TCR and inflammatory signals tune human MAIT cells to exert specific tissue repair and effector functions

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ABSTRACT

MAIT cells are an abundant T cell population that are highly enriched in epithelial tissues such as the liver. They are known to be activated both through TCR-dependent and -independent mechanisms. However, the specific functional responses of MAIT cells to these signals remain elusive. We examined the impact of combinations of TCR-dependent and -independent signals in blood and tissue-derived human MAIT cells. TCR-dependent and -independent signals drive MAIT cells to exert overlapping and unique effector functions. Importantly, this includes several novel functions that are relevant not only to host defence but also tissue homeostasis. While TCR triggering is insufficient to drive sustained activation, gene expression signatures of TCR-triggered MAIT cells also showed specific enrichment of tissue-repair functions. Activation of certain effector and antimicrobial functions may be triggered further with specific innate cytokine signals, such as the TNF superfamily member TL1A. In addition, gut-resident cells exposed to commensal-derived ligands showed evidence of persistent activation *in vivo*. Taken together, this study demonstrates the pivotal role of MAIT cells in controlling the balance between healthy and pathological processes of tissue inflammation and repair.

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Author contributions

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INTRODUCTION

Human innate and adaptive immune systems form a critical partnership in immune defence against microorganisms. Recent studies have revealed several types of unconventional T lymphocyte that sit at the bridge between innate and adaptive immunity, including mucosal associated invariant T (MAIT) cells (Godfrey et al., 2015). MAIT cells are abundant in human blood, and enriched most substantially in the liver (Treiner et al., 2003). They are marked by high surface expression of the C-type lectin molecule CD161, and bear the semi-invariant TCR Vα7.2-Jα33/12/20, which restricts them to the evolutionary conserved, non-polymorphic MHC class I-related protein 1, MR1 (Kjer-Nielsen et al., 2012; Reantragoon et al., 2013). MAIT cells recognize microbially derived riboflavin synthesis intermediates presented by MR1 (López-Sagaseta et al., 2013; Ussher et al., 2014). Recently, MR1-tetramers loaded with riboflavin and folate intermediates have been developed, enabling the specific detection and characterization of human and mouse MAIT cells (López-Sagaseta et al., 2013; Rahimpour et al., 2015; Reantragoon et al., 2013).

Despite this specific antigen recognition as an effector T cell, the MR1-TCR signaling alone is insufficient to fully activate MAIT cells (Turtle et al., 2015). In order to achieve sufficient activation, TCR signaling is supported by other co-stimulatory signals such as CD28 and notably by cytokines, for example, interleukin (IL)-18 and IL-12 (Ussher et al., 2013). This is true in mouse cells examined *in vivo* – normal expansion is only seen if ligand is delivered with a TLR-stimulus (Chen et al., 2017).

This behavior has prompted investigation into the responsiveness of MAIT cells to innate signals, including IL-12, IL-18, IL-15, and type I interferons (IFNs) (Sattler et al., 2015; Ussher et al., 2013; Wilgenburg et al., 2016). *In vitro* studies in human cells have shown that cytokines, such as IL-12 and IL-18, can, in combination, activate MAIT cells in a fully TCR-independent manner (Ussher et al., 2013). Cytokine-stimulated $CD161^{++}CD8^{+}$ T cells, including MAIT cells, may exert effector functions by secretion of cytokines and upregulation of Granzyme (Gr)B (Billerbeck et al., 2010; Kurioka et al., 2014). We and others recently highlighted a role for MAIT cells in viral infections, where MAIT cell activation was TCR-independent, but dependent on IL-18 in synergy with IL-12, IL-15, and/or type I interferons (IFN-α/β)

(Loh et al., 2016; Wilgenburg et al., 2016). Thus it is clear that MAIT cells can be activated via TCR-dependent and -independent pathways. However, the diversity of functions triggered by different cytokines and by TCR signaling has yet to be defined.

The discussion of specific functions of MAIT cells elicited by cytokines is particularly relevant in mucosal tissues, such as the gut, where local signaling may be critical in defining the balance between host defence responses and tolerance. Recent data on IL-17-expressing skin-homing mouse $CD8^+$ T cells, an innate-like T cell population which mirrors some critical features of MAIT cells, indicate that they display a tissue repair rather than a pure inflammatory phenotype in response to TCR triggering via commensal associated ligands (formyl peptides restricted by H2-M3) (Linehan et al., 2018). The authors propose that responses to commensals driven by TCR could support a role for such T cells in tissue homeostasis. Indeed this behaviour may extend to more broadly include innate-like T cells restricted by MHC1b molecules, which are evolutionarily ancient (Klenerman and Ogg, 2018).

TNF-like protein 1A (TL1A)/TNF superfamily member 15 (TNFSF15) is a gut-associated proinflammatory cytokine originally characterized in a screen for TNF-α homologous molecules. It is expressed by activated T cells, dendritic cells, and monocytes, and signals through death receptor-3 (DR3) (Meylan et al., 2008; Migone et al., 2002; Shih et al., 2009). TL1A is particularly relevant as it has previously been described to activate a subset of CD4+ memory T cells expressing IL-18Rα and DR3 (Gudjonsson et al., 2015). More specifically, it has been shown to increase production of IFN-γ and TNF-α by $CD161^+CD4^+$ T cells in the presence of anti-CD3 or IL-12+IL-18 (Jin et al., 2012). This may be relevant to MAIT cell functions as we have previously identified a phenotypic, functional, and transcriptional program shared by CD161-expressing cells (Fergusson et al., 2014).

Here, we addressed how TCR-dependent and -independent signals synergize and drive the activation of *in vitro* blood- and gut-derived MAIT cells. We find that IL-12 and IL-18, in synergy with TCR triggering, promotes the activation of MAIT cells in a dose-dependent manner and that TL1A also potently induces specific MAIT cell functions. Interestingly, triggering with TCR alone drives a set of functions linked to a tissue repair signature. Overall, our data provide insight into the precise nature of

TCR- and cytokine- mediated human MAIT cell activation, characterized by a broad range of effector functions including not only emergency host defense but also maintaining ongoing homeostasis. This feature may be also relevant to other innate-like T-cell subsets found at barrier sites in humans.

MATERIALS AND METHODS

Samples. All samples were collected with appropriate patient consent and NHS REC provided ethical approval (reference numbers 09/H0606/5 for IBD patients and 16/YH/0247 for controls). Healthy PBMCs were isolated from leukocyte cones (NHS Blood Services). For long-term storage, PBMCs were kept in liquid nitrogen with freezing media (10% DMSO, 90% fetal calf serum, both Sigma-Aldrich). Colonic tissues were collected from uninvolved mucosa of patients with colorectal cancer. Patient information is shown in **Table 1**. All patients involved gave written consent. Colonic tissues were digested at 37°C for overnight with Collagenase A (Roche) and DNase I (Sigma-Aldrich). Colonic lymphocytes were then isolated from the cell suspension using Percoll (GE Healthcare). A detailed protocol has been described by Geremia *et al*. (Geremia et al., 2011).

Table 1. Characteristics of the CRC patients.

CRC patients	n = 21
Age (Average, SD*)	68, 12
Sex (Male/Female)	12/9
Uninflamed, tumour-free tissue origin Small intestine Large intestine	 0 21
Time since diagnosis (years) Average, SD	 1, 1

* SD: standard deviation

Cell culture. PBMCs were thawed, washed and maintained in RPMI 1640 with 10% fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin (R10) (all Sigma-Aldrich). $CD8^+$ T cells were positively labeled with CD8 Microbeads (Miltenyi Biotech, purities were ≥ 90%), and enriched from PBMCs using MS or LS columns following the manufacturer's instructions (Miltenyi Biotech). Colonic lymphocytes were used without prior enrichment by CD8 microbeads, and were maintained in R10

supplemented with 25ng/mL amphotericin B (Gibco), 40µg/mL gentamicin (Gibco), and 10µg/mL ciprofloxacin (Sigma-Aldrich).

***In vitro* stimulations.** For TCR triggering, PBMCs, enriched CD8 T cells, sorted cells or colonic lymphocytes were stimulated with plate bound anti-CD3/28 antibodies (Miltenyi), or anti-CD3/28 beads (Miltenyi) at 1:1 ratio. ELISA plates (Greiner) were coated with 5µg/mL anti-CD3/28 with the final volume of 100µL at 4°C for overnight. Antibody mix was washed off the next morning, and plates were used after 1-hour 37°C incubation with R10. Anti-CD3/28 beads were prepared following the manufacturer's instructions.

For cytokine triggering, cells were stimulated for 20 hours with IL-12 (Miltenyi) at 2ng/mL, IL-18 (MBL) at 50ng/ml, IL-15 (Miltenyi) at 25ng/ml, TL1A (R&D) at 100ng/ml, unless otherwise stated.

Flow cytometry. Brefeldin A (eBioscience, 1000x) was added into the cell culture for the last 4 hours before intracellular staining.

Dyes/human antibodies used were: viability dye Live/Dead fixable-Near-IR (Invitrogen), CD3-eFluor450 (OKT3, eBioscience), CD4-VioGreen (M-T466, Miltenyi), CD4 PerCP-Cyanine5.5 (OKT4, eBioscience), CD8-VioGreen or -Vio770 (REA734, Miltenyi), CD161-PE or -PE-Vio770 (191B8, Miltenyi), Vα7.2-APC or -PE/Cy7 (3C10, BioLegend), Pan Vγδ-FITC (IM1571U, Beckman Coulter), Vγδ-APC-Cy7 (11F2, Miltenyi), IFN-γ-FITC (45-15, Miltenyi), -PE/Cy7 (4S.B3, BioLegend), TNF-α-PerCP-Cy5.5 or -FITC (MAb11, BioLegend), GrB-APC (GB12, Invitrogen), CD39-PE (A1, Biolegend), PD-1-BV421 (EH12.2H7, BioLegend), MR1 tetramer-PE (NIH).

Data were acquired on a MACSQuant cytometer (Miltenyi) or LSRII (BD Biosciences) and analyzed on FlowJo (Tree Star Inc.). Gating strategy is shown in **Supplementary Fig. 4A**.

RNA sequencing (RNAseq). $CD8^+$ T cells were enriched from PBMCs of three healthy individuals and were rested overnight prior to sorting. On the next day, MAIT

($CD161^{++}$ $V\alpha7.2^{+}$) cells were sorted using a Beckman Coulter MoFlo XDP and stimulated with a range of conditions including anti-CD3/28, cytokines (IL-12/IL-18/IL-15/TL1A), or the combination of both, or left untreated in R10 media for 24 hours. RNA was then extracted from these 12 samples using an RNeasy Micro kit (Qiagen). The quantity and quality of extracted RNA was first evaluated using both a nanodrop spectrophotometer and the Agilent 2100 bioanalyzer. All samples had RNA integrity (RIN) values greater than 9 and were free from contaminating protein and organic compounds. RNAseq was performed by Wellcome Trust Centre for Human Genetics (University of Oxford) on a HiSeq4000v platform. Gene lists that were differentially expressed (>4 fold, $P<0.01$, $FDR<0.05$) between various conditions and their normalised expression values, as well as the principle component analysis (PCA) plots, were generated with Partek® Flow®, an online analysis platform for Next Generation Sequencing data (http://www.partek.com/partek-flow/), following the user's guide. Volcano plots were generated with Prism software. Heat maps were generated using normalised counts with Heat Mapper (http://www.heatmapper.ca/expression/) with the averaged linkage clustering method and Pearson distance measurement method. Venn diagrams were drawn with an online diagram drawing platform developed by Ghent University, Belgium (http://bioinformatics.psb.ugent.be/webtools/Venn/). Gene set enrichment analysis (GSEA) was performed using GSEA version 3.0 (Subramanian et al., 2005), comparing gene expression data as a whole with the reference gene list obtained from the publication by Linehan *et al*. (Linehan et al., 2018).

qPCR. $CD161^{++}$ $V\alpha7.2^{+}$ (MAIT) and $CD161^{-}$ $V\alpha7.2^{-}$ cells were sorted from pre-enriched blood $CD8^{+}$ T cells. These cells were then stimulated with anti-CD3/28, cytokines (IL-12/IL-18/IL-15/TL1A), or the combination of both, or left untreated in R10 media for 20 hours. The total RNA of sorted T cells was extracted with an RNeasy Micro kit (Qiagen) and reverse transcribed using reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen). For detection of IL-26 mRNA, a 20X IL-26 human TaqMan® probe was used (Hs00218189_m1) with 2X TaqMan® Fast Advanced Master Mix (both from ThermoFisher Scientific). OSM and HEBGF cDNA quantification was performed with Roche ® hydrolysis probes (*OSM*: forward primer sequence, 5'-cttccccagtgaggagacc-3', reverse sequence, 5'-ctgctctaagtcggccagtc-3'; HBEGF: forward primer sequence, 5'-tggggcttctcatgtttagg-

3', reverse sequence, 5'-catgcccaacttcactttctc-3'), with GAPDH as the internal control (forward primer sequence, 5'-ccccggtttctataaattgagc-3', reverse sequence, 5'-cttccccatggtgtctgag-3').

Data analysis. All graphs and statistical analyses, except RNAseq data analysis, were completed using GraphPad Prism Software Version 6.0b (La Jolla, CA). Statistical significance was assessed using paired Student's t-test, or repeated-measures two-way analysis of variances, with Bonferroni's correction for multiple comparison assays. All data were presented as means with s.e.m.

RESULTS

MAIT cells respond to combinations of cytokines and TCR triggering and enhance effector functions in a dose-dependent manner.

Studies have addressed the hypo-responsiveness of $CD8^+$ MAIT cells to anti-CD3 by comparing their ability to proliferate and produce cytokines *in vitro* to their $CD161^-$ $CD8^+$ counterparts (Turtle et al., 2015). We hypothesized that this could be due to a lack of complementary inflammatory signals. Thus, we first asked whether the TCR signaling, insufficient on its own, could sensitize MAIT cells and further promote *in vitro* MAIT cell activation in a pro-inflammatory milieu. To minimize the impact of the other cells in the adult peripheral blood on $CD8^+$ MAIT cell activation, CD8+ T cells were magnetically enriched from peripheral mononuclear blood cells (PBMCs) of healthy donors, with a post-enrichment purity > 90% (**Fig. 1A**). The synergy of IL-12 and IL-18 has been shown to robustly induce IFN-γ expression in human adult MAIT cells (Ussher et al., 2013), while TL1A is a potent driver of activation for $CD161^+$ $CD4^+$ memory T cells in the presence of other cytokines (Gudjonsson et al., 2015). We therefore assessed if these cytokines could additionally promote MAIT cell activation in the presence of the TCR trigger. The proportion of MAIT cells releasing IFN-γ is markedly increased by higher doses of IL-12, but not by IL-18 or TL1A (**Fig. 1 B, C**).

We next investigated whether the TCR signaling could enhance the effector functions of MAIT cells in the presence of a suboptimal cytokine trigger. Enriched CD8+ T cells were stimulated singly by suboptimal concentrations of IL-12 (2ng/ml)

and IL-18 (2ng/ml), or in combination with anti-CD3/CD28 beads (with bead-to-cell ratios ranging from 0 to 10:1) (**Fig. 1D, E**). The frequency of MAIT cells that responded to anti-CD3/CD28 by producing IFN-γ or TNF-α positively correlated to the bead-to-cell ratio. TCR signaling and cytokines had a profound synergy in $CD8^+$ MAIT cells. It is interesting to note that IFN-γ expression correlated with that of CD161 – the proportion of IFN-g-producing CD8+T cells with intermediate CD161 expression ($CD161^+$) also increased moderately in response to the combined trigger. This is consistent with previous findings by Fergusson *et al.* that expression of CD161 defines shared transcriptional and functional features across human T cells lineages, including $CD161^+$ $CD8^+$ T cells, $CD161^+$ $CD4^+$ T cells, and γδ T cells (Fergusson et al., 2015, 2014).

TL1A alone does not activate MAIT cells, as we observed no change in expression levels of CD69, IFN-γ, or TNF-α between TL1A-stimulated and unstimulated MAIT cells (**Fig. 1F**). It also poorly synergized with TCR triggering to further drive effector functions of MAIT cells (**Fig. 1C**). To probe the role of TL1A in activating MAIT cells, we thus interrogated its potential in synergizing with other cytokines to mediate MAIT cell activation. Enriched $CD8^+$ T cells were then treated with suboptimal doses of IL-12 (2ng/ml) and IL-18 (2ng/ml), alone or in combination with increasing doses of TL1A. Higher doses of TL1A at 10 and 100 ng/ml significantly enhanced IL-12/IL-18 -induced MAIT cell activation, shown by percentages of MAIT cells producing IFN-γ (**Fig. 1G**), TNF-α (**Fig. 1H**), and CD69 (**Fig. 1I**). The dose-dependent response to TL1A was only present in MAIT cells, and was not found in $CD161^{+/-}$ $CD8^+$ T cells.

TL1A and IL-15 enhance effector functions of human MAIT cells.

To further explore the impact of cytokine triggering of MAIT cells we examined combinatorial signaling in more detail. The ability of TL1A and IL-15 to promote T cell activation in the presence of a suboptimal IL-12 and IL-18 trigger has been shown in the CD161-expressing $CD4^+$ T cells (Cohavy et al., 2011; Jin et al., 2012; Sattler et al., 2015, 2009). We therefore addressed whether MAIT cells possess similar responsiveness.

Expression of IFN-γ, TNF-α, and Granzyme (Gr) B by stimulated MAIT cells from a representative donor is shown in **Fig. 2A**. IL-15 (50ng/ml), TL1A (100ng/ml), or the

combination of both markedly promoted IL-12 (2ng/ml) /IL-18 (50ng/ml) -induced MAIT cell activation, measured by MAIT cell expression of IFN-γ (**Fig. 2B**), TNF-α (**Fig. 2C**), and GrB (**Fig. 2D**). We found that TL1A and IL-15, individually increased MAIT cell expression of IFN-γ and TNF-α, and upregulated GrB expression. IL-15 was more potent than TL1A when added singly to the IL-12+IL-18 culture. The peak level of MAIT cell activation was achieved by a combination of both cytokines in the presence of IL-12 and IL-18. TL1A, when added to the IL-12+IL-15+IL-18 culture, resulted in a robust increase of the percentage of MAIT cells expressing IFN-γ (**Fig. 2C**) and GrB (**Fig. 2D**), and its effect was more pronounced for TNF-α expression (**Fig. 2B**). Of note, TL1A and IL-15 cannot drive MAIT cell activation when used singly or in combination in the absence of IL-12 and IL-18 (**Fig. 1C, Fig. 2E**). This suggests that a maximal activation of MAIT cells is only reached by the collective presence of all four cytokines.

$CD161^+$ $V\alpha7.2^-$ and $CD161^-$ $V\alpha7.2^-$ $CD8^+$ T cells were also investigated for their responsiveness to innate cues (**Supplementary Fig. 1A**). TL1A and IL-15 also profoundly enhanced IFN-γ production by $CD161^+$ $V\alpha7.2^-$ $CD8^+$ T cells (**Supplementary Fig. 1B-D**), although to a lesser extent compared to MAIT cells (**Supplementary Fig. 1B**). However, these cytokines had a minimal role in promoting the production of TNF-α or GrB by $CD161^+$ $V\alpha7.2^-$ $CD8^+$ cells (**Supplementary Fig. 1E, F**), or, in general, activating $CD161^-$ $V\alpha7.2^-$ $CD8^+$ T cells (**Supplementary Fig. 1B, G-I**).

MAIT cells possess distinct transcriptional signatures upon activation.

To further explore the effector functions of MAIT cells elicited by the TCR or cytokine signals, we utilized RNAseq to characterize transcriptional profiles of MAIT cells under different treatments, including the TCR (anti-CD3/CD28, labeled here as “T”), Cytokines (IL-12/IL-15/IL-18/TL1A, labeled here as “C”), and a combination thereof (labeled here as “TC”). Transcriptional profiles of differentially stimulated MAIT cells were compared to those of untreated (UT) cells. TCR beads were used at 1:1 bead-to-cell ratio, and cytokines were used at the concentrations specified in Figure 2.

The mRNA levels of 132, 1124, and 1375 genes were significantly modulated ($p < 0.01$, $|\text{fold change}| > 4$, false discovery rate (FDR) < 0.05, including upregulation and

downregulation) by TCR, cytokines, or combined TCR and cytokine stimulation, respectively. Venn diagrams highlight the overlapping and unique transcriptional signatures elicited by these 3 different stimulations (**Fig. 3A-C, Supplementary Table 1-3)**. We found that stimulating MAIT cells with TCR beads and/or cytokines resulted in the significant alteration of 89 mRNA transcripts in MAIT cells, including 88 upregulated genes (**Fig. 3B**, **Supplementary Table 1**), and 1 downregulated gene (**Fig. 3C**). In addition, Gene Ontology (GO) enrichment analysis on 88 upregulated genes by MAIT cells predicts that they are involved in cytokine production (IRF8, TNFRSF9, IRF4, MYB, NFKB1, NFKB2, FURIN, RELB, CCL3, CCL20, TNF, SEMA7A, SPHK1, CD83, EBI3), IL-17 signaling (NFKB1, NFKBIA, CCL20, TRAF4, TNF, IL23R), IL-12-mediated signaling (IL23R, EBI3, IL2RA, RELB, NFKB1, NFKB2, CCL3), and TNF signaling (TNF, NFKB1, NFKBIA) (**Supplementary Table 4**).

Analysis of the other genes unique to TCR and cytokines indicate diverse physiological functions that these two signals induce in MAIT cells (**Fig. 3A**). Among 1594 modulated genes (**Supplementary Table 5**), 960 (60.2%) were upregulated and the rest were downregulated (**Fig. 3A, B**). MAIT cells stimulated with TCR beads and cytokines shared 572 upregulated genes with their counterparts only stimulated with cytokines. They constitute 82.1% of upregulated gene transcripts elicited by cytokines, and 70.8% elicited by TCR beads and cytokines (**Fig. 3B**).

The 1594 genes with significantly altered expression levels between conditions were then plotted in a heat map according to their normalized expressions using average linkage hierarchical clustering (**Fig. 3D**). We also performed a principal component analysis (PCA) using the first two principal components of the 1594 mRNA transcripts and visualized the correlation of the transcriptional profiles of differentially stimulated MAIT cells (**Fig. 3E**). Clustering of C- and TC- conditions confirmed that cytokine stimulation at this time-point had a dominant impact on MAIT cell activation. However, the finding of 221 upregulated genes (**Fig. 3B**) and 208 downregulated genes (**Fig. 3C**) unique to TC stimulation also suggests a strong synergy between the TCR signaling and cytokines to further drive MAIT cell activation and promote their effector functions. Furthermore, the PCA analysis indicates that a TCR stimulus

alone can trigger a pronounced level of activation, as the T clustering was clearly separate from UT.

To further confirm our findings, MAIT cells from the same donors were examined for their release of IFN-γ, TNF-α and expression of GrB in response to stimulations consistent with those used in the RNAseq analysis (**Supplementary Fig. 2A-C**). Cytokines robustly increased IFN-γ and TNF-α release by MAIT cells, compared to cells singly stimulated by a TCR trigger alone. The addition of an additional TCR trigger did not significantly increase the proportion of MAIT cells releasing IFN-γ and TNF-α. This corroborates the overlap in MAIT cell responses to cytokines ± TCR trigger. TCR signaling enhanced GrB expression by MAIT cells in the presence of cytokines, in line with previous observations (Kurioka et al., 2014).

We next analyzed volcano plots that show differentially expressed transcriptional profiles of stimulated MAIT cells compared to their unstimulated counterparts. A limited number of genes were significantly altered by a single dose of TCR stimulation, after filtering for transcripts with p value <0.01 and fold change > 4 (**Fig. 3F**). The transcriptional profiles of MAIT cells in the presence of cytokines were remarkably distinct from that of unstimulated (**Fig. 3G, H**). Genes that are known to be associated with MAIT cell activation are highlighted, including IFNG, GZMB, and TNF.

Transcriptional signatures of activated MAIT cells predict not only anti-microbial but also tissue repair functions.

We here discuss 3 genes of interest identified through further investigation of gene regulation in strongly activated MAIT cells (stimulated by cytokines ± TCR), namely IL-26, oncostatin M, and Heparin binding early growth factor (HBEGF) (**Fig. 3G, H**, marked in blue).

To confirm our findings from RNAseq, IL-26 mRNA transcript levels were subsequently quantified by qPCR analysis on RNA samples extracted from activated MAIT cells (**Fig. 4A).** MAIT cells significantly upregulated their IL-26 mRNA level in response to cytokines (an average 89-fold increase compared to unstimulated

samples). *IL26* upregulation was independent of TCR triggering, as *IL26* expression in MAIT cells treated with TCR beads was the same as that in unstimulated MAIT cells. Furthermore, the addition of TCR beads to cytokine-stimulated MAIT cells did not significantly alter *IL26* expression. Therefore, activated MAIT cells may potentially exert their antibacterial functions via not only GrB and perforin release, but also IL-26 production. IL-26 is an emerging member of IL-10 family cytokines recognized for its versatile role in antiviral, antimicrobial, and autoimmune responses (Stephen-Victor et al., 2016). It has been reported to directly kill bacteria by mediating membrane-pore formation (Brown et al., 2016), or in an indirect manner by recruiting neutrophils to bacteria at the lungs, or triggering the production of IFN-α by plasmacytoid dendritic cells (pDCs) (Che et al., 2014; Meller et al., 2015).

Similarly, cytokines profoundly increased OSM and HBEGF mRNA transcript levels in MAIT cells, contributing to a >1000-fold increase for both genes compared to unstimulated samples (**Fig. 4B, C)**. HBEGF is a member of the ligand family of EGF receptor (EGFR) discovered in human macrophages (Besner et al., 1990), and regulates several biological processes, notably angiogenesis, would healing, and tumour progression (Singh et al., 2016; Taylor et al., 2014). OSM is an IL-6 family cytokine mainly produced by activated T cells, monocytes, and dendritic cells. OSM plays an important role in hematopoiesis, immunity, bone remodelling, and inflammation (Tanaka and Miyajima, 2003). It is also involved in intestinal epithelial restitution by enhancing STAT3-dependent cells proliferation, thus promoting intestinal barrier functions (Beigel et al., 2014).

Given the broad range of responses seen after TCR and cytokine mediated activation we speculated that barrier functions of MAIT cells extended beyond conventional antimicrobial responses.The recent discovery of a skin-homing Tc17 subset in mouse responsive to commensal ligands has shed light on a unique form of adaptive immunity where antimicrobial functions and tissue repair are coupled within the same subset of unconventional T cells (Linehan et al., 2018). These commensal-specific T cells elicited a tissue repair signature and accelerated wound closure, in addition to promoting protection to pathogens. MAIT cells are commensal responsive and similarly have been associated with a type-17 phenotype (Billerbeck et al., 2010; Dusseaux et al., 2011). Therefore, we investigated functional overlap

using a genomic comparison between activated human MAIT cells and mouse skin-homing Tc17 cells. Genes significantly differentially expressed compared to unstimulated MAIT cells were identified from T, C, and TC –stimulated MAIT cells and compared to the tissue repair gene data set (Linehan et al., 2018). Remarkably, gene set enrichment analysis demonstrated significant enrichment ($p<0.0002$) of these tissue repair related genes in MAIT cells stimulated by TCR ± cytokines (**Fig. 4D, E**), but not by cytokines alone (**Fig. 4F**). These data suggest that TCR triggering by MAIT cells may be important in driving a tissue repair program.

The comparator tissue repair gene set included TNF, and studies have confirmed its role in dermal wound healing (Fedyk et al., 2001), as well as liver repair and regeneration (Galun and Axelrod, 2002; Krafts, 2010). We therefore examined further the TCR-dependence of TNF production in MAIT cells. MAIT cells express TNF-α in response to fixed *E.coli* in a time-dependent manner, and such response was largely dependent on MR1 signaling, as the blocking antibody against MR1 abrogated the release of TNF-α production by MAIT cells at investigated time points (**Fig. 4G**).

Taken together these findings suggest *in vitro* evidence for the proposed model whereby MAIT cells exert tissue repair and wound healing functions via MR1-dependent triggering.

Gut-derived MAIT cells also respond to a range of cytokine and TCR stimulations

For such a model to be tenable, similar functionality should be observed in cells derived from barrier tissues. We therefore next examined MAIT cells in the gut to address the role of signal integration in tissue-derived MAIT cells. A gating strategy for MAIT cells on a representative sample is shown (**Supplementary Fig. 3A**). An additional staining for γδ T cells was included to show that the number of γδ TCR-bearing $CD8^+$ T cells is minimal. For all subsequent experiments, the γδ T cell population was excluded from analysis using a dump channel. Control lymphocytes were isolated from uninvolved mucosa from colorectal cancer resection tissue.

Compared to MAIT cells in PBMCs, the percentage of MAIT cells was lower in the $CD8^+$ T cells derived from healthy gut tissues (**Fig. 5A**).

To further characterize the role of TL1A in the gut, we examined the responsiveness of healthy gut-derived MAIT cells to TL1A. LPLs were cultured overnight with a low dose of IL-12 (2ng/ml) and IL-18 (50ng/ml), in the absence or presence of 100ng/ml TL1A. MAIT cell activation was assessed by their expression of IFN-γ, TNF-α and GrB (**Fig. 5B**). TL1A increased IFN-γ and TNF-α expression in MAIT cells in the presence of suboptimal trigger of IL-12+IL-18, but the effect was not pronounced for GrB.

Furthermore, MAIT cells from control colonic mucosa responded to a range of stimulations, and significantly enhanced their expression of IFN-γ (**Fig. 5C**), GrB (**Fig. 5D**), and TNF-α (**Fig. 5E**), in a similar pattern to those derived from healthy blood (**Supplementary Fig. 3**). The addition of anti-CD3/CD28 beads to the IL-12+IL-18 culture promoted the activation of MAIT cells, leading to a 35% increase of IFN-γ production by gut MAIT cells. Similarly, IL-15+TL1A also promoted IL-12+IL-18-induced MAIT cell activation, the percentage of MAIT cells producing IFN-γ increased 40%. We also found that the combination of IL-12, IL-18, IL-15, and TL1A already maximally activated MAIT cells, thus an additional anti-CD3/CD28 signal could not promote IFN-γ or GrB production (**Fig. 5C, D**), but, consistent with the data above, it enhanced the release of TNF-α (**Fig. 5E**) by MAIT cells.

Gut-derived MAIT cells show evidence of sustained *in vivo* activation

To provide *in vivo* context for our observations, we next sought evidence that human MAIT cells at a barrier surface exposed to commensal cues would show certain evidence of ongoing activation. MAIT cells in blood are low in GrB but rapidly upregulate this in response to TCR and cytokine signals. When we examined MAIT cells from the gut we observed a consistent upregulation of GrB (**Fig. 6A**).

Chronic TCR stimulation e.g. in viral infections such as LCMV, HCV and HIV can lead to upregulation of both CD39 and PD-1 on CD8+ T cells (Gupta et al., 2015). PD1 and CD39 were both significantly elevated on MAIT cells derived from the

control gut (**Fig. 6B, C**), and consistent findings were made by measuring MAIT cell PD-1 and CD39 geometric mean of fluorescence intensity (**Fig. 6D, E**). We then examined the co-expression of these two markers and found a significantly elevated proportion of $CD39^+$ $PD\text{-}1^+$ MAIT cells in the control mucosa than the blood (**Fig. 6F, G**).

Co-expression of PD-1 and CD39 has been associated with decreased responsiveness of T cells following persistent stimulation. Consistent with this, directly comparing the production of IFN-γ, TNF-α and GrB we found a lower response from gut-derived MAIT cells compared to those from the blood in stimulation (**Fig. 6H**). We confirm that this lower responsiveness was not due to discrete preparation conditions (**Supplementary Fig. 4B**) and these data taken together suggest ongoing, sustained triggering of MAIT cells in a healthy gut, with phenotypic adaptation.

Discussion

CD161-expressing human T lymphocytes possess shared transcriptional and functional phenotypes, and their enhanced innate ability to respond to inflammatory cues has been investigated with transcriptomic approaches (Fergusson et al., 2014). MAIT cells comprise a large proportion of these CD161-expressing T cells, and have previously been described to show limited responses to conventional TCR signals (Slichter et al., 2016; Turtle et al., 2015). To further dissect the differential signals that promote and sustain MAIT cell effector function, it is therefore of central importance to fully understand the role of MAIT cells in both health and disease. Here we probed the contribution of innate and adaptive signals to MAIT cell activation in the blood and gut, describing and segregating novel functions of MAIT cells following activation.

We defined MAIT cells in both human blood and gut as $CD161^{++}V\alpha7.2^+CD8^+CD4^-$ $CD3^+$ T cells, a common approach used by a number of studies to dissect MAIT cell effector function elicited by cytokines or the TCR(Kurioka et al., 2014, 2017; Sattler et al., 2015; Ussher et al., 2013; Wilgenburg et al., 2016). MR1 tetramers, combined with CD161 staining, identify MAIT cells unequivocally, but these reagents only

became available at the end of this study. We also stained freshly isolated colonic lymphocytes using the MR1 tetramer, with Va7.2 and CD161 antibodies on the same panel. These provide similar estimates of frequency – an example is shown (**Supplementary Fig. 4C).**

Responses following a TCR stimulation of MAIT cells and conventional CD8 T cells differ greatly. We and others (Slichter et al., 2016) have found that a single TCR stimulation with anti-CD3/CD28 beads could induce effector function of both conventional CD8 T cells and MAIT cells at early time points. In contrast, it is only sustained at later time points in conventional $CD8^+$ T cells, but not MAIT cells. Genes controlling the TCR signaling pathway have shown to be differentially regulated in MAIT cells and conventional $CD8^+$ T cells ($CD161^-$ cells) (Turtle et al., 2015), yet biological reasons for a dampened response by TCR-triggered MAIT cells are still unclear. The conserved MAIT TCR consisting of the invariant TCRα chain and a restricted repertoire of β chains allows MAIT cells to specifically recognize bacterial metabolites, which can be derived from either pathogenic or commensal bacteria. Given the abundance of both MAIT cells and commensal bacteria in the human gut (Serriari et al., 2014), we speculate that MAIT cells could tune TCR signals to optimise immunological responses when encountering commensal-derived antigens.

Although a TCR signal is not sufficient to sustain the full range of effector functions in MAIT cells, we here demonstrate that cytokine signals, or a combined TCR+cytokine signal can strongly promote activation. Resting MAIT cells express a higher level of IL-18Rα on the cell surface, compared to their $CD161^{++}$Vα7.2^- or CD161- counterparts (Fergusson et al., 2014; Ussher et al., 2013). However, we found that IL-18 had a very limited role in enhancing MAIT cell activation in the presence of TCR triggering at 20 hours. Studies also showed that other human lymphocytes, including NK cells and T cells, did not produce IFN-γ when singly treated with IL-18 (Jampel et al., 2009; Tominaga et al., 2000). The IL-18 receptor is a heterodimer comprising IL-18Rα (IL-1Rrp) and IL-18Rβ (AcPL) chains. Both subunits are markedly upregulated in cytokine-activated NK and T lymphocytes, including IL-12 and IFN-α (Sareneva et al., 2000), but not by a single TCR signal. Thus, the observed low response of MAIT cells to TCR+IL-18 stimulation maybe due to insufficient expression of IL-18Rβ, which is important for downstream signaling

(Cheung et al., 2005). A signal combining IL-12 and IL-18 has been described to activate a range of lymphocytes, including Th1 cells, B cells, NK cells, and more recently, MAIT cells, independently from the TCR signaling pathway (Sareneva et al., 2000; Ussher et al., 2013; Yoshimoto et al., 2015). We show here that titrating anti-CD3/CD28 beads into a suboptimal IL-12+IL-18 coculture restored effector function of MAIT cells. IL-12 and TCR synergy likely results from IL-12-mediated phosphorylation of Lck, a key player in the TCR signaling (Vacaflores et al., 2016). To summarize, our work provides evidence that inflammatory cytokines and the TCR signal synergise to enhance MAIT cell effector function.

Our data also show evidence of TL1A mediated MAIT cell activation. In addition to its pro-inflammatory and costimulatory role in the human blood, TL1A is a gut-associated cytokine, and has been linked to IBD (Jin et al., 2012; Shih et al., 2009). Patients with IBD have higher levels of DR3 and TL1A expression in their mucosal T cells and macrophages (Bamias et al., 2003; Prehn et al., 2004). We highlight that TL1A enhanced the effector function of gut MAIT cells in the presence of a suboptimal dose of IL-12+IL-18, this suggests that TL1A may contribute to the amplification the inflammatory responses. Interestingly we found that TL1A impacted on MAIT cell TNF-α production in response to inflammatory stimuli but blockade did not impair responsiveness to bacteria. Thus TL1A blockade *in vivo* could potentially achieve an anti-inflammatory response while maintaining barrier function.

RNAseq data on differentially stimulated MAIT cells show that the transcriptional profiles of MAIT cells share common features yet also possess unique elements under various treatments. The extent of overlap of transcriptional regulation between C- and TC- stimulated MAIT cells was striking, yet both conditions elicited unique genes that were differentially regulated. Our data suggest that the addition of a TCR trigger played a role in driving further MAIT cell transcriptional changes.

In addition to bacterial killing, IL-26 promotes production of pro-inflammatory cytokines in the gut, thus is also associated with IBD pathogenesis. We here report that in addition to intestinal intraepithelial lymphocytes, colonic Th17 cells, and innate lymphoid cells (ILCs) from Crohn's Disease patients (Dambacher et al., 2009; A. Geremia et al., 2011), MAIT cells also contribute to the pool of IL-26 producing cells.

Further investigation of the activation profile of gut MAIT cells could perhaps shed light on their functions in IBD pathogenesis and disease progression.

In contrast to their roles in host defence and in inflammation there is yet to be a consensus established around the function of CD8+ T cells in wound healing and tissue repair. The precise function of different T cell types and subsets are largely unknown, and differs from tissue to tissue and between species. While γδ T cells are commonly believed to promote tissue healing process (Havran and Jameson, 2010), studies around CD4+ Tregs and CD8+ cytotoxic cells have suggested their diverse and distinct roles in wound healing. In the context of bone, fracture healing is accelerated in mice depleted with T and B cells (Toben et al., 2011), whereas other studies have shown that CD4+ Tregs have critical to repair and regenerate several tissues including skin and lung (Julier et al., 2017), and that CD8+ T cells aid skeletal muscle regeneration (Zhang et al., 2014) and wound closure in mice (Linehan et al., 2018). Our findings of upregulated expression of HBEGF and OSM, as well as a statistically enriched tissue repair transcriptional signature in MAIT cells supports the argument that at least a subset of CD8+ T cells can directly or indirectly promote tissue repair in mucosal layers and at barriers sites. Our finding that MAIT cells expressed a high level of HBEGF when maximally activated indicates that MAIT cells could also be a direct mediator of wound healing in breach of epithelial integrity. In addition, HBEGF is also involved in cancer metastases; the level of HBEGF is elevated in cancers including hepatocellular cancer, breast cancer, and CRC (Jayne et al., 2000). Whether activated gut MAIT cells could lead to differential outcomes in disease development and progression via release of HBEGF is yet to be defined.

Expression of an IBD-associated gene OSM was also increased in cytokine-activated blood-derived MAIT cells. OSM has been reported to be present in colonic tissues of IBD patients, and recently its role in eliciting inflammatory responses in the gut has been studied. West *et al.* demonstrated that OSM is a gut-related cytokine by contributing to inflammation in mouse intestinal stroma, particularly in synergy with TNF. In addition, OSM is upregulated in severe anti-TNF-therapy-refractive IBD patients (West et al., 2017). Similar to HBEGF, OSM has also been shown to induce migration of keratinocytes *in vitro* and skin repair *in vivo* (Boniface et al., 2007;

Hoffmann et al., 2011). Our finding of OSM upregulation in activated MAIT cells suggests an additional function for MAIT cells at the mucosal barrier.

The association of TCR-driven signals – potentially derived from interaction with commensal bacteria in the gut – with a tissue repair signature similar to that recently reported in IL17+ innate like T cells in murine skin models is intriguing (Linehan et al., 2018). We propose a model whereby in the human gut and potentially also in the liver, MAIT cells are continuously exposed to MR1-bound ligand derived from the commensal bacteria present in the microbiome. In the absence of inflammatory signals, this serves only to drive the minimal transcriptional signature associated with local homeostatic function. Consistent with this, gut MAIT cells show evidence of persistent TCR-driven signalling (upregulation of CD39 and PD1) together with downregulation of function compared to blood-derived cells. During loss of bowel integrity associated with inflammatory bowel disease, MAIT cells have been shown to be further activated in some studies, but whether this is a response to tissue injury or they play a causative role is yet to be defined. Our data would suggest that during tissue injury, the innate cytokines induced would drive the full activation signal seen, which includes a broad inflammatory response. We note that for example IL17F, which has been recently shown to be pathogenic in IBD models (Tang et al., 2018), is not induced by TCR signals alone but is strongly induced in the presence of inflammatory cytokines (fold change >400, $p < 10^{-6}$). Of note, our data are consistent with a similar finding of this same tissue repair signature of human MAIT cells activated by the stimulatory ligand 5-OP-RU TCR, as well as mouse MAIT cells during acute *Legionella longbeachae* infection (Hinks et al, Manuscript in preparation).

Together, our data demonstrate that a combined signal of cytokines and the TCR triggering elicit sustained effector function in human MAIT cells derived from the blood or the gut. We report TL1A as another potent inducer of specific MAIT cell functions. Furthermore, a comprehensive RNAseq study produced a set of genes that are significantly upregulated by fully activated blood MAIT cells. These genes suggest that in addition to their anti-bacterial, anti-viral, and cytotoxic roles, MAIT cells could potentially impact on direct bacterial killing, wound healing and an ongoing role in tissue remodelling.

FIGURE LEGENDS

Figure 1. **TCR signalling and cytokines combine to promote MAIT cell effector functions.** CD8 from healthy donors (n=6-9), enriched by CD8 magnetic beads, were analysed by flow cytometry following an overnight incubation with a range of cytokines in the presence or absence of αCD3/CD28. (**A**) Representative FACS plots show the CD8 population in PBMCs prior to (left panel) and post (right panel) CD8 magnetic bead enrichment, after gating on live CD3+ cells. (**B**) Representative histogram showing IFN-γ production by MAIT cells following αCD3/CD28+IL-12 trigger (top to bottom: increasing IL-12 concentration). (**C**) Proportion of $CD8^+$ MAIT cells producing IFN-γ following varying concentrations of cytokines, including IL-12 (n=8), IL-18 (n=8), or TL1A (n=7), respectively in the presence of plate-bound anti-CD3/CD28 antibodies. (**D, E**) Proportion of $CD8^+$ MAIT ($CD161^{++}V\alpha7.2^+$)/$CD161^+$/$CD161^-$ cells (n=8) producing IFN-γ and TNF-α following overnight incubation with suboptimal concentrations of IL-12 and IL-18, plus anti-CD3/CD28 beads at various bead-to-cell ratios. (**F**) Proportion of $CD8^+$ MAIT cells producing CD69, IFN-γ, and TNF-α when left untreated, or stimulated singly with 100ng/ml TL1A (n=9). (**G-I**) Proportion of $CD8^+$ MAIT/$CD161^+$/$CD161^-$ cells (n=6) producing IFN-γ, TNF-α, and CD69 following overnight incubation with suboptimal concentrations of IL-12 and IL-18, plus TL1A at varying concentrations. Each symbol represents the average response of tested donors, and bars represent s.e.m.. Results were analysed by repeated-measures one-way analysis of variance (ANOVA), with Bonferroni's multiple comparisons test. Data shown are pooled from two or three individual experiments. **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant.

Figure 2. **IL-15 and TL1A enhance the activation of MAIT cells sub-optimally stimulated with IL-12 and IL-18.** $CD8^+$ T cells were enriched from healthy PBMCs (n=7) and stimulated overnight with different combinations of cytokines: IL-12 used at 2ng/mL, IL-18 at 50ng/mL, IL-15 at 25ng/mL, and TL1A at 100ng/mL. (**A**) Representative histograms showing expression of IFN-γ, TNF-α, and GrB by MAIT cells following different cytokine triggering. (**B-D**) Proportion of $CD8^+$ MAIT cells

producing IFN-γ, TNF-α, and GrB when treated with conditions specified below the horizontal axis. (**E**) Proportion of $CD8^+$ MAIT cells producing IFN-γ, TNF-α, and GrB when treated with IL-15, TL1A, or the combination of both. Data shown are pooled from two individual experiments. Differences between conditions were analysed by one-way ANOVA with Bonferroni's multiple comparisons test. *p<0.05, ***p<0.001, ****p<0.0001.

Figure 3. **TCR- and Cytokine-activated MAIT cells possess distinct transcriptional profiles.** (**A**) Venn diagram showing genes that are significantly differentially modulated (p<0.05, fold change>4) in TCR (T)- / Cytokines (C)- / TCR+Cytokines (TC)- treated $CD8^+$ MAIT cells compared with untreated (UT) MAIT cells of three healthy individuals. The cytokine stimulation consisted of a cocktail of 4 cytokines including IL-12 (2 ng/ml), IL-18 (50 ng/ml), IL-15 (25 ng/ml), and TL1A (100 ng/ml). Genes with significantly altered expression levels are further divided into two sets, those that are upregulated (**B**), and downregulated (**C**) upon stimulation. (**D**) Heatmap showing 1,594 significantly (p < 0.05, fold change >4) differentially expressed transcripts between T/C/TC-stimulated and UT $CD8^+$ MAIT cells amongst the same three healthy individuals. (**E**) Visualisation of the $CD8^+$ MAIT cell transcripts elicited by differential stimulations in the subspace of the first two principle components. Each colored circle represents a sample and they are colour-coded in accordance with the conditions with which cells were stimulated, as illustrated on the right-hand side of the graph. (**F-H**) Volcano plots to visualize differentially expressed transcriptional profiles of activated $CD8^+$ MAIT cells compared with their unstimulated counterparts. Each point represents a single gene, and genes expressed at higher levels when stimulated are depicted on the upper-right corner of each plot. Genes upregulated by C and TC conditions include IFNG, TNF and GRMB, whose presence in activated MAIT cells on the protein level have been previously confirmed.

Figure 4. **Gene signatures in activated MAIT cells predict their antimicrobial and tissue repair functions.** (**A-C**) HBEGF, IL-26, and OSM expression levels in $CD8^+$ MAIT cells (n=5) examined by qPCR. Their expression levels are benched marked against GAPDH or β-actin, depending on the probe chosen for each gene. Data shown are pooled from two individual experiments. Differences between

conditions were analysed by one-way ANOVA with Bonferroni's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (**D-F**) Gene set enrichment summary plots for cytokine-stimulated (C), TCR-stimulated (T), or TCR+cytokine(TC)-stimulated sorted MAIT cell versus unstimulated (UT) cell-ranked genes. Non-significant for C vs UT, NES = 1.63, p < 0.0002 for T vs UT, NES = 1.57, p < 0.0002 for TC vs UT. (**G**) Frequencies of $CD161^{++}$/MAIT $CD8^{+}$ T cells expressing TNF-α in response to fixed *E.coli* trigger in the presence or absence of the anti-MR1 blocking antibody.

Figure 5. **MAIT cells in the gut respond to innate and adaptive signals.** (**A**) Right: Representative plots showing the percentage of MAIT cells as a proportion of $CD8^{+}$ T cells derived from the blood and control colon tissue (right). Left: Proportion of MAIT cells in $CD8^{+}$ T cells derived from the healthy blood (n = 32) and control colonic mucosa (n=13). (**B**) Paired comparison of the production of IFN-γ, TNF-α, and GrB by gut-derived $CD8^{+}$ MAIT cells (n=11, the number of samples used for each condition and measurement vary) in the presence or absence of TL1A in the presence of IL-12 (2ng/ml) and IL-18 (50ng/ml). (**C-E**) Proportion of $CD8^{+}$ MAIT cells from colon tissues (n=17) producing IFN-γ, GrB, and TNF-α when treated with conditions specified under the horizontal axis. Differences between conditions were analysed by two-way ANOVA with Bonferroni's multiple comparisons test. *p<0.05, ***p<0.001, ****p<0.0001, ns = not significant.

Figure 6. **MAIT cells derived from the gut display differential phenotypes to those from the blood**. Healthy PBMCs (n=32) and colonic LPLs (n=13) were analysed by flow cytometry. (**A-C**) Proportion of MAIT cells from the blood and tissue expressing GrB, PD-1 or CD39 at rest. (**D, E**) the gMFI of PD-1 and CD39 in these MAIT cells. (**F**) Representative plots showing the co-expression of PD-1 and CD39 in MAIT cells derived from the blood and the gut. (**G**) Proportion of $PD\text{-}1^{+}CD39^{+}$ MAIT cells from the blood and gut. Differences between conditions were analysed by Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplementary Figures

SFigure 1. **IL-15 and TL1A weakly enhance IFN-γ production by CD161$^+$ Vα7.2$^-$ CD8$^+$ T cells, and have no impact on promoting effector functions of CD161$^-$ CD8$^+$ T cells.** CD8$^+$ T cells were enriched from healthy PBMCs (n=7) and stimulated overnight with combinations of cytokines specified under the horizontal axis. (**A**) Gating strategy for CD8$^+$ MAIT (CD161^{++}Vα7.2$^+$) / CD161$^+$ Vα7.2$^-$ / CD161$^-$ Vα7.2$^-$ cells (**B**) Proportion of CD8$^+$ MAIT (CD161^{++}Vα7.2$^+$), CD161$^+$ Vα7.2$^-$, or CD161$^-$ Vα7.2$^-$ cells producing IFN-γ when stimulated with IL-12, IL-15, IL-18 and TL1A. (**C**) Proportion of CD8$^+$ CD161$^+$ Vα7.2$^-$ cells producing IFN-γ when stimulated with a range of conditions. (**D-F**) Proportion of CD8$^+$ CD161$^+$ Vα7.2$^-$ cells producing IFN-γ, TNF-α, and GrB when treated with conditions specified below the horizontal axis. (**G, I**) Proportion of CD8$^+$ CD161$^-$ Vα7.2$^-$ cells producing IFN-γ, TNF-α, and GrB when treated with conditions specified below the horizontal axis. Data shown are pooled from two individual experiments. Differences between conditions were analysed by one-way ANOVA with Bonferroni's multiple comparisons test. *p<0.05, ***p<0.001, ****p<0.0001.

SFigure 2. **Functional studies on the samples used for RNAseq confirmed by FACS.** Proportion of CD8$^+$ MAIT cells producing IFN-γ, TNF-α, and GrB when treated with identical conditions in the RNAseq experiment. Each dot corresponds to a donor in the RNAseq study. Differences between conditions were analysed by one-way ANOVA with Bonferroni's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant.

SFigure 3. **MAIT cells in the blood respond to innate and adaptive signals.** The percentage of MAIT cells expressing IFN-γ (**A**), GrB (**B**), or TNF-α (**C**) in response to various stimulations listed on the x-axis. Differences between conditions were analysed by one-way ANOVA with Bonferroni's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant.

SFigure 4. **DNase and Collagenase digestion does not impair MAIT cell response to combined TCR and cytokine stimulations.** (**A**) Gating strategy to identify CD8$^+$ MAIT cells from gut LPLs. (**B**) Proportion of CD8$^+$ MAIT cells producing CD69, IFN-γ, and TNF-α after overnight stimulations. CD8$^+$ MAIT cells were derived from PBMCs, which, prior to stimulation, were either rested in the normal media or

stirred in the digestion media for 12 hours. (**C**) Representative plot showing how to identify MAIT cells from the gut by using either a conventional Vα7.2 TCR staining antibody or the MR1-tetramer staining antibody, in combination with CD161 staining.

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Figure 1

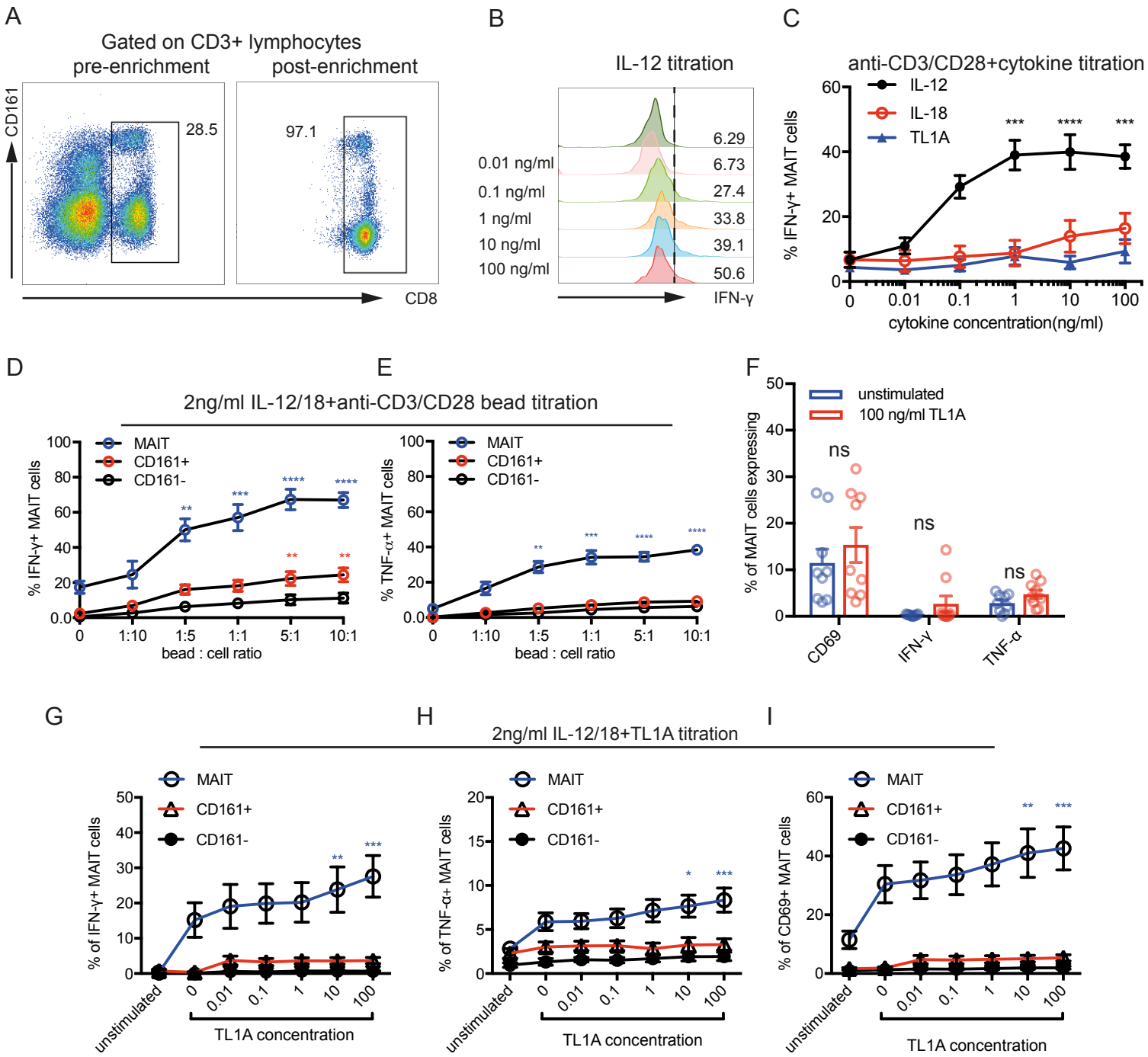

A
Gated on CD3+ lymphocytes
pre-enrichment
post-enrichment
CD161
28.5
97.1
CD8
B
IL-12 titration
0.01 ng/ml
0.1 ng/ml
1 ng/ml
10 ng/ml
100 ng/ml
6.29
6.73
27.4
33.8
39.1
50.6
IFN-γ
C
anti-CD3/CD28+cytokine titration
IL-12
IL-18
TL1A
% IFN-γ+ MAIT cells
cytokine concentration(ng/ml)
D
2ng/ml IL-12/18+anti-CD3/CD28 bead titration
MAIT
CD161+
CD161-
% IFN-γ+ MAIT cells
bead : cell ratio
E
% TNF-α+ MAIT cells
F
unstimulated
100 ng/ml TL1A
% of MAIT cells expressing
ns
CD69
IFN-γ
TNF-α
G
H
I
2ng/ml IL-12/18+TL1A titration
% of IFN-γ+ MAIT cells
% of TNF-α+ MAIT cells
% of CD69+ MAIT cells
unstimulated
TL1A concentration

Figure 2

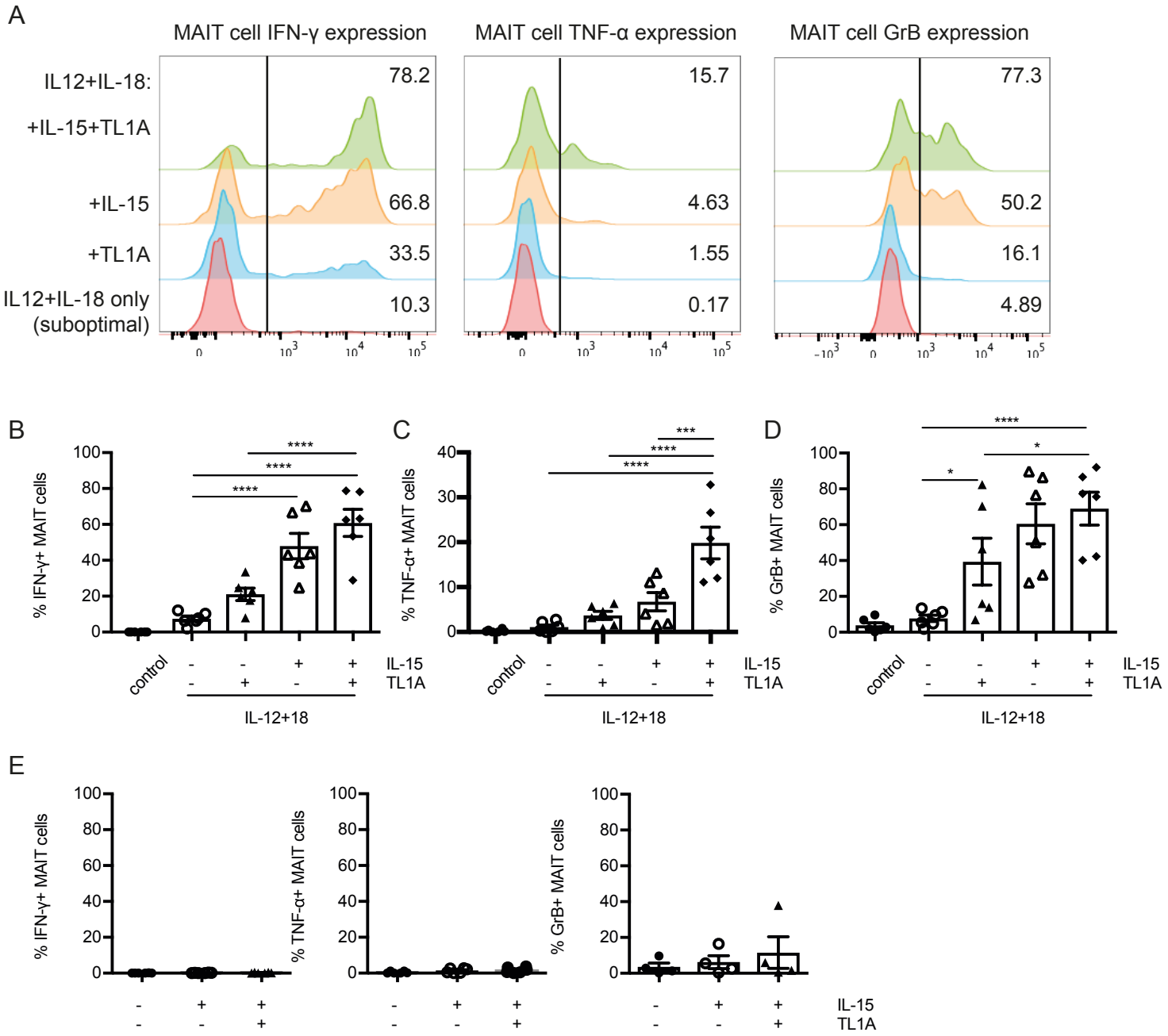
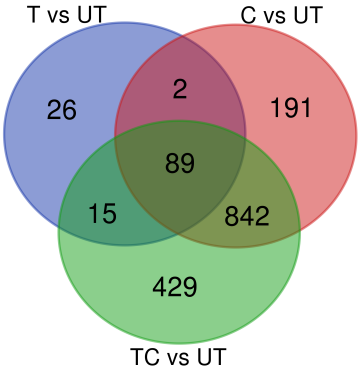

A
MAIT cell IFN-γ expression
MAIT cell TNF-α expression
MAIT cell GrB expression
IL12+IL-18:
+IL-15+TL1A
+IL-15
+TL1A
IL12+IL-18 only
(suboptimal)
78.2
66.8
33.5
10.3
15.7
4.63
1.55
0.17
77.3
50.2
16.1
4.89
B
% IFN-γ+ MAIT cells
C
% TNF-α+ MAIT cells
D
% GrB+ MAIT cells
control
IL-15
TL1A
IL-12+18
E
% IFN-γ+ MAIT cells
% TNF-α+ MAIT cells
% GrB+ MAIT cells
IL-15
TL1A

Figure 3

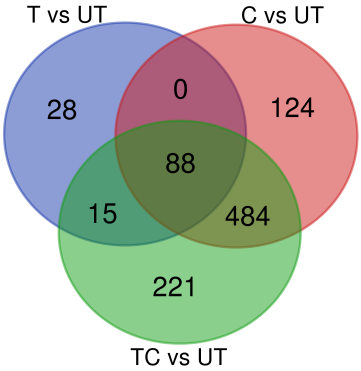
A

significantly altered genes



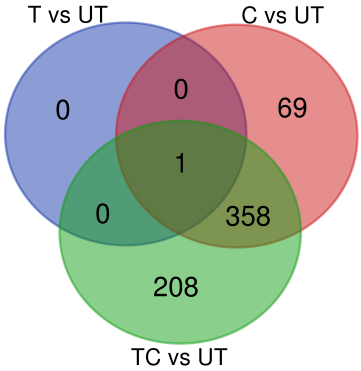
B

genes upregulated in stimulation

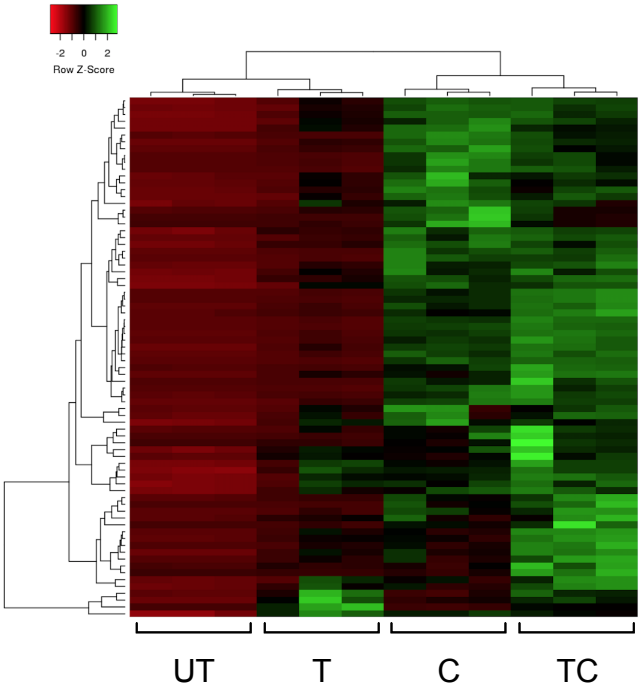


C

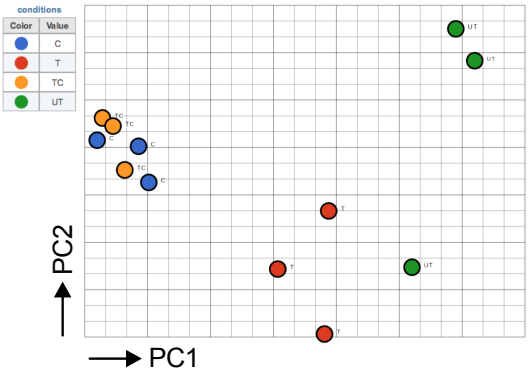
genes downregulated in stimulation



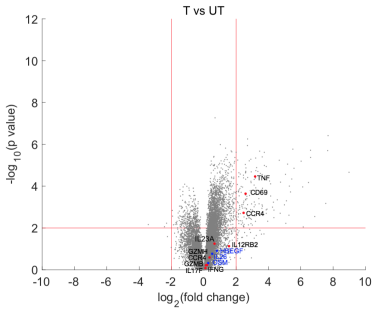
D



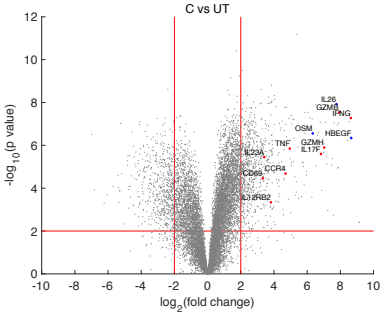
E



F



G



H

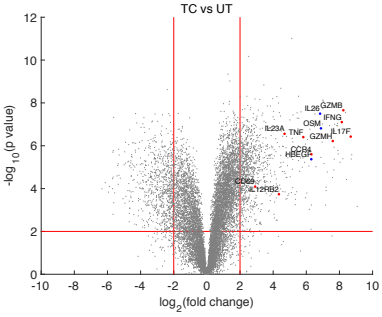
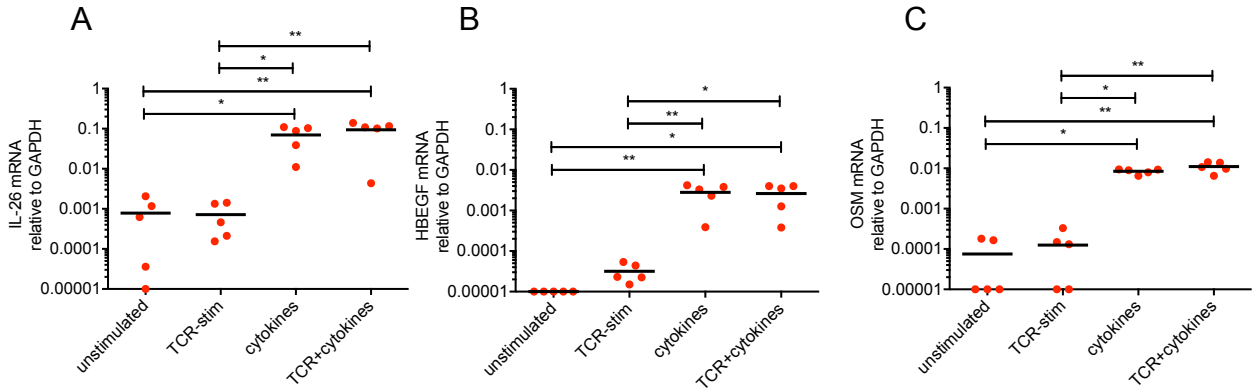
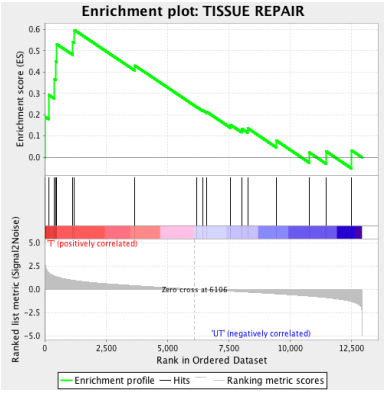


Figure 4

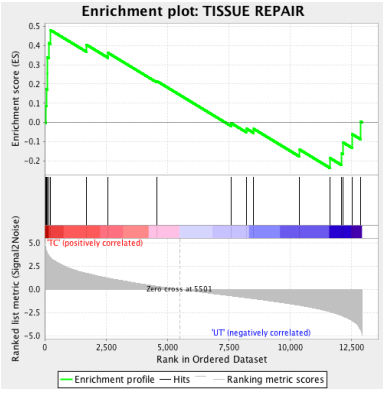


D



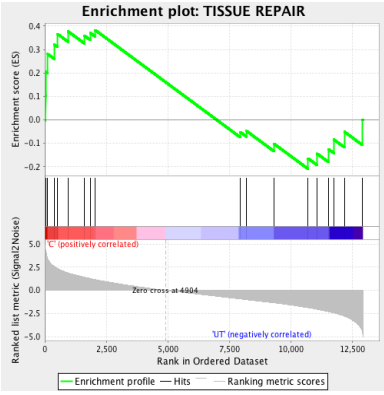
Data set: T vs UT
Enrichment Score (ES): 0.6
Normalised ES (NES): 1.63
Nominal P value: <0.0002

E



Data set: TC vs UT
Enrichment Score (ES): 0.48
Normalised ES (NES): 1.57
Nominal P value: <0.0002

F



Data set: C vs UT
Enrichment Score (ES): 0.38
Normalised ES (NES): 1.34
Nominal P value: 0.304

G

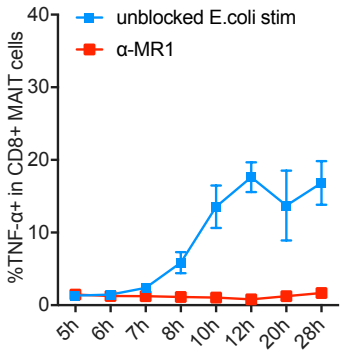


Figure 5

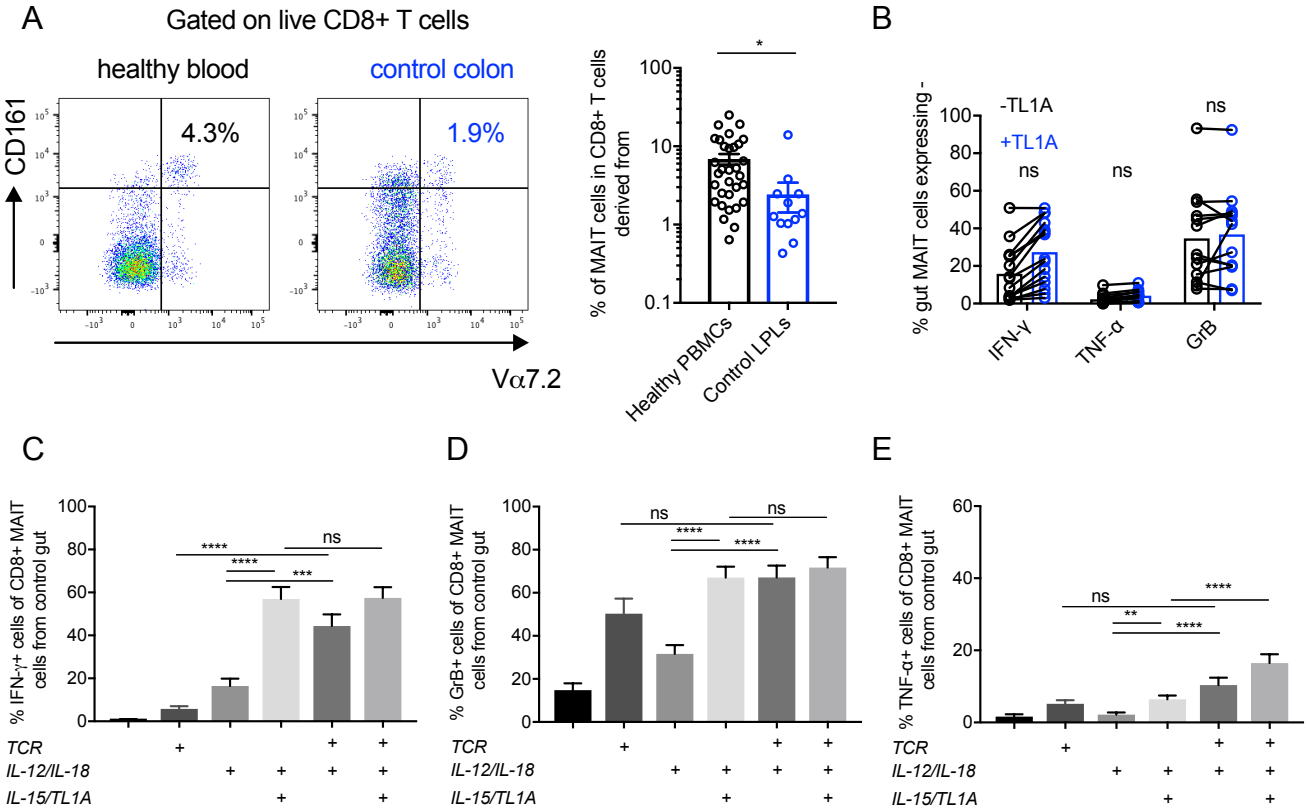
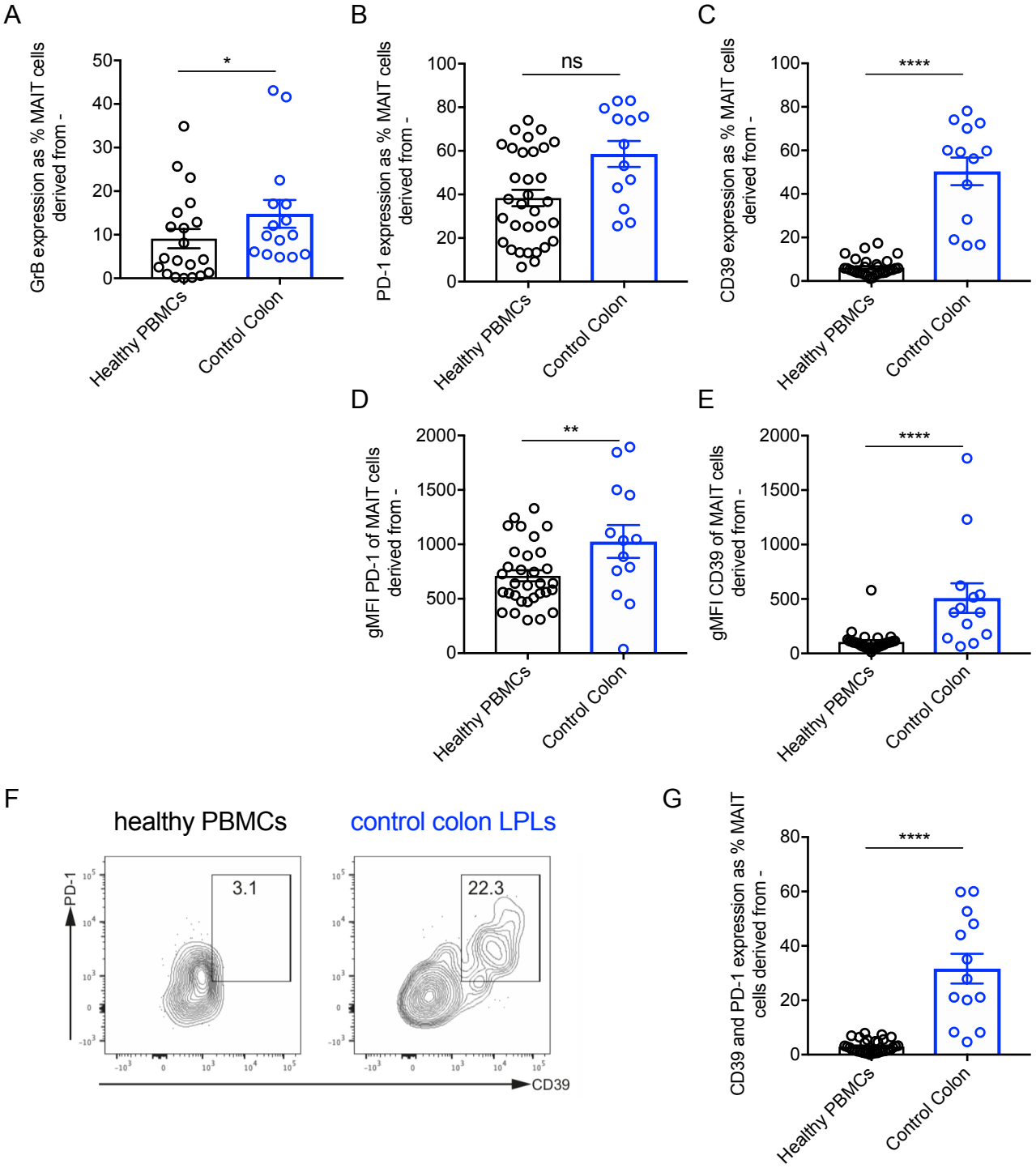

A
Gated on live CD8+ T cells
healthy blood
control colon
CD161
4.3%
1.9%
Vα7.2
% of MAIT cells in CD8+ T cells derived from
Healthy PBMCs
Control LPLs
B
% gut MAIT cells expressing -
-TL1A
+TL1A
ns
IFN-γ
TNF-α
GrB
C
% IFN-γ+ cells of CD8+ MAIT cells from control gut
D
% GrB+ cells of CD8+ MAIT cells from control gut
E
% TNF-α+ cells of CD8+ MAIT cells from control gut
TCR
IL-12/IL-18
IL-15/TL1A

Figure 6


A
GrB expression as % MAIT cells derived from -
50
40
30
20
10
0
*
Healthy PBMCs
Control Colon
B
PD-1 expression as % MAIT cells derived from -
100
80
60
40
20
0
ns
Healthy PBMCs
Control Colon
C
CD39 expression as % MAIT cells derived from -
100
80
60
40
20
0
****
Healthy PBMCs
Control Colon
D
gMFI PD-1 of MAIT cells derived from -
2000
1500
1000
500
0
**
Healthy PBMCs
Control Colon
E
gMFI CD39 of MAIT cells derived from -
2000
1500
1000
500
0
****
Healthy PBMCs
Control Colon
F
healthy PBMCs
control colon LPLs
PD-1
3.1
22.3
CD39
G
CD39 and PD-1 expression as % MAIT cells derived from -
80
60
40
20
0
****
Healthy PBMCs
Control Colon