations of increased use of lipid as a fuel source in tissue resident memory T cells which favors long-term survival and renders T cells more capable of undergoing metabolic reprogramming later [46-48]. Interestingly, IL-12 signaling, which is enriched in ld-MAIT cells, might also increase the mitochondrial potential, and reduce the dependence on glycolysis [49]. Overall, our observation of enhanced fatty acid oxidation and OXPHOS in ld-MAIT cells was in line with previous observation with tissue resident T cells [50]. Enhanced cholesterol biosynthesis was also observed in ld-MAIT cells and could be important for maintaining cell membrane stiffness for cell-to-cell membrane interaction/tissue surveillance, proliferation, and could potentially fine-tune receptor signaling by modulating different conformational states of membrane receptors [51].

The transcriptional response of ld-MAIT cells stimulated by TCR stimuli or by cytokines were similar to those of blood MAIT cells, which we recently reported [22]. In both studies, *E. coli* or 5-A-RU ± MG stimulated MAIT cells showed considerable overlap in their transcriptome and clustered separately from unactivated and cytokine activated MAIT cells. Differential cytokine production (TNFα vs IFNγ) and cytotoxic granule content (granzyme A vs granzyme B) in blood MAIT cells and ld-MAIT cells upon stimulation by TCR and cytokines were also evident in the transcriptional response. Consistent with this, modulation of various transcription factors and histone demethylase KDM6B expression in ld-MAIT cells with different modes of activation, were also similar to blood MAIT cells [22]. The TCR-dependent enrichment of tissue repair genes and bacterial regulation of type I interferon signalling, which were reported following studies on blood MAIT cells, were also evident in ld-MAIT cells [22, 26]. Although the transcriptome of blood and ld-MAIT cells were similarly modulated upon activation via TCR and cytokine signals, tissue specific differences were evident in the heat-map analysis of genes associated with a tissue residency or circulatory signature, as well as in the combined transcriptomic analysis, reflecting some basic differences in unstimulated blood and ld-MAIT cells. This is consistent with the recent single cell sequencing study by Zheng et al which showed sub-grouping of blood and ld-MAIT cells within the main MAIT cell cluster [52].

Some of the differences observed between untreated blood and ld-MAIT cells could be due to the different subsets of MAIT cells used in the RNA sequencing studies ($CD3^+CD8^+$ MAIT cells from blood versus $CD3^+$ MAIT cells from liver). While the data is likely to reflect the transcriptional signature of the majority $CD8^+$ population (~80 % of ld-MAIT cells, Figure 1B),

some differences may be due to the presence of minority $CD4^-CD8^-$ (~17 %), $CD4^+CD8^-$ (1 %), and $CD4^+CD8^+$ (2 %) populations. $CD4^-CD8^-$ MAIT cells were recently shown to be functionally distinct but developmentally related to $CD8^+$ MAIT cells [27]. However, our observation of enhanced IL-12 signaling in ld-MAIT cells compared to blood MAIT cells is very unlikely to reflect the presence of minority MAIT cell populations, as in blood enhanced IL-12 signaling has been reported in $CD8^+$ MAIT cells compared to $CD4^-CD8^-$ MAIT cells [27]. Also, it is unlikely due to the contamination with non-MAIT cells as the sorted cells expressed very high levels of MAIT cell TCR.

A recent comparison of blood and thoracic duct lymph infiltrating MAIT cells, demonstrated equivalent TCR diversity despite basic differences in their phenotype, suggesting recirculation between blood and tissues [53]. Similarly, equivalent TCR diversity was observed when MAIT cells from blood and liver perfusate were compared [33]. Furthermore, the observation of comparable responses to TCR stimulation between blood and ld-MAIT cells in our study would also be consistent with re-circulation of MAIT cells between blood and liver. In contrast, Salou et al reported that human ld-MAIT cells express a tissue residency signature, arguing against recirculation [54]. While our study found no enrichment of a tissue residency signature in ld-MAIT cells on GSEA, despite using the same circulatory and tissue-residency gene lists [55], we observed tissue specific clustering of MAIT cells in the heat-map analyses of genes associated with a tissue residency/circulatory signature and of the combined transcriptomic analysis.

In summary, MAIT cells are enriched in liver and undergo rapid changes following TCR or cytokine stimulation, similar to blood MAIT cells. Ld-MAIT cells were more responsive to cytokine-mediated activation whereas the response of blood and ld-MAIT cells to TCR stimulation was comparable. The increased responsiveness towards cytokine stimulation and enhanced cytokine signaling in ld-MAIT cells accompanied by enrichment of pathways associated with fatty acid metabolism and oxidative phosphorylation compared to blood MAIT cells, suggests that the hepatic environment may modulate MAIT cell function.

## Materials and Methods

### Blood and liver mononuclear cells isolation

Patients undergoing liver resection for cancer metastasis at Dunedin Hospital were recruited; patient information is shown in S. Table 1. Collection of liver and blood from patients undergoing hepatic resection was approved by the New Zealand Health and Disability Ethics

Committee (16/STH/83) and collection of blood from healthy donors was approved by the University of Otago Human Ethics Committee (H14/046). Written informed consent was obtained from each donor. A piece of normal liver tissue as far away from the tumour as possible (usually >10 mm) was excised fresh from the main surgical specimen. Mononuclear cells were isolated from liver as previously described [26]. Peripheral blood mononuclear cells (PBMCs) were isolated from blood (taken on the same day prior to liver resection) by Lymphoprep density gradient centrifugation, as previously described [22]. Isolated cells were rested overnight in RPMI 1640 pre-supplemented with L-glutamine (Life Technologies Corporation, NY, USA) with 10 % fetal calf serum (Gibco, New Zealand) and Penicillin-Streptomycin (Sigma-Aldrich) (now onwards referred as R10) and stimulated the next day.

### Preparation of bacterial stocks and 5-A-RU/MG

An overnight culture of *Escherichia coli* HB101 in Luria-Bertani (LB) broth was washed with phosphate buffered saline (PBS) (Oxoid, England), then fixed with 2% paraformaldehyde for 20 mins at 4 °C. Fixed bacteria were washed three times with PBS and counted using 123 count eBeads (Invitrogen, Carlsbad, USA) using a FACSCanto II (BD biosciences, San Jose, CA, USA).

5-amino-6-D-ribitylaminouracil (5-A-RU) was synthesized as previously described [22]. Immediately prior to use, 5-A-RU was mixed with methylglyoxal (MG) (Sigma) at a molar ratio of 1:50 (referred to as 5-A-RU/MG) and diluted with milliQ water to the desired concentration.

### In vitro activation of MAIT cells from liver and blood

A total of 0.2-1x$10^6$ liver derived mononuclear cells (LDMCs) or PBMCs were treated with fixed *E. coli* (25 bacteria per cell (BpC)) or 5-A-RU/MG (100 nM) for 6 hours to stimulate MAIT cells via their TCR, or the combination of 50 ng/mL IL-12 (Miltenyi Biotec, Bergisch Gladbach, Germany) and 50 ng/mL IL-18 (R & D Systems, Minneapolis, USA) for 24 hours to activate MAIT cells by cytokines alone; equal numbers of LDMCs or PBMCs from a donor were used for each treatment. Brefeldin A (10 µg/mL) (BioLegend, San Diego, USA) was added for the final four hours of activation in all experiments where intracellular production of effector molecules (TNFα, IFNγ, granzyme A, granzyme B, and perforin) was measured. Anti-CD107a PE antibody was added at the start of the experiment to capture CD107a trafficked to the surface.

### Antibodies and flow cytometry

Antibodies used were anti-CD3 PE-Cy7 (UCHT1, BioLegend) or BV510 (OKT3, BioLegend), anti-CD8 eFluor450 (RPA-T8, eBioscience), anti-TCR Vα7.2 PE or PE-Cy7 or AF700 (3C10,

BioLegend), anti-CD161 APC (191B8, Miltenyi Biotech) or BV605 (HP-3G10, BioLegend), anti-TNFα FITC (Mab11, BioLegend), anti-IFNγ PerCP-Cy5.5 (4S.B, BioLegend), anti-CD107a PE (H4A3, BioLegend), anti-granzyme A PE (CB9, BioLegend), anti-granzyme B FITC (QA16AO2, BioLegend), anti-perforin PerCP-Cy5.5 (B-D48, BioLegend), anti-CD40L FITC (24-31, BioLegend), CD69 FITC (FN50, BioLegend), anti-4-1BB PE (4B4-1, BioLegend), anti-RORγt PE (Q21-559, BD Biosciences, San Jose, CA, USA), anti-PLZF AF647 (R17-809, BD Biosciences), anti-T-bet PECy7 (4B10, BioLegend), anti-EOMES eFluor660 (WD1928, eBiosciences), and anti-Blimp1 PE-CF594 (6D3, BD Biosciences).

All samples were stained with live/dead fixable near IR (Invitrogen) to exclude dead cells and acquired on a BD FACSCantoII, a BD LSRFortessa, or, for sorting, a BD FACSAriaII (all BD Biosciences). Flow cytometric analysis was performed in FlowJo$^{TM}$ V10 (TreeStar, Ashland, OR, USA). Ld-MAITs activated by *E. coli,* 5-A-RU/MG, or by IL-12+IL-18, or unstimulated were flow sorted from LDMCs as $CD3^{+}CD161^{++}Vα7.2^{+}$ cells by FACSAriaII (gating strategy is shown in Figure 1A and S. Figure 1). MAIT cells from blood were isolated as $CD8^{+}CD161^{++}Vα7.2^{+}$ cells as previously described [22].

### Real time RT-PCR

RNA was isolated from the sorted cells using Nucleospin RNA isolation kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol and first strand cDNA was synthesized using the Superscript IV (SSIV) system (Invitrogen). Briefly, Oligo-$d(T)_{20}$ primers were annealed to template RNA at 65 °C for 5 mins followed by 1 min incubation on ice and mixed with reverse transcription (RT) mix (SSIV reverse transcriptase, SSIV buffer, DTT, ribonuclease inhibitor). This was followed by incubation at 55 °C for 10 mins and 80 °C for 10 mins, before the addition of RNase H and incubation at 37 °C for 20 mins. To assess the expression of the MAIT cell TCR (Vα7.2-Jα33/12/20) and β2-microglobulin, KAPA PROBE FAST mastermix (KAPA Biosystems) was used. To assess the expression of cytokines (TNFα, IFNγ, IL-17A and IL-22) and GAPDH, KAPA SYBR FAST mastermix (KAPA Biosystems) was used. Primers used for MAIT cell TCR and cytokines assessment were from Kurioka et al. [25] and Lamichhane et al. [22] respectively. Real time RT-PCR was performed on a Viia7 or QS6 Real-Time system (Applied Biosystems, Foster City, USA) with a PCR program of 95 °C for 3 mins followed by 40 cycles of 95 °C for 3 secs and 60 °C (MAIT cell TCR and β2-microglobulin assays) or 61 °C (cytokines and GAPDH assays) for 20 secs; for the assessment of cytokine gene expression and GAPDH melt-curve analysis was performed to confirm the specificity of the product. Relative expression of MAIT cell TCR and β2-microglobulin or cytokines and GAPDH were measured by the comparative CT method ($2^{\Delta Ct}$) [56].

### RNA Sequencing of MAIT cells and data analysis

LDMCs were treated with *E. coli* (25 BpC) or 5-A-RU/MG (100 nM) for 6 hours or IL-12+IL-18 (50ng/mL each) for 24 hours and MAIT cells ($CD3^+CD161^{++}V\alpha7.2^+$ lymphocytes) from treated and untreated LDMCs were flow-sorted by BD FACSAria and stored in RA1 lysis buffer from the Nucleospin RNA isolation kit (Macherey-Nagel) at -80 °C. This was performed on different days for each donor (n=4). RNA was isolated as described above and stored at -80 °C. RNA Sequencing was performed as described previously [22]. Briefly, libraries were prepared with the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit and were sequenced on an Ion S5 system. R-Bioconductor DESeq2 package was used for downstream analysis to obtain differentially expressed genes (DEG) from raw gene counts; $\log_2$ fold change and adjusted p-value (Padj) were set at 1 (fold change 2) and 0.05 respectively as cut-offs [57]. Strict filtering of lowly expressed genes was performed by setting an arbitrary cut-off for mean gene count from four donors of > 5, provided the count was > 10 in one donor. DEGs were used to generate Venn-diagram in an online tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). Multidimensional scaling analysis was performed using normalized gene expression of all genes. Heatmapper (http://www.heatmapper.ca/expression/), employing the average linkage clustering and Spearman rank correlation distance measurement methods, was used to make heat-maps [58]. Gene Set Enrichment Analysis (GSEA) was performed using normalized gene counts versus either a curated database (Reactome) provided in the software or tissue repair gene list obtained from the publication by Linehan et al. [59].

For transcriptomic comparison of MAIT cells from blood and liver, raw read counts of untreated ld- ($CD3^+$) and blood ($CD8^+$) MAIT cells were merged and analyzed in DESeq2. Untreated and treated blood and ld-MAIT cells were also merged assuming the treatments were comparable. For RNA-sequencing of blood, PBMCs were treated with *E. coli* (10 BpC) or 5-A-RU (5 µM) for 6 hours or IL-12+IL-18 (50 ng/mL each) for 24 hours and MAIT cells were flow-sorted as $CD3^+CD8^+CD161^{++}V\alpha7.2^+$ lymphocytes by BD FACSAria [22]. DEGs between untreated Ld-MAIT cells against blood MAIT cells were also analysed by Enrichr, an online gene list enrichment analysis tool [60]. Heat-maps were generated by Heatmapper online tool and GSEA analysis was performed between non-activated blood and ld-MAIT cells against the available Reactome and KEGG pathways in the software as well as the circulatory and tissue-residency gene lists obtained from the publication by Milner et al [55].

### Statistical analysis

GraphPad Prism V7.03 was used for statistical analysis. Data were first tested for normality with the Shapiro-Wilk test. To test statistical significance, repeated measures one-way

ANOVA with Sidak's multiple comparison post hoc test was used for multiple comparisons. Paired or unpaired two tailed t-test, Wilcoxon test or Mann-Whitney test was performed for comparison between two groups. $p < 0.05$ was considered significant. Data are presented as mean ± standard error of mean (SEM).

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**Author contributions**

JEU, JLM, and RL designed the project. JEU and RL designed the experiments. JEU managed the study. FM and JLM provided blood and liver samples. SMH and AV synthesised 5-A-RU. TWH and PKD provided support for the transcriptomic analysis. RL performed the experiments and analysed the data. RL and JEU wrote the manuscript. All authors revised and contributed to the editing of the manuscript.

**Conflicts of interest**

The authors declare no commercial or financial conflict of interest.

**Data availability statement**

The data that support the findings of this study can be available upon reasonable request to the corresponding author.

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## Figure legends

Figure 1. MAIT cells are enriched in liver and can be robustly activated via both TCR dependent and independent mechanisms. (A) Representative flow cytometry gating for distinguishing MAIT cells (CD3+CD161++Vα7.2+ lymphocytes) from other blood and liver mononuclear cells. Cumulative data showing the frequency of MAIT cells of total T cells and of CD8+ T cells in blood and liver. (B) Representative flow cytometry plot and cumulative frequency of CD8+, CD8-CD4- and CD4+ MAIT cell subsets of total MAIT cells (CD3+CD161++Vα7.2+) in blood and liver. (C-H) Expression of (C and D) CD69, (E and F) 4-1BB, and (G-H) CD40L on blood and ld-MAIT cells following (C, E and G) TCR activation by formaldehyde-fixed E. coli (25 BpC) or 5-A-RU/MG (100 nM) for 6 hours or (D, F and H) cytokine stimulation with 50 ng/mL IL-12 + 50 ng/mL IL-18 for 24 hours and measured by flow cytometry. Data are presented as mean ± SEM and are pooled from 4-13 independent experiments performed with one donor per experiment (n=13 donors for A, n=4 donors for B, G and H, and n=7 donors for C-F). Repeated measures one-way ANOVA with Sidak multiple comparison tests (C, E and G), two tailed paired t-tests (D, F and H) and Wilcoxon test (A) were used to assess statistical significance. *p<0.05, **p<0.01, ***p<0.001, ns = non-significant. The p value is shown for near-significant comparison. MFI = mean fluorescent intensity. Numbers on flow cytometry plots indicate frequency of parent.

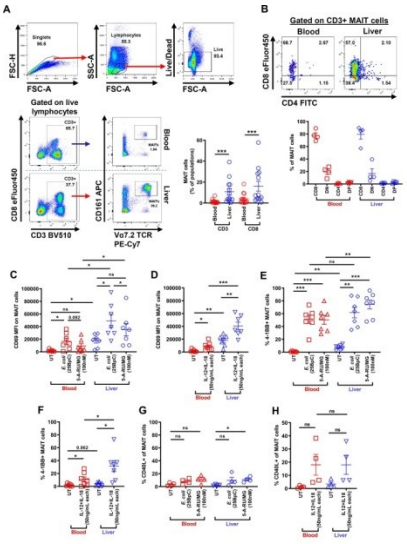

Figure 2. Ld-MAIT cells produce more pro-inflammatory cytokines in response to IL-12+IL-18. Production of (A and B) TNFα and (C and D) IFNγ by blood and ld-MAIT cells following (A and C) TCR activation by formaldehyde-fixed E. coli (25 BpC) or 5-A-RU/MG (100 nM) for 6 hours or (B and D) cytokine stimulation with 50 ng/mL IL-12 + 50 ng/mL IL-18 for 24 hours and measured by flow cytometry. (E) Representative flow cytometry flow plots and cumulative pie charts showing the proportion of MAIT cells producing TNFα only, IFNγ only, or both TNFα and IFNγ following TCR and cytokine stimulation, as in A-D. Data are presented as mean ± SEM (A-D) or average (E) and are pooled from 5-6 independent experiments performed with one donor per experiment (n=6 donors for liver and n=5 donors for blood). Repeated measures one-way ANOVA with Sidak multiple comparison test (A and C), paired/unpaired t tests, Wilcoxon test or Mann-Whitney test (B and D) were used to assess statistical significance. *p<0.05, **p<0.01, ****p<0.0001. The p value is shown for near-significant comparisons. Numbers on flow cytometry plots indicate frequency of parent.

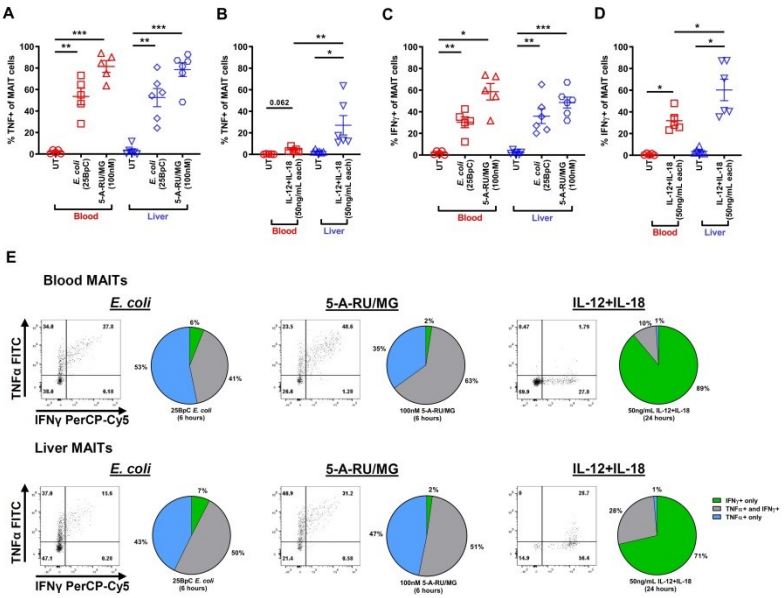

Figure 3. Changes in cytotoxic protein content of blood and ld-MAIT cells is similar following TCR or cytokine stimulation. Expression of (A-C) granzyme A, (D-F) granzyme B, (G-I) perforin, and (J-L) CD107a by blood and ld-MAIT cells following (B, E, H, and K) TCR activation by formaldehyde-fixed E. coli (25 BpC) or 5-A-RU/MG (100 nM) for 6 hours or (C, F, I, and L) cytokine stimulation with 50 ng/mL IL-12 + 50 ng/mL IL-18 for 24 hours. Representative flow cytometry histograms for ld-MAIT cells are also shown (A, D, G, and J). Data are presented as mean ± SEM and are pooled from 4-5 independent experiments performed with one donor per experiment (n=4 donors for granzyme A and CD107a and n=5 donors for granzyme B and perforin). Repeated measures one-way ANOVA with Sidak multiple comparison test (B, E, H, and K) and two tailed paired t-tests (C, F, I, and L) were used to assess statistical significance. *p<0.05, **p<0.01, ***p<0.001, ns = non-significant. The p-value is shown for near-significant comparisons.

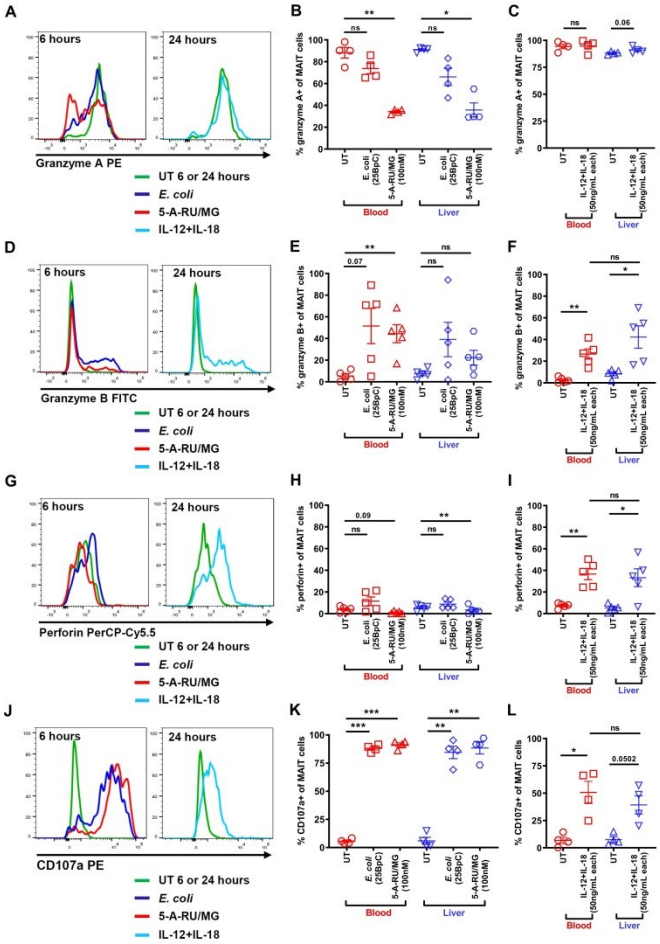

Figure 4. TCR and cytokine-activated ld-MAIT cells have distinct transcriptional profiles. Liver derived mononuclear cells (LDMCs) were treated with 25 BpC E. coli or 100 nM 5-A-RU/MG for 6 hours or 50 ng/mL IL-12 + 50 ng/mL IL-18 for 24 hours; untreated LDMCs were used as a control; this was done separately for four donors on four different occasions. Activated and non-activated MAIT cells (ranging from 3,190 to 255,815) were flow-sorted and RNA sequencing was performed; none of the isolated MAIT cells were pooled for any experiment. (A) Venn-diagram of common and unique differentially expressed genes (DEGs) in E. coli, 5-A-RU/MG, or IL-12+IL-18 stimulated ld-MAIT cells obtained using fold change >2 and adjusted p-value <0.05 as cut-offs; separate Venn-diagrams of total (left), upregulated (middle), and downregulated (right) DEGs with each treatment are shown. (B) Multi-dimensional analysis of normalized whole gene expression of activated and non-activated ld-MAIT cells. Each dot represents a sample and color represents different treatments as indicated. (C) Heat map and dendrogram of the normalized expression of total DEGs (1193) with all treatments compared to untreated control. (D) Gene set enrichment analysis for the tissue repair gene list (obtained from Linehan et al. [29]), comparing the normalized gene expression of E. coli, 5-A-RU/MG, or cytokine activated ld-MAIT cells against untreated MAIT cells. Enrichment score (ES), normalized enrichment score (NES) and nominal p-value from the GSEA analysis are shown.

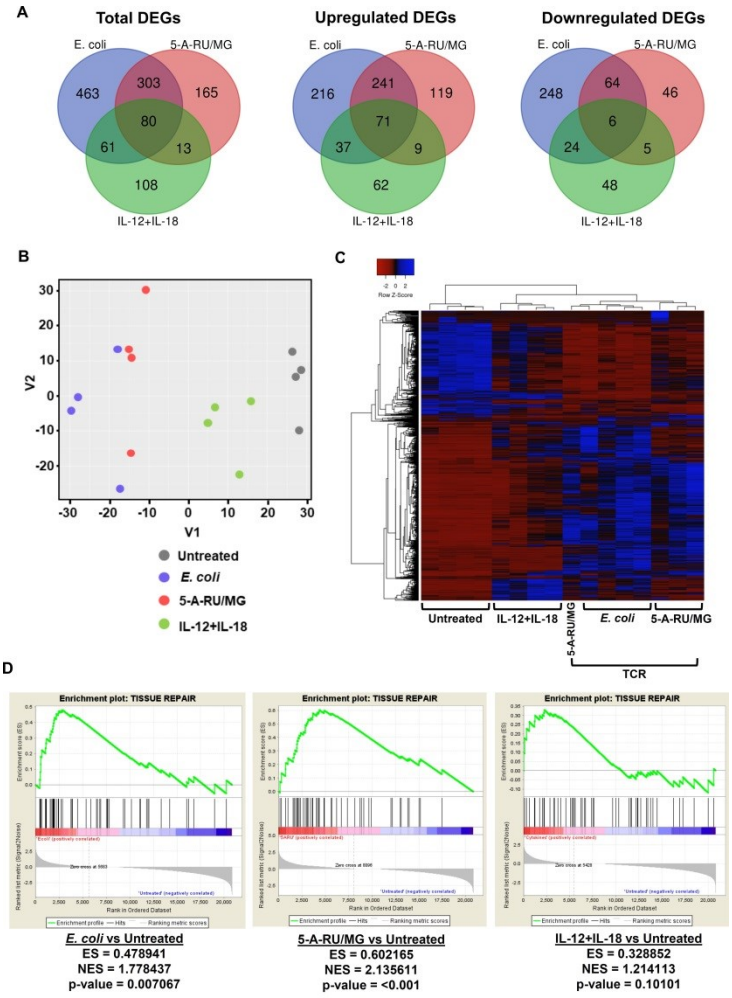

Figure 5. Blood and ld-MAIT cells differ in the expression of genes associated with cellular metabolism and cytokine signaling. (A) Heat map and dendrogram of the normalized expression of total DEGs (1883) in untreated ld-MAIT cells compared to untreated blood MAIT cells. (B-E) Gene set enrichment analysis for (B) oxidative phosphorylation and glycolysis gluconeogenesis, (C) IL-12 signaling, and (E) Interferon gamma signaling, comparing the normalized gene expression of untreated ld-MAIT cells to blood MAIT cells; Enrichment score (ES), normalized enrichment score (NES) and nominal p-value from the GSEA analysis are shown for each pathway. (D) Gene set enrichment analysis generated heat-map comparing expression of genes involved in IL-12 signaling between blood and ld-MAIT cells.