IL-7 Licenses Activation of Human Liver Intrasinusoidal Mucosal-Associated Invariant T Cells

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Human mucosal-associated invariant T (MAIT) cells are a T cell population characterized by the expression of a semi-invariant TCR capable of recognizing bacterial products in the context of MR1. MAIT cells are enriched in the human liver, which is constantly exposed to bacterial products from the intestine. Whether this specific parenchymal localization influences their function remains unknown. We analyzed MAIT cells resident in the vascular bed of livers and showed that they represented the majority of T cells expressing NK markers and the dominant IL-17A$^+$ T cell subset in the human liver sinusoids. In comparison with MAIT cells purified from peripheral blood, intrasinusoidal MAIT cells expressed markers of T cell activation; however, TCR-mediated cytokine production was equally suppressed in both circulating and intrasinusoidal MAIT cells. MAIT cells also expressed high levels of IL-7R, and we showed that IL-7, a cytokine produced by hepatocytes during inflammation, regulated TCR-mediated activation of MAIT cells, licensing them to dramatically increase Th1 cytokines and IL-17A production. Our quantitative and functional data indicate that MAIT cells are a specialized cell population highly adapted to exert their immune functions in the vascular network of the liver. ***The Journal of Immunology*****, 2013, 190: 3142–3152.**

The vast liver vascular bed, comprising narrow fenestrated capillaries, is home to a large population of T cells expressing NK markers (1, 2). In mice, these T cells are predominantly invariant NKT (iNKT) cells expressing an invariant TCRαβ, formed by the Vα14 chain paired with the Vβ2, Vβ7, or Vβ8 chain. These cells patrol the murine liver sinusoids where they represent ∼20% of resident lymphocytes and can release proinflammatory cytokines (2, 3). iNKT cells recognize glycolipid Ags presented by the nonpolymorphic CD1d molecule (4) and play an important role in the pathogenesis with different etiologies (5–8). However, in humans, the identity of intrahepatic T cells expressing NK markers is controversial (1, 2, 9).

The frequency of CD1d-restricted human iNKT cells expressing the invariant TCR Vα24-Jα18 chain is <1% of total T cells in the pathological livers (1, 9, 10). Although classical TCR αβ T cells with virus specificity and coexpressing NK markers have also been detected in the liver (11), their intrahepatic frequency is small. Nonetheless, recent studies described a population of lymphocytes, enriched in the human liver and intestine, which has limited TCR repertoire diversity and shares some functional features with iNKT cells (12–15). These T cells are called mucosal-associated invariant T (MAIT) cells, and they express TCR Vα7.2-Jα33 chain (12) and high levels of the NK marker CD161 (16). MAIT cell development is linked to microbial colonization during early life (13, 17), but unlike iNKT cells, MAIT cells are stimulated by ligand(s), recently suggested to be microbial vitamin B metabolites (18), presented by the MHC class I–like molecule MR1 (14). Activated peripheral MAIT cells produce large quantities of IFN-γ and TNF-α but also possess a Th17-signature profile, because they release IL-17A after mitogen stimulation and express the Th17-associated transcription factor *RORC* and the Th17-related chemokine receptor CCR6 (16, 17). The functional and developmental profile of MAIT cells, together with their preferential localization in the vascular bed of the gut and liver, indicate that MAIT cells may play a major role in controlling the dissemination of pathogens that could escape the gut mucosal barrier (19) or be implicated in liver pathological processes of different etiologies (20). However, the objective difficulties in obtaining samples from human parenchymal organs, particularly in nonpathological situations, have hampered the functional characterization of MAIT cells from the human liver. Indeed, in most of the studies, MAIT cells from cord and peripheral blood compartments were used, whereas liver-resident MAIT cells have been scarcely investigated (16, 17).

Taking advantage of the availability of cells purified from nonpathological liver grafts preceding liver transplantations, we show that the large majority of intrasinusoidal T cells expressing NK markers (CD3$^+$CD161$^+$CD56$^+$) are MAIT cells, and we provide a detailed profile of their phenotypic and functional features.

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Abbreviations used in this article: B-MAIT, blood-derived mucosal-associated invariant T; iNKT, invariant NKT; Liv-MAIT, liver-derived mucosal-associated invariant T; MAIT, mucosal-associated invariant T.

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Materials and Methods

Human samples

Liver-associated mononuclear cells were collected from living donors (n = 20) after portal flush using cold (4°C) preservation solution following removal of the right lobe of the donors' livers. Collection was performed according to the standard protocol preceding liver transplantation (21). Blood samples were obtained from age-matched healthy controls (age 23–48 y; n = 10). Subsequently, liver resident/intrasinusoidal cells and PBMCs were isolated by density-gradient centrifugation on Ficoll-Hypaque. The study was approved by the Gleneagles Hospital Ethics Committee, and all patients gave written informed consent.

Abs and reagents

mAbs of anti–human-CD3-eFluor 605NC (clone OKT3) or -FITC (UCHT1), anti–CD4-eFluor 650NC (RPA-T4), anti–CD7-FITC (4H9), anti–CD127-allophycocyanin-eFluor 780 (eBioRDR5), anti–CD161-PerCP-Cy5.5 (HP-3G10), anti–Vα24-Jα18 TCR-PE (6B11), anti–HLA-DR-Alexa Fluor 700 (LN3), anti–CCR6-PE-Cy7 (R6H1), anti–IFN-γ–PE–Cy7 (4S.B3), and anti–IL-17A–Alexa Fluor 488 (eBio64DEC17) were obtained from eBioscience (San Diego, CA). Anti–CD3-PE and -Biotin (SK7), anti–CD8-V500 or –PE-Cy7 (RPA-T8), anti–CD8β-allophycocyanin (2ST8.5H7), anti–CD11c-allophycocyanin (B-1y6), anti–CD14-PE-Cy7 (M5E2), anti–CD20-V450 (L27), anti–CD25-V450 (M-A251), anti–CD38-V450 (HIT2), anti–CD45RA-allophycocyanin (L48), anti–CD45RO-PE-Cy7 (UCHL-1), anti–CD56-PE-Cy7 (B159), anti–Bcl2-FITC (Bcl-2/100), anti–Ki67-FITC (B56), anti–PD1-PE-Cy7 (EH12.1), anti–CCR4-V450 (1G1), anti–IFN-γ–V450 (B27), anti–TNF-α–PE–Cy7 (MAb11), anti–IL-2–PerCP–Cy5.5 (MQ1-17H12), anti–IL-4–FITC (MP4-25D2), anti-IL-10–allophycocyanin (JES3-19F1), and anti–IL-17A–V450 (N49-653) Abs were obtained from Becton Dickinson (BD, San Jose, CA). Anti–CD3-FITC (HIT3a), anti–CD16-allophycocyanin-Cy7 (3G8), anti–CD27-allophycocyanin-Cy7 (O323), anti–CD28-Alexa Fluor 700 (CD28.2), anti–CD56-FITC or –allophycocyanin-Cy7 (HCD56), anti–CD69-Alexa Fluor 700 (FN50), anti–CD123-PerCP-Cy5.5 (6H6), anti–Vα7.2 TCR-PE or -allophycocyanin (3C10), and anti–CXCR6-Alexa Fluor 647 (TG3/CXCR6) Abs were obtained from BioLegend (San Diego, CA). Anti–TCRγδ-FITC or -PE (5A6.E9), anti–Granzyme B-allophycocyanin (GB11), and streptavidin-Qdot605 Abs were obtained from Invitrogen (Carlsbad, CA). Anti–CCR10-PE (314305) and anti–IL-22–allophycocyanin (142928) Abs were obtained from R&D Systems (Minneapolis, MN). Anti–CD161-Biotin (191B8) Ab was obtained from Miltenyi Biotec (Auburn, CA). Live/Dead Fixable Dead Cell Stain Kits (yellow and near infrared) were obtained from Invitrogen. Recombinant human IL-1β and IL-7 cytokines were obtained from R&D Systems. Recombinant human IL-23 cytokine was obtained from eBioscience. IL-1β and IL-23 were used at 50 ng/ml, whereas IL-7 was used at 10 ng/ml.

Flow cytometry

Mononuclear cells were washed in PBS and stained with Live/Dead Fixable Dead Cell Stain. They were then washed in staining buffer (PBS, 1% BSA [Roche, Basel, Switzerland] and 0.1% sodium azide [Sigma-Aldrich, St. Louis, MO]) and stained for expressed cell surface molecules. Stained cells were analyzed on a BD FACSCanto or LSR II cytometer. MAIT cells in total liver/blood mononuclear populations were gated based on $CD3^+$ $CD161^{high}V\alpha7.2^+$ expression. Following overnight stimulation with PMA (2 ng/ml) and ionomycin (1 μg/ml) or anti-CD3/CD28–coupled beads (Invitrogen, 1:1 bead/cell ratio) in the presence of brefeldin A (2 μg/ml), surface-stained cells were fixed and permeabilized (Cytofix/Cytoperm; BD) before being stained for produced cytokines. Cells were then washed in staining buffer with 0.1% saponin (Sigma-Aldrich) prior to acquisition on a FACSCanto or LSR II cytometer. Data were analyzed using FACSDiva software (BD). All flow cytometry plots and graphs are displayed as $\log_{10}$ fluorescence.

Cell-sorting strategy

MAIT cells were sorted based on $CD3^+CD161^{high}V\alpha7.2^+$ expression and confirmed to be $CD4^-\gamma\delta\text{-}TCR^-$ (13). Conventional CD8 T cells were sorted from the $MAIT^-$ fraction based on CD8 expression. These cells were confirmed to be $CD3^+CD8^+$ and not $V\alpha7.2^+CD161^{high}$.

MR1 knockdown and bacterial stimulation

MR1-specific small interfering RNA (Sigma-Aldrich) was transfected using Lipofectamine 2000 (Invitrogen) into the THP-1 cell line. After 2 d, THP-1 cells were coincubated with sorted MAIT cells in AIM-V media (Life Technologies) supplemented with 2% AB serum (Invitrogen). *Pseudomonas aeruginosa* was added to the culture at a multiplicity of infection 10 per THP-1 cell and incubated overnight. Brefeldin A (10 μg/ml) was added 5 h before cells were washed and stained for intracellular cytokine responses. Total RNA was isolated from transfected THP-1 cells using an RNeasy Mini Kit (QIAGEN, Germantown, MD), according to the manufacturer's protocol. cDNA was synthesized using SMART MMLV Reverse Transcriptase (Clontech, Mountain View, CA), and 20-mer oligo-dT primers were synthesized from 1st BASE (Singapore). cDNA was used for quantitative PCR using TaqMan Gene Expression Assays specific for MR1, TaqMan Universal PCR Master Mix, and the 7500 Real-Time PCR System (all from Applied Biosystems, Foster City, CA).

Cytokine multiplex bead–based assay and ELISA

Sorted MAIT cells and $CD4^+$ and $CD8^+$ T cells were cultured for 18 h at 300,000 cells/well in 96-well plates and were stimulated or not with either PMA and ionomycin or anti-CD3/CD28–coupled beads. Cytokine concentrations within supernatants were assessed by cytokine multiplex bead–based assay, according to the manufacturer's instructions (Luminex, Austin, TX). ELISA of IL-22 and CCL20 was performed according to the manufacturer's instructions (R&D Systems).

Human immunology gene-expression analysis

Cell lysates from a minimum of 10,000 cells were analyzed using the preassembled nCounter GX Human Immunology Kit and the nCounter system (NanoString Technologies, Seattle, WA), according to the manufacturer's instructions. A cut-off of 2 times the mean of the negative controls supplied in the kit was used to discriminate against nonspecific probe binding (noise). Samples were then normalized based on the geometric means of both the supplied positive controls and the panel of housekeeping genes, as recommended by the manufacturer. The coefficient of variation (SD of the normalized counts across all samples/mean normalized counts across all samples, expressed as a percentage) of each gene was also calculated, and the mean coefficient of variation of the housekeeping genes was used as a cut-off to filter out genes that remain stable across all samples analyzed. Log_2 mean normalized count values were used to generate the heatmap using the Matrix2png web interface (22).

Clustering

Log_2 mean normalized counts were used for clustering analysis. Data were normalized (mean centering of genes), and hierarchical clustering of genes was produced with Cluster 3.0 (similarity metric: Euclidean distance, Clustering method: Average linkage) and visualized in TreeView (23).

IL-7 treatment

Cells were left untreated or were treated with one dose of IL-7 or a combination of IL-1β and IL-23 and cultured overnight or for 2 or 4 d. Cells were subsequently stimulated or not with anti-CD3/CD28–coupled beads (Invitrogen) overnight and then analyzed with NanoString and flow cytometry. For expression of Vα7.2, CD8αα, and CD8αβ molecules, whole cells were either treated or not with one dose of IL-7 and subsequently monitored for 4 d. Cells were then immediately stained for respective surface molecules and analyzed by flow cytometry.

Statistical analysis

The nonparametric Mann–Whitney U test was used to determine the statistical significance of differences, and p values <0.05 were considered statistically significant.

Results

MAIT cells are present in large numbers in normal human livers

We first analyzed a potential contamination of peripheral blood on the hepatic perfusion in the first three donor samples by measuring the frequency of $CD56^{bright}$ NK, $CD4^+$ T, $CD8^+$ T, and B cells in the first 500 ml of portal vein flush in comparison with subsequent flushes. Supplemental Fig. 1 shows that the cells collected from perfusion were enriched in $CD56^{bright}$ NK cells; they also had higher frequencies of $CD8^+$ T cells compared with $CD4^+$ T cells and few B cells, a signature of intrahepatic immune cells (1). There were minimal differences between the first and subsequent flushes, suggesting that peripheral blood contamination

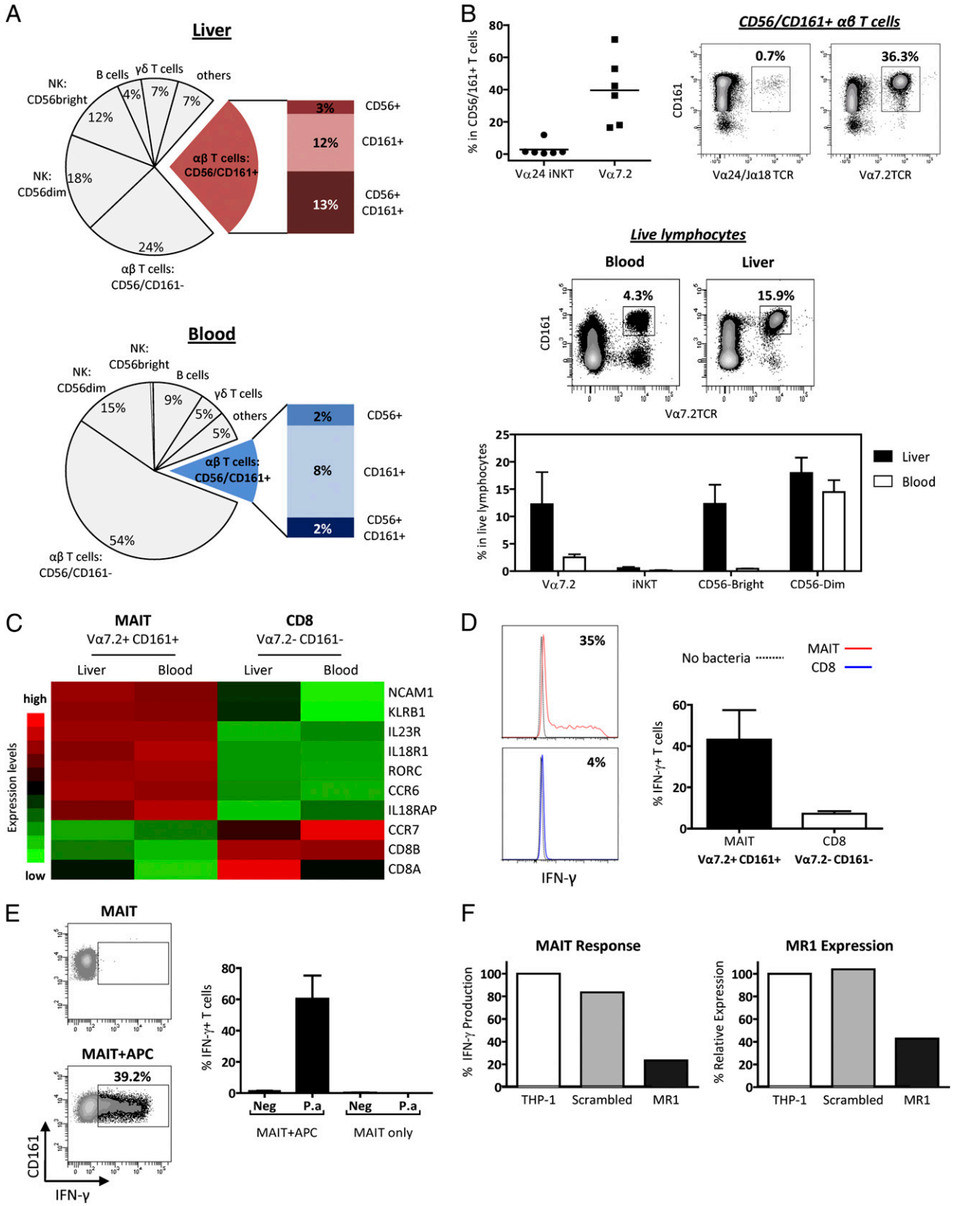

FIGURE 1. MAIT cells preferentially home to the liver and constitute a majority of the intrasinusoidal $CD56^{+}CD161^{+}$ T cells. (**A**) Comparison of lymphocyte distribution in the intrasinusoidal ($n = 6$, *upper panel*) and blood compartments ($n = 7$, *lower panel*). Bars show the frequency of CD56- and/or CD161-expressing T cells among total lymphocytes. (**B**) Frequency of Vα24-Jα18^{+} iNKT cells and $V\alpha7.2^{+}CD161^{+}$ cells among T cells expressing CD56 and CD161 in the liver (*top left panel*, summary plot; *top right panels*, representative dot plots). Dot plots show the frequency of $V\alpha7.2^{+}CD161^{+}$ cells among total live lymphocytes from both compartments (*middle panels*). Bars show the mean frequency of $V\alpha7.2^{+}CD161^{+}$, $CD56^{bright}$, and $CD56^{dim}$ NK and iNKT cells among total live lymphocytes purified from both compartments (*bottom panel*). (**C**) NanoString gene expression analysis of previously characterized MAIT-associated genes in sorted ($CD3^{+}CD161^{high}V\alpha7.2^{+}CD4^{-}\gamma\delta\text{-}TCR^{-}$) MAIT cells ($n = 4$) and $CD8^{+}$ T cells ($CD3^{+}CD8^{+}$ and not $V\alpha7.2^{+}CD161^{high}$) ($n = 4$) from both compartments (each two donors). (**D**) Liv-MAIT cell ($n = 3$) and $CD8^{+}$ T cell ($n = 3$) responses against *P. aeruginosa* (multiplicity of infection 10). Representative graphs are shown (*left panel*). (**E**) Liv-MAIT (*Figure legend continues*)

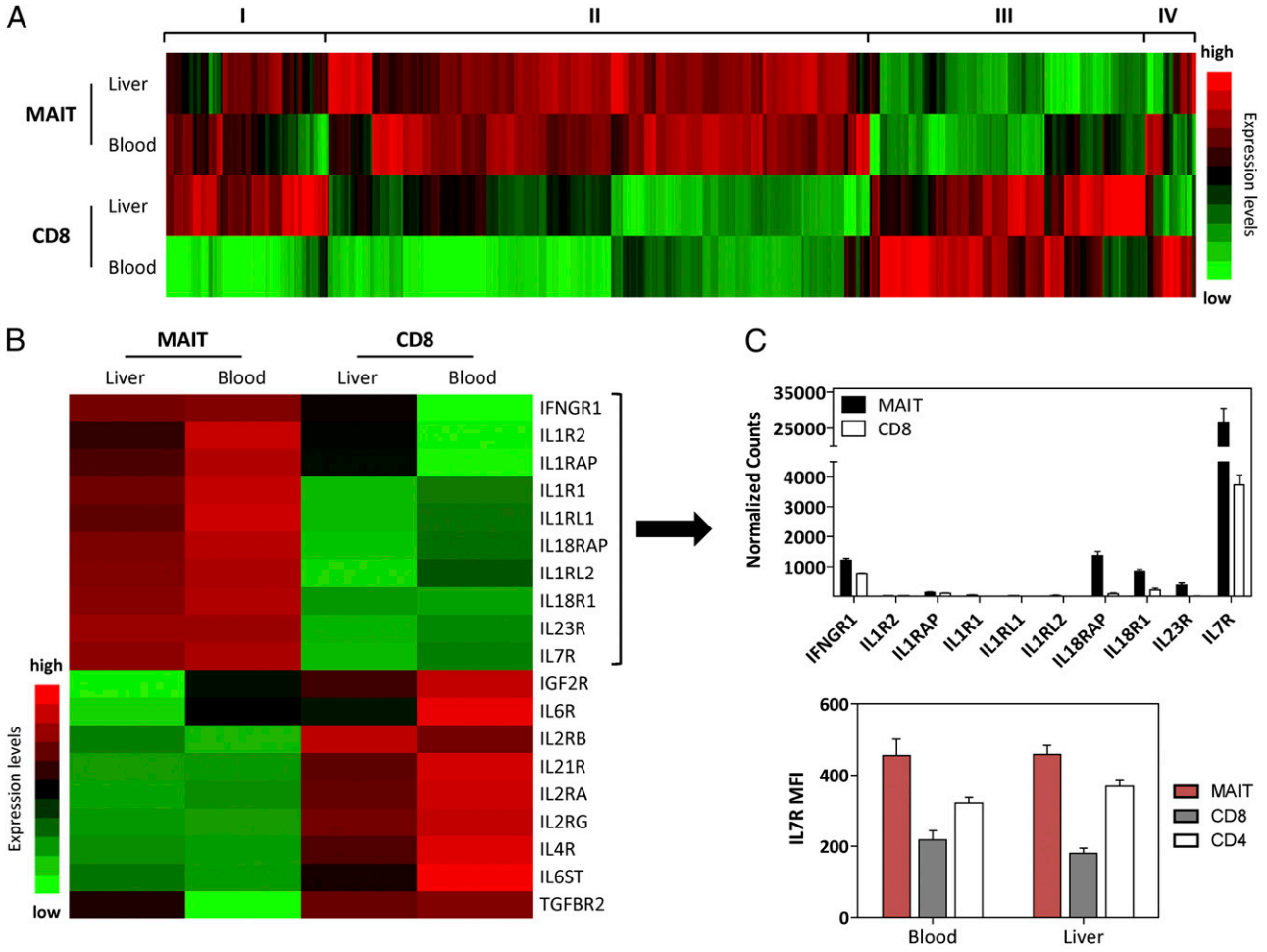

FIGURE 2. MAIT cells express a distinct profile of immune genes. Sorted MAIT cells ($CD3^+CD161^{high}V\alpha7.2^+CD4^-\gamma\delta\text{-}TCR^-$) and conventional $CD8^+$ T cells ($CD3^+CD8^+$ and not $V\alpha7.2^+CD161^{high}$) from the liver and blood compartments were analyzed. NanoString expression analysis of all immune genes (**A**), including cytokine receptors (**B**), in sorted MAIT cells ($n = 4$) and $CD8^+$ T cells ($n = 4$) from the liver and blood compartments (two donors from each compartment). (**C**) Bar graphs show the mean normalized counts of cytokine receptor mRNAs that are expressed more frequently in MAIT cells than in $CD8^+$ T cells (*upper panel*). The mean fluorescence intensity (MFI) of IL-7R in MAIT cells, $CD8^+$, and $CD4^+$ T cells in the liver and blood are shown (*lower panel*).

was negligible. Note that we did not obtain matched blood samples from the liver donors because of ethical/safety reasons; hence, peripheral blood from age-matched healthy individuals was used.

The percentage of the intrasinusoidal TCR αβ T cell population expressing NK markers CD161 and CD56 was investigated, together with that of other lymphocyte populations ($CD56^{bright/dim}$ NK, B, TCR γδ cells) in six donors (Fig. 1A, Supplemental Fig. 2). T cells expressing CD161 and/or CD56 were highly enriched in the intrasinusoidal lymphocytes, with ∼28% of the total intrasinusoidal T cells expressing at least one of the NK markers compared with only 12% in the blood of healthy subjects ($n = 7$, Fig. 1A). The frequency of intrasinusoidal T cells expressing NK markers was similar to that of NK cells (∼30% of total lymphocytes) and slightly higher than that of conventional $CD56^-CD161^-$ T cells (Fig. 1A).

To determine the identity of intrasinusoidal $CD161^+CD56^+$ T cells, mononuclear cells from six donors were analyzed for the expression of the invariant TCR chain Vα24-Jα18, a marker of iNKT cells, as well as for the expression of CD161 and TCR Vα7.2, characteristic of MAIT cells. Although iNKT cells were barely detectable, MAIT cells represented ∼40% of the intrasinusoidal T cells expressing NK markers (Fig. 1B). The frequency of MAIT cells in intrasinusoidal lymphocytes was ∼15%, considerably more than what we observed in the blood samples (∼3%, Fig. 1B). Quantitatively, within the healthy human livers, MAIT cells ($CD3^+V\alpha7.2^+CD161^+$) were found in similar quantities to $CD56^{bright}$ or $CD56^{dim}$ NK cells (Fig. 1B).

Next, we defined the molecular identity of liver-derived $V\alpha7.2^+CD161^+$ T cells by directly quantifying published MAIT-associated gene expression (17) in sorted cells from liver and blood and compared them with those of conventional $CD8^+$ T cells ($CD3^+CD8^+V\alpha7.2^-$). Sorted $CD3^+V\alpha7.2^+CD161^+CD4^-\gamma\delta\text{-}TCR^-$ T cells expressed higher levels of MAIT-signature genes (*KLRB1*, *IL23R*, *IL18R1*, *RORC*, *CCR6*) than did conventional $CD8^+$ T cells, irrespective of their anatomical origin (Fig. 1C). Furthermore, despite being predominantly $CD8^+$, liver-

cell ($n = 2$) response against *P. aeruginosa* in the presence or absence of APCs. Representative dot plots are shown (*left panel*). (**F**) Liv-MAIT cell response against *P. aeruginosa* in the presence of MR1-knockdown THP-1 cells (*left panel*). The MR1 mRNA levels in THP-1 cells before and after knockdown experiments (*right panel*).

derived Vα7.2$^+$CD161$^+$ T cells expressed lower levels of *CD8α* and *CD8β* genes in comparison with classical CD8$^+$ T cells (Fig. 1C).

We also assessed whether liver-derived MAIT (Liv-MAIT) cells responded to bacterial infection, as described for blood-derived MAIT (B-MAIT) cells (14). After stimulation with APCs infected with the Gram-negative bacteria *P. aeruginosa*, Liv-MAIT cells, but not conventional CD8$^+$ T cells, produced large quantities of IFN-γ (Fig. 1D). MAIT cell activation also required the presence of APCs (Fig. 1E) and MR1 molecules, because small interfering RNA knockdown of MR1 expression on APCs suppressed MAIT cell activation (Fig. 1F). In addition, analysis of Vβ-chains of Liv-MAIT and B-MAIT cells revealed a similar repertoire to that previously described (12, 17), with most MAIT cells from both compartments expressing Vβ2, 3, or 13.2 (data not shown).

Thus, in humans, a large proportion of lymphocytes resident in the intrasinusoidal compartment of healthy livers is composed of MAIT cells, which represent, on average, 15% of intrasinusoidal lymphocytes.

Intrasinusoidal MAIT cells display a "partially activated" phenotype

We determined whether the functional and phenotypic profile of Liv-MAIT cells differed from their blood counterparts by analyzing the immune gene expression and the phenotypic characterization of chemokine/cytokine receptors and T cell memory/activation markers.

Immune genes (n = 394) were differentially expressed between paired MAIT and CD8$^+$ T cells sorted from the intrasinusoidal and peripheral compartments (two different subjects from each compartment, Fig. 2A). MAIT cells were remarkably similar, irrespective of their anatomical origin, but they possessed an expression profile distinct from conventional CD8$^+$ T cells. Clustering analysis showed four gene clusters corresponding to genes uniquely upregulated in MAIT cells (cluster II, n = 206) or in CD8$^+$ T cells (cluster III, n = 105), as well as genes that were not distinctly enriched in either population (cluster I and IV, n = 63 and n = 20). In addition, the majority (~80%) of the differentially expressed genes were clustered into either a MAIT cell– or CD8$^+$ T cell–specific immune gene-expression profile. A major difference between MAIT cells and conventional CD8$^+$ T cells was detectable in the expression of cytokine and chemokine receptors (Fig. 2B, Supplemental Fig. 3A, 3B, respectively). Cytokine receptors important in the differentiation of Th17 cells (*IL-1BR* and *IL-23R*) were upregulated in Liv-MAIT and B-MAIT cells but not in conventional CD8$^+$ T cells. MAIT cells also preferentially expressed CCR6 and CXCR6 but not CCR7 or CXCR3 (Supplemental Fig. 3A, 3B). In particular, MAIT cells presented high levels of *IL-7R* mRNA, with values ≥1-log higher than the expression of cytokine receptors known to be characteristic of MAIT cells (*IL-23R* and *IL-18R1*, Fig. 2B, 2C). Importantly, these expression profiles were confirmed at the protein level (cytokine receptors, Fig. 2C; chemokine receptors, Supplemental Fig. 3B). Thus, MAIT and conventional CD8$^+$ T cells expressed a profoundly different array of immune genes, whereas Liv-MAIT and B-MAIT cells were remarkably similar. Only 18 of the 394 differentially expressed genes analyzed displayed ≥2-fold differential expression between Liv-MAIT and B-MAIT cells (Supplemental Fig. 3C).

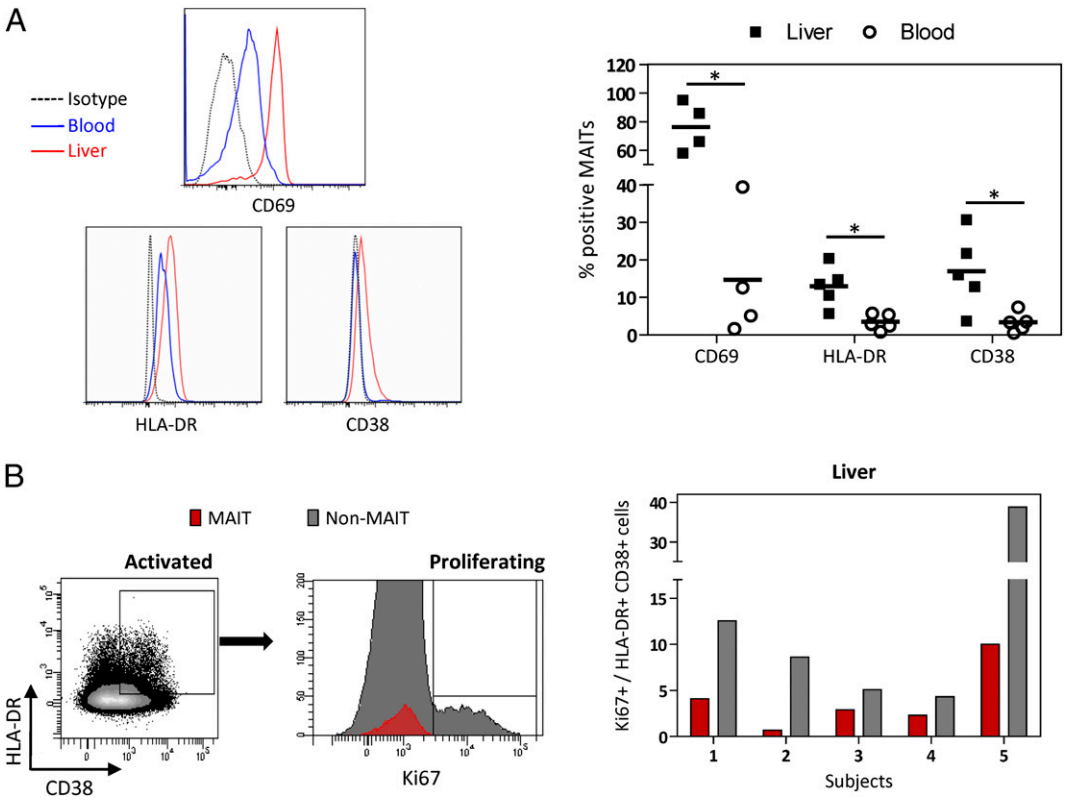

FIGURE 3. Liv-MAIT cells possess a "partially activated" phenotype. Cells purified from blood and liver compartments were stained directly ex vivo with the indicated markers. (**A**) Graphs of activation marker (CD69, HLA-DR, and CD38) expression in Liv-MAIT and B-MAIT cells (CD3$^+$CD161^{high} Vα7.2$^+$) of representative subjects (*left panels*). The frequency of MAIT cells expressing activation markers in the liver and blood (five subjects) (*right panel*). (**B**) Representative dot plots of the frequency of Ki67$^+$ Liv-MAIT cells and conventional T cells (non-MAIT) within the respective activated (HLA-DR$^+$CD38$^+$) populations. Bar graph shows the frequencies of all analyzed subjects (*right panel*).

We next analyzed lymphocyte memory and activation markers. Liv-MAIT cells, similar to their circulating counterpart (16), displayed a memory effector phenotype, being $CD45RO^+CD45RA^-CD28^+CD27^+$ both at the mRNA and protein levels (data not shown). Liv-MAIT cells displayed a more activated phenotype and expressed higher levels of activation markers than did B-MAIT cells. Approximately 80% of Liv-MAIT cells expressed very high levels of CD69; CD38 and HLA-DR were also upregulated, although at lower levels (Fig. 3A). The high expression of CD69 molecules on MAIT cells is in line with what was reported for intrahepatic (21) and intraepithelial gut lymphocytes (24). Despite their activation phenotype, Liv-MAIT cells were in a nonproliferating state, being almost completely negative for Ki-67 (<1% of MAIT, data not shown), similar to the feature described in B-MAIT cells (16) but in contrast to a large proportion of intrahepatic activated conventional T cells (Fig. 3B).

MAIT cell response to mitogens or TCR-mediated stimulation

B-MAIT cells are known to produce high quantities of Th1 and Th17 cytokines in response to mitogens (16, 17). However, in the absence of innate cytokines or costimulatory signals, B-MAIT cells demonstrated poor cytokine production upon TCR stimulation (25). We analyzed the cytokine production of Liv-MAIT and B-MAIT cells after TCR-dependent or -independent stimulations. We sorted MAIT and conventional $CD8^+$ T cells from both compartments and stimulated them with PMA/ionomycin or anti-CD3/CD28–coupled beads. After overnight culture, mRNA and protein levels of different cytokines were analyzed. Fig. 4A shows the expression levels of cytokine mRNAs in Liv-MAIT, B-MAIT, and conventional $CD8^+$ T cells. High mRNA levels of Th1 cytokines (IFN-γ, TNF-α, IL-2) and Th17 cytokines (IL-17A, IL-17F), but not of IL-22 or Th2 cytokines (IL-4, IL-5, IL-9, IL-10, IL-13), were detected after mitogen stimulation of MAIT cells, irrespective of their anatomical derivation. In contrast, stimulation with anti-CD3/CD28–coupled beads was poor in MAIT cells and only efficient in conventional $CD8^+$ T cells (Fig. 4A, 4B). To further investigate the disparity between mitogen- and TCR-mediated activation, we quantified the protein levels of Th1, Th2, and Th17 cytokines in sorted MAIT cells after stimulation (Fig. 4C). Mitogen activation clearly induced a greater production of Th1 cytokines and IL-17A in Liv-MAIT cells than in B-MAIT cells (Fig. 4C), but such differences were not evident after anti-CD3/CD28–coupled beads' activation (Fig. 4C).

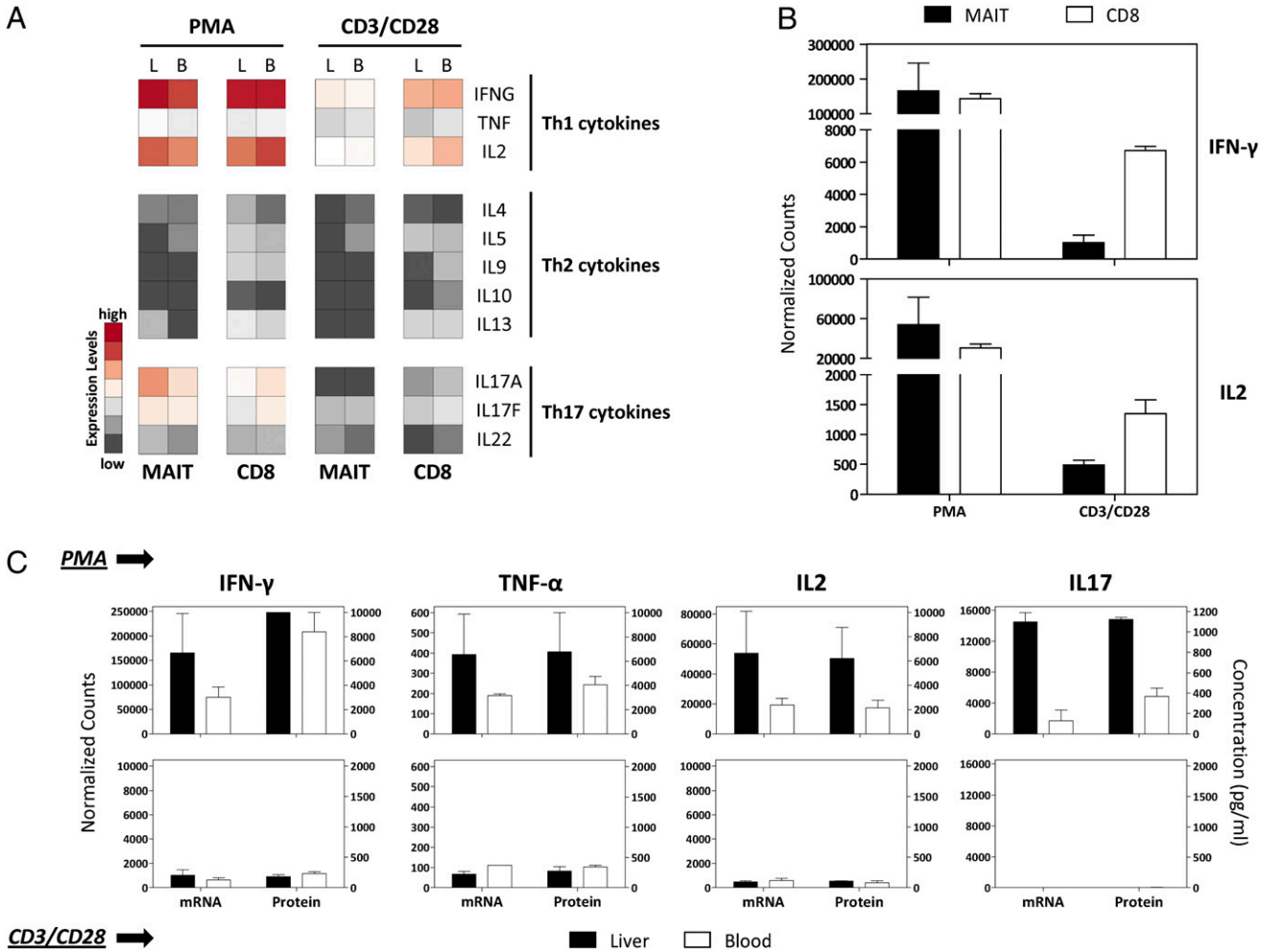

FIGURE 4. MAIT cells mainly produce Th1/Th17 cytokines, but they respond poorly to TCR-mediated activation. Sorted MAIT cells ($CD3^+CD161^{high}$ $V\alpha7.2^+CD4^-\gamma\delta$-$TCR^-$) and conventional $CD8^+$ T cells ($CD3^+CD8^+$ and not $V\alpha7.2^+CD161^{high}$) from the liver and blood compartments were studied upon PMA/ionomycin and anti-CD3/CD28–coupled bead stimulation. mRNA levels were measured with NanoString. Protein levels were measured in the supernatants of stimulated cells. (**A**) Heatmap of gene expression analysis of Th1, Th2, and Th17 cytokines in sorted MAIT cells and $CD8^+$ T cells from the liver (L) and blood (B) compartment (each two donors). (**B**) IFN-γ and IL-2 mRNA expression levels in liver MAIT cells and $CD8^+$ T cells upon stimulation. (**C**) mRNA and protein levels of IFN-γ, TNF-α, IL-2, and IL-17A from sorted liver (n = 2) and blood (n = 3) MAIT cells upon stimulation.

IL-17A–producing T cells in the intrahepatic environment are mainly MAIT cells

Because of the importance of IL-17A–producing cells in inflammatory and autoimmune phenomena (26), we investigated the quantity of IL-17A–producing MAIT cells in the liver and blood compartments. The frequencies of IL-17A$^+$, IFN-γ$^+$, and IL-22$^+$ T cells were first analyzed by intracellular cytokine staining in cells purified from blood or liver compartments and activated with PMA/ionomycin (Fig. 5A). Although B-MAIT cells represented a small minority of the IL-17A–producing T cells (<4%), most of the IL-17A$^+$ T cells in the liver were MAIT cells (~65% of CD3$^+$ cells, Fig. 5A). Virtually all IL-17$^+$ MAIT cells in the liver also released IFN-γ but not IL-22 (Fig. 5A).

Measurement of IL-17A and IL-22 in the supernatants of sorted cell populations (Fig. 5B) confirmed the results obtained with intracellular cytokine staining. We also observed that intrahepatic MAIT cells were the T cell subset primarily able to secrete CCL20 (Fig. 5B), the known ligand of CCR6, which is expressed on Th17 (27, 28) and MAIT cells (Supplemental Fig. 3). Taken together, these data suggest that MAIT cells can be a critical promoter of IL-17A–mediated inflammation in the liver.

IL-7 licenses MAIT cells to produce large quantities of IFN-γ after TCR-mediated stimulation

The refractory response of B-MAIT cells to TCR-mediated stimulation was associated with lower levels of TCR-signaling components (*CD8A*, *CD8B*, *IKBKE*, *LCK*) and higher levels of negative regulators (*SOCS1*, *SOCS3*) in comparison with conventional CD8$^+$ T cells (25). We compared the transcriptional levels of these genes in sorted Liv-MAIT and B-MAIT cells and in conventional CD8$^+$ T cells from both compartments. We observed lower levels of the aforementioned TCR-signaling components, as well as higher levels of *SOCS1* and *SOCS3*, in Liv-MAIT cells compared with intrasinusoidal conventional CD8$^+$ T cells (data not shown), in agreement with previous studies (25).

Previous work showed that the low responsiveness of cells to TCR stimulation can be partially rescued by innate cytokines. For example, IL-1β increases the cell response to anti-CD3 stimulation and encourages maturation toward IL-17A production in CD161$^+$ T cells (25). Similarly, conventional CD4$^+$ T cells with a Th17 signature possess extended functional plasticity, and IL-7 (29, 30) or a combination of IL-1β and IL-23 (31, 32) may reduce the T cell–activation threshold and positively influence differentiation toward Th17 cells. Because Liv-MAIT cells produced high quantities of IL-17A only after mitogen stimulation, we tested whether inflammatory cytokines influence the maturation and responsiveness of Liv-MAIT cells to TCR-mediated stimulation. Emphasis was placed on testing the effect of IL-7 for three reasons: MAIT cells express high levels of IL-7R (Fig. 2B); hepatocytes are the primary source of inducible IL-7 during inflammatory processes in the liver (33); and IL-7 is capable of

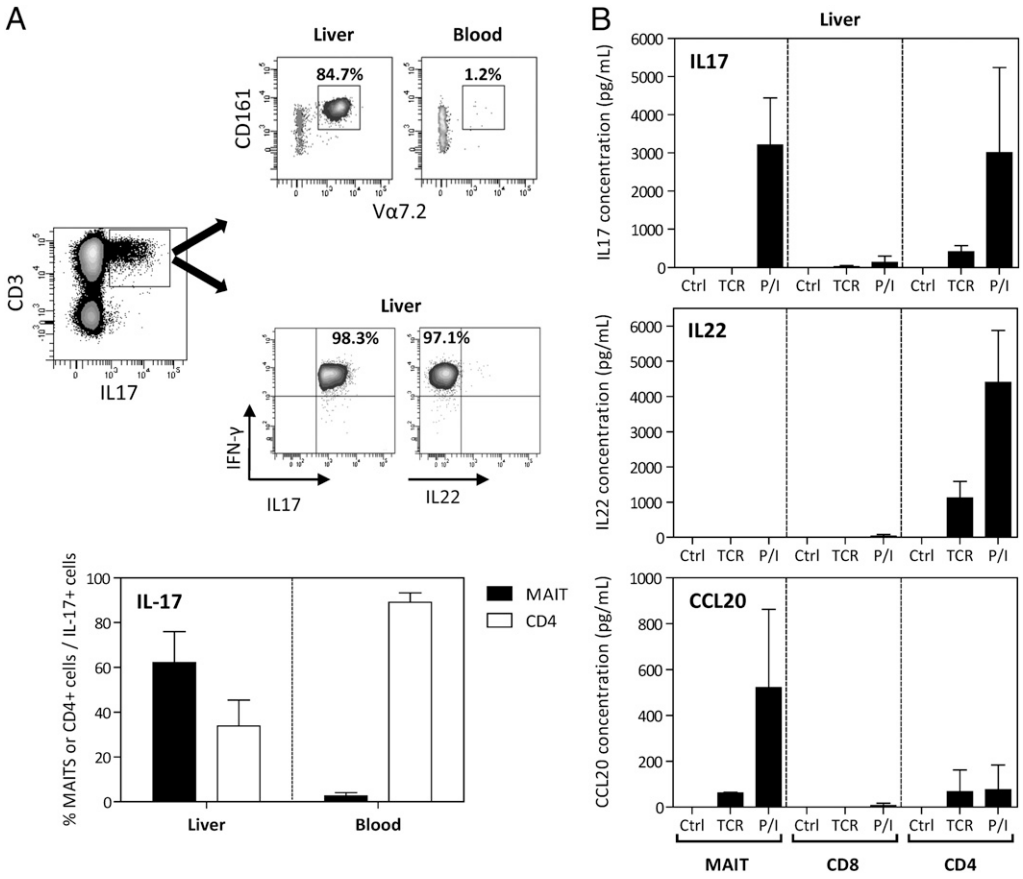

FIGURE 5. Intrasinusoidal MAIT cells are the main producers of IL-17A and CCL20. (**A**) Cells purified from blood and liver compartments were activated with PMA/ionomycin and analyzed with intracellular cytokine staining. Staining was performed using anti-CD3, anti-CD4, anti-Vα7.2, anti-CD161, anti–IFN-γ, anti–IL-22, and anti–IL-17A Abs. Dot plots of a representative subject display the frequency of MAIT cells and the IFN-γ and IL-22 producers within the IL-17A–producing T cells. Bar graph shows the mean frequencies of MAIT and CD4$^+$ T cells within the IL-17A–producing T cells in both compartments (each three donors) (*bottom panel*). (**B**) Cytokine production in the supernatant of equal numbers (3×10^5 cells/well) of sorted intrasinusoidal MAIT cells and CD8$^+$ and CD4$^+$ T cells ($n = 2$) after anti-CD3/CD28–coupled beads (TCR-mediated) or PMA/ionomycin (non–TCR-mediated) activation.

modulating IL-17A production in memory CCR6$^+$CD4$^+$ T cells and in γδ T cells (34), as well as increasing T cell responsiveness (30).

Intrasinusoidal and circulating mononuclear cells (n = 5 each) were incubated with IL-7 or a combination of IL-1β and IL-23 for 12, 48, or 96 h, and the frequencies of IFN-γ–, IL-22–, and IL-17A–producing MAIT and non-MAIT T cells were analyzed after stimulation with anti-CD3/CD28–coupled beads. Functions of both Liv-MAIT and B-MAIT cells were dynamically regulated by the presence of IL-7, which strongly enhanced their responsiveness to TCR-mediated stimulation. Note that IL-7 alone did not activate MAIT cells, and its boosting effect was clearly less evident in non-MAIT T cells. Moreover, the boosting effects of IL-7 on MAIT cells were higher than those detected with the combined IL-1β and IL-23 treatment (Fig. 6A, 6B). IL-7 was also able to boost IFN-γ production after activation with bacteria (data not shown).

In addition to IFN-γ, IL-7 treatment enhanced the anti-CD3/CD28–mediated production of IL-17A by MAIT cells (Fig. 6A, 6B), increasing the frequency of IFN-γ/IL-17A double producers (Supplemental Fig. 4). However, such an effect on IL-17A production was not detected in bacteria-stimulated MAIT cells (data not shown). IL-7 also boosted the production of TNF-α and IL-2 by MAIT cells, whereas IL-22 remained absent (data not shown). Experiments repeated with sorted MAIT cells showed similar augmentation of cytokine production (data not shown), indicating the direct effect of IL-7 on MAIT cells.

IL-7 mediates reinvigoration of MAIT cells

Next, we investigated the possible mechanisms underlying the IL-7–induced increased responsiveness of MAIT cells. This phenomenon seems to be independent of cell division, because sorted MAIT cells remain Ki-67$^-$ during incubation with IL-7 (data not shown). Because previous work showed that IL-7 increases T cell cytokine production and induces Th17 maturation through the inhibition of *SOCS3* (30), we analyzed the expression levels of IL-7–related genes in sorted Liv-MAIT cells. IL-7 treatment of Liv-MAIT cells caused the downregulation of *SOCS3* and upregulation of *Bcl-2*, as described previously (35) (Fig. 7A). We also found that TCR-signaling components were upregulated upon IL-7 treatment (Fig. 7A).

To confirm the gene-expression data obtained with sorted cells, we analyzed the expression levels of TCR and CD8 coreceptors in IL-7–treated Liv-MAIT and B-MAIT cells present in the bulk population of cells purified from blood and liver. IL-7 treatment progressively upregulated the surface expression of TCR and the CD8αβ heterodimer but not that of the CD8αα homodimer (Fig. 7B, 7C).

Discussion

The realization that bacterial colonization of the gut influences immune system development and its association with immune-related diseases sparked an interest in the peculiar populations of immune cells that home to the gastrointestinal system (20). The liver is considered a part of this system, because it drains ~1–2 l of blood/min, of which 75% is portal venous blood that has perfused the gut (36). Information regarding the immune cells that reside in the intrahepatic vascular bed is scarce and derived principally from studies performed in mice (2). Because we had access to a large number of intrasinusoidal lymphocytes from healthy indi-

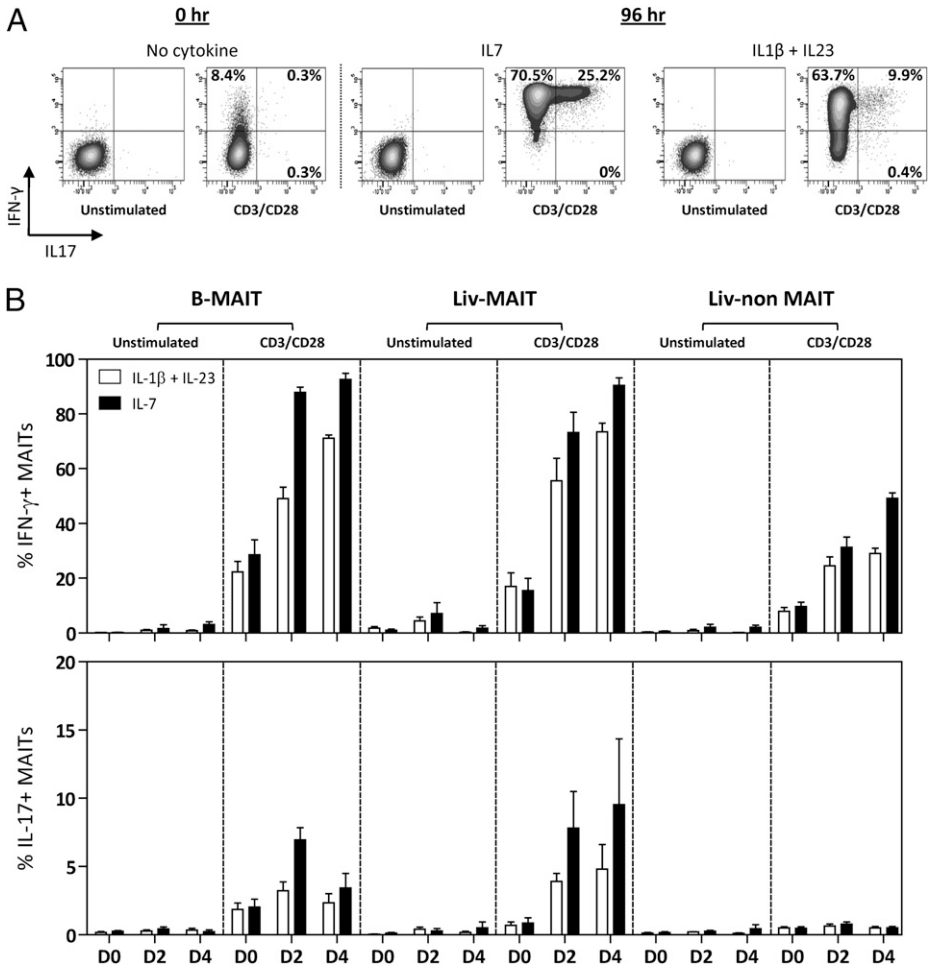

FIGURE 6. IL-7 enhances IFN-γ and IL-17A production by MAIT cells upon TCR stimulation. Longitudinal analysis of IFN-γ and IL-17A production by liver and blood cells (MAIT and CD3$^+$ non-MAIT T cells) in the presence of the indicated cytokines. (**A**) Dot plots of a representative subject show IFN-γ and IL-17A production by Liv-MAIT cells at days 0 and 4 in the presence of IL-7 or IL-1β and IL-23 in stimulated (with anti-CD3/CD28–coupled beads) and unstimulated cells. (**B**) Bars show the mean frequencies of IFN-γ$^+$ or IL-17A$^+$ MAIT (CD3$^+$CD161^{high}Vα7.2$^+$) and non-MAIT cells in indicated compartments. Cells from five subjects were analyzed upon TCR stimulation at the various time points after addition of the respective cytokines.

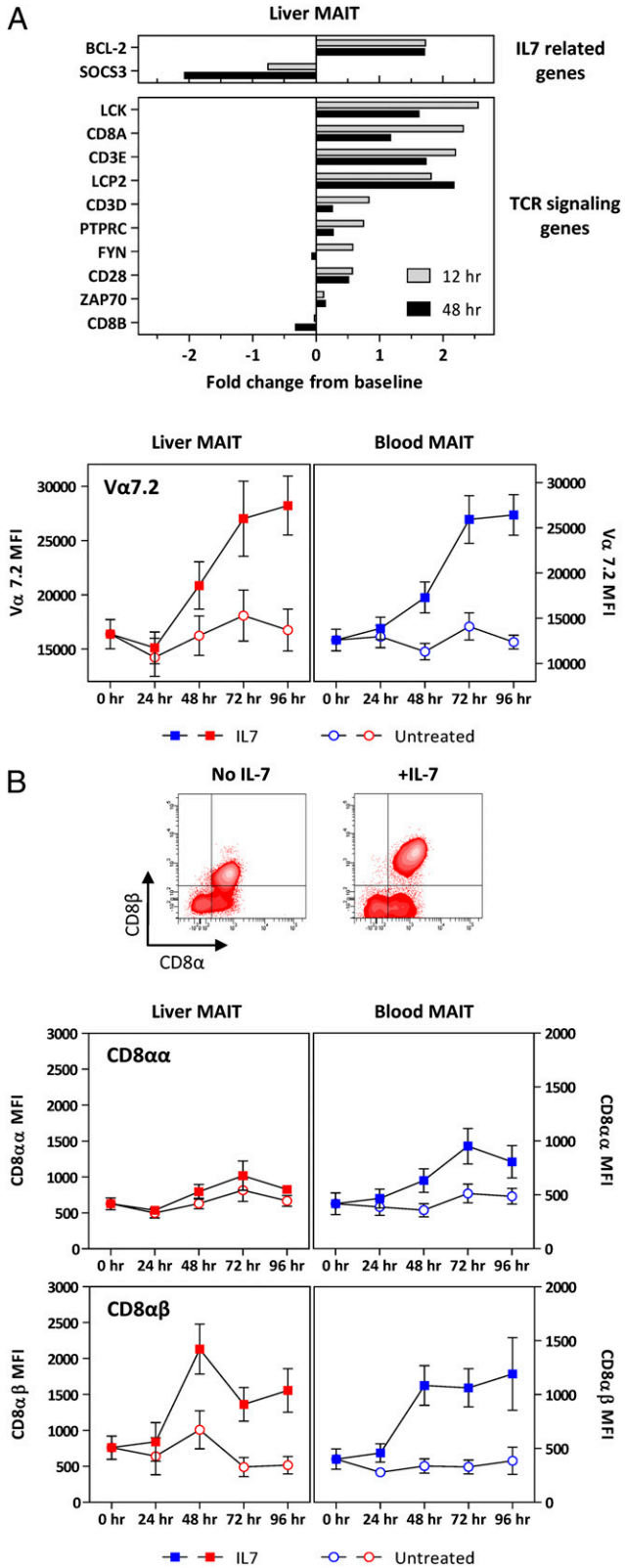

FIGURE 7. IL-7 upregulates the TCR signaling components and increases the expression of CD8αβ in MAIT cells. (**A**) Longitudinal gene expression analysis of TCR-signaling components and IL-7–related genes in a sorted Liv-MAIT cell sample after IL-7 treatment. Data are expressed as the fold change in expression levels at 0 h (*upper panel*). Longitudinal Vα7.2 mean fluorescence intensity (MFI) on Liv-MAIT cells (n = 5) and B-MAIT cells (n = 5) after IL-7 addition (*lower panels*). (**B**) Dot plots show CD8αα and CD8αβ expression on gated ($CD3^+CD161^{high}V\alpha7.2^+$) Liv-MAIT cells for 4 d after treating a bulk population of intrasinusoidal mononuclear cells with one dose of IL-7 (*top panel*). Graphs show the longitudinal CD8αα (*middle panel*) and CD8αβ (*bottom panel*) MFI on gated Liv-MAIT and B-MAIT cells (each n = 5) after the addition of IL-7 at the indicated time points.

viduals, we studied the composition and function of the large population of T cells expressing NK markers present in the liver. We showed that MAIT cells are a lymphocyte population highly enriched in this environment and that, in addition to their ability to produce IFN-γ, IL-2, and TNF-α, they represent the major source of IL-17A in the normal liver and can secrete CCL20, the chemokine specific for the Th17-associated chemokine receptor CCR6 (27). We also demonstrated that intrasinusoidal MAIT cell function is regulated not only by the innate cytokines IL-1β and IL-23, but also by IL-7, a cytokine that is secreted by hepatocytes under inflammatory conditions (33). Overall, these findings indicate that MAIT cells are a specialized cell population highly adapted to exert specific immune functions in the liver.

The initial analysis of the composition of intrasinusoidal lymphocytes clearly revealed that the majority of T cells expressing NK markers in healthy human livers was MAIT cells and not iNKT cells, as detected in murine livers (2). The reason for this lack of correlation between murine and human data remains unclear; one possibility is that it is due to the different enteric microbiome occurring in human and in experimental animals, a variable that can shape iNKT and MAIT cell populations (13, 37). The difference also raises the question whether murine iNKT and human MAIT cells perform similar effector functions. MAIT and iNKT cells are activated by Ags presented by distinct Ag-presenting molecules (4, 14), and although they were shown to be activated during bacterial infections (4, 14), their cytokine production can differ. Murine iNKT cells can produce Th1 and Th17 cytokines, as well as IL-4, IL-10, and IL-13 (38). In contrast, human MAIT cells displayed a preferential ability to produce Th1 cytokines and IL-17A, which is in line with their predominantly expressed transcription factors *Tbx21* and *RORC* (Th1- and Th17-specific, respectively) (16). Thus, available data indicate that these two cell populations perform different specialized functions and can be activated by stimuli that overlap only partially. As such, the importance of iNKT cells in human liver pathologies, such as acute hepatitis B virus infection (7), should be considered with caution, because iNKT cells are extremely rare in the human liver.

Enrichment of MAIT cells in the healthy human liver results in two clear consequences. First, a tight control over MAIT cell responsiveness to Ags is crucial to prevent wanton responses within the liver, yet still allow MAIT cells to respond swiftly and effectively to pathogenic Ags. Indeed, we observed that Liv-MAIT cells expressed markers of T cell activation (CD69, CD38, HLA-DR) but were in a noncycling state and were poorly responsive to TCR-mediated stimulation; this may be a "partial activation" state similar to that described in intestinal intraepithelial lymphocytes (24). It is worth mentioning that beyond its use as an early activation marker, CD69 was also associated with inflammatory infiltrates (39), TGF-β1 production (40), and regulation of the Th17 response (41). As such, the functional significance of the elevated expression of CD69 on Liv-MAIT cells compared with B-MAIT cells remains to be investigated. In addition, we showed that the state of MAIT cell partial activation can be rescued through innate signals via cytokines, such as IL-1β and IL-23 or IL-7. The ability of IL-7 to license full functional capacity of MAIT cells is of particular interest because this cytokine is produced by hepatocytes under inflammatory conditions in response to IFN-β production by activated liver

accessory cells (33). Thus, the functional link between IL-7 and MAIT cells suggests a scenario in which MAIT cells might be fully activated only in the presence of the ongoing innate responses of other intrahepatic cells (e.g., Kupffer or dendritic cells) that induce IL-7 production by hepatocytes. Mechanistically, IL-7 licenses MAIT cells by decreasing *SOCS3* expression, as previously found with other T cell populations in mice (30), and by upregulating the expression of the TCR complex and other molecules involved in TCR triggering. In addition, IL-7 boosted the production of IL-17A by MAIT cells upon anti-CD3/CD28–mediated TCR activation, providing evidence that TCR stimulation, in addition to mitogen stimulation (16, 17), can induce the release of this cytokine. However, IL-7 was unable to promote IL-17A production by MAIT cells when stimulated with bacteria-infected APCs, supporting the hypothesis of Dusseaux et al. (16) that IL-17 inhibitory molecules or cytokines could be secreted by monocytes upon bacterial infection.

Second, MAIT, but not $CD4^+$ T, cells were the predominant cells able to produce IL-17A within the normal liver. IL-17A production triggers several inflammatory conditions (26) and has been implicated in the establishment of liver fibrosis (42) and in the pathogenesis of alcoholic, autoimmune, and hepatitis B virus– or hepatitis C virus–related hepatitis (42–45). Importantly, in all these chronic inflammatory conditions, IL-17–producing $CD4^+$ T (Th17) cells were reported to be the major source of IL-17A in the liver, with few reports describing IL-17A–producing $CD161^+CD8^+$ T cells (11, 45). This apparent discrepancy can be resolved by the possibility that MAIT cells are responsible for the initial acute IL-17A production that can trigger the subsequent recruitment of classical inflammatory $CD4^+$ Th17 cells during chronic damage. This scenario is supported by recent data in mice showing that the expansion and differentiation of Th17 cells in the gut were controlled by an IL-17A–dependent production of CCL20 in gut mucosa (46). Indeed, we observed that Liv-MAIT cells produced both IL-17A and CCL20, supporting the possible role for MAIT cells in the liver-driven recruitment of inflammatory $CCR6^+$ Th17 cells (27, 28).

In conclusion, the access to intrasinusoidal lymphocytes from healthy individuals provided us with a unique opportunity to demonstrate that MAIT cells are a specialized T cell population that is well adapted to exert distinct functions within the human liver. We revealed the existence of regulatory mechanisms of MAIT cell activation requiring IL-7 and provided a benchmark profile of intrasinusoidal MAIT cells that will be indispensable in evaluating their role in liver pathological processes of different etiologies.

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Disclosures

The authors have no financial conflicts of interest.

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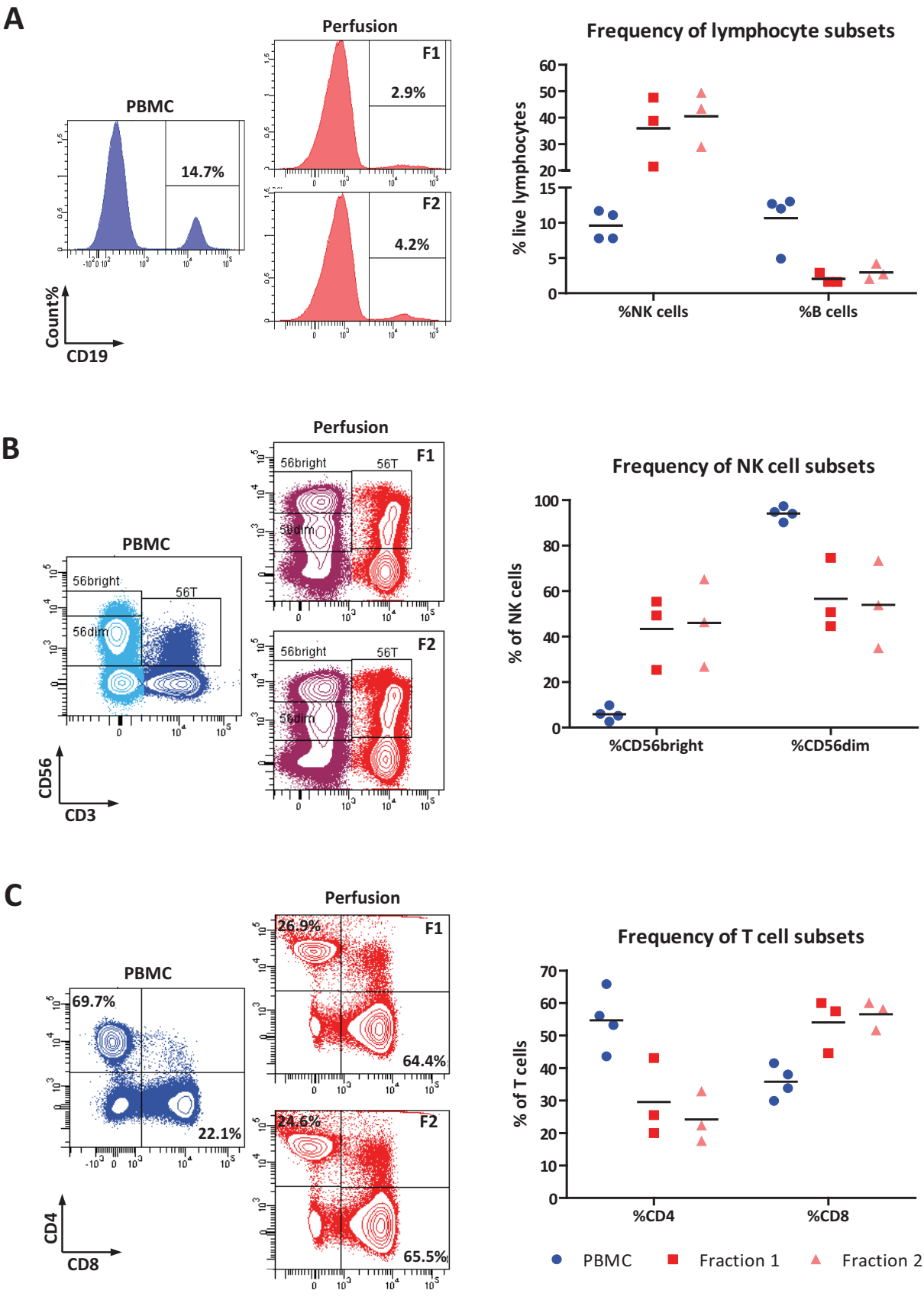
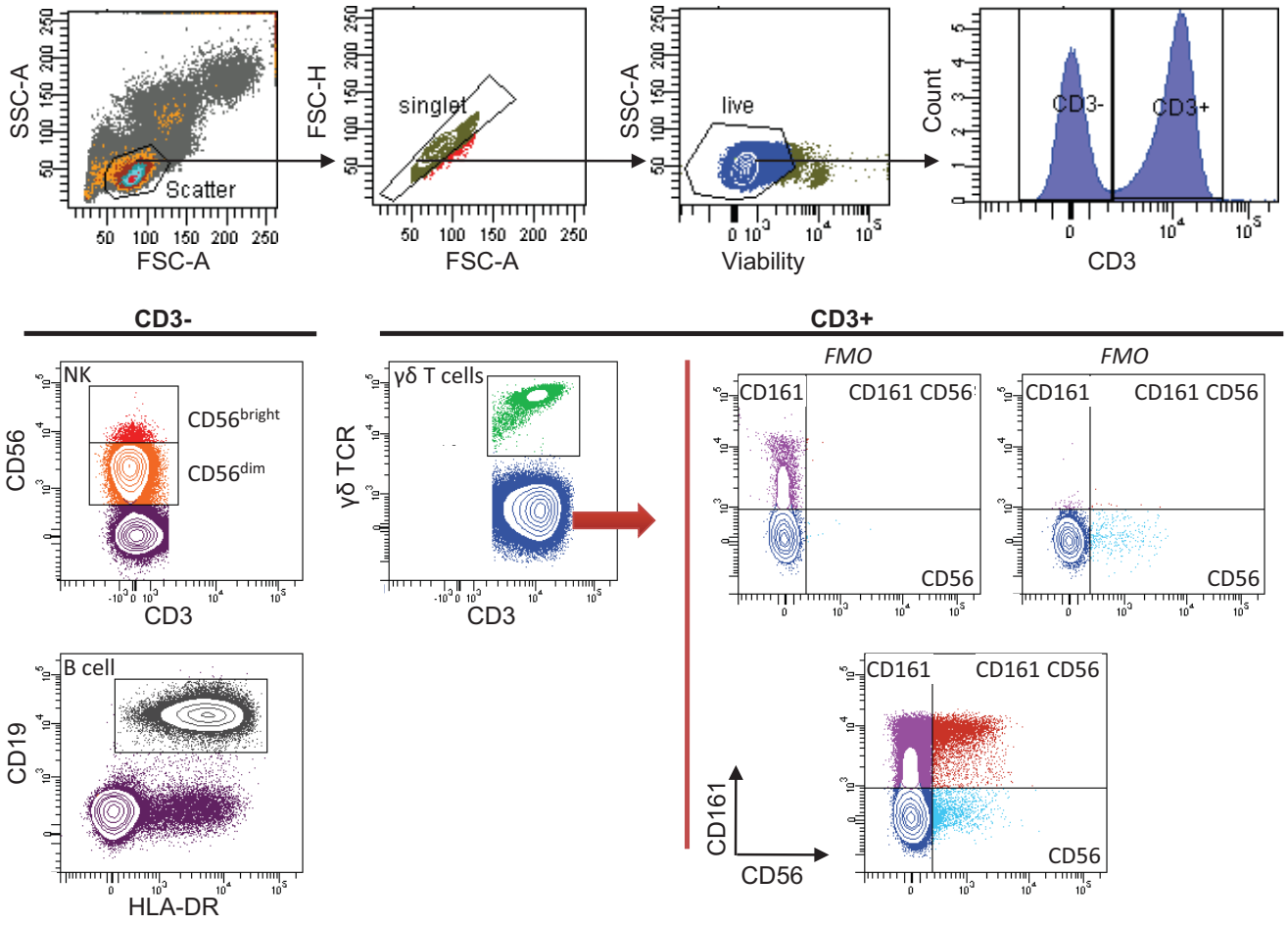
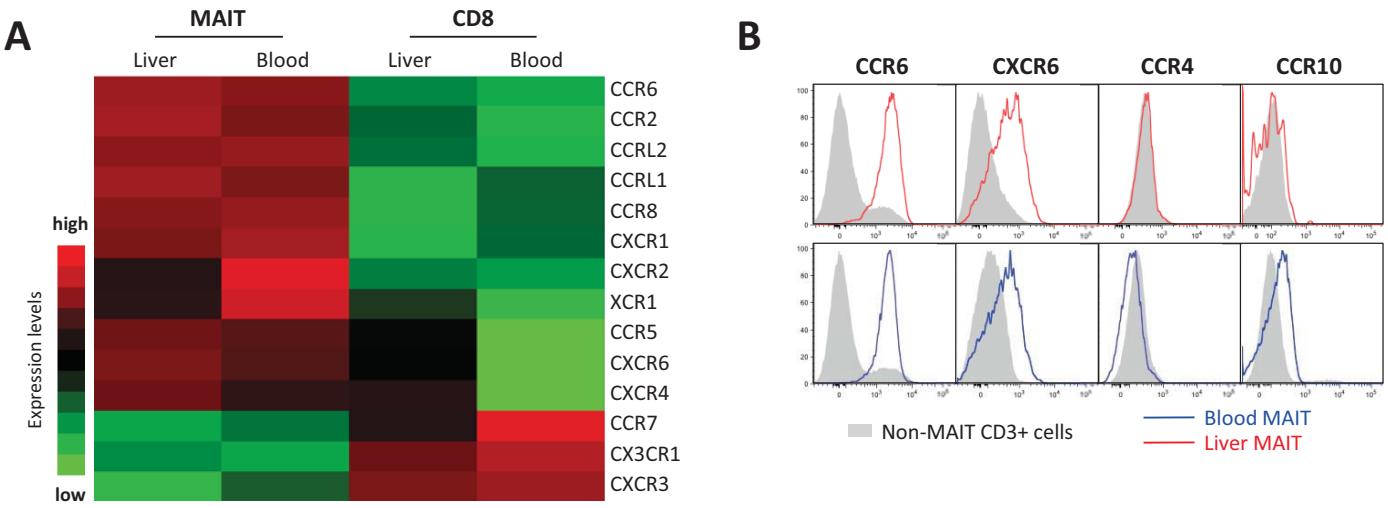


Supplementary Figure 1. Minimal differences of lymphocyte populations in first and subsequent fractions of liver perfusion. Frequencies of NK and B cells (A), CD56^{bright} and CD56^{dim} (B), and CD4^{+} and CD8^{+} T cells (C) in the blood (n=4) and in the 2 fractions of liver perfusion (n=3). Representative dot plots are shown on the left while the mean frequencies of 3 liver perfusions are shown on the right.



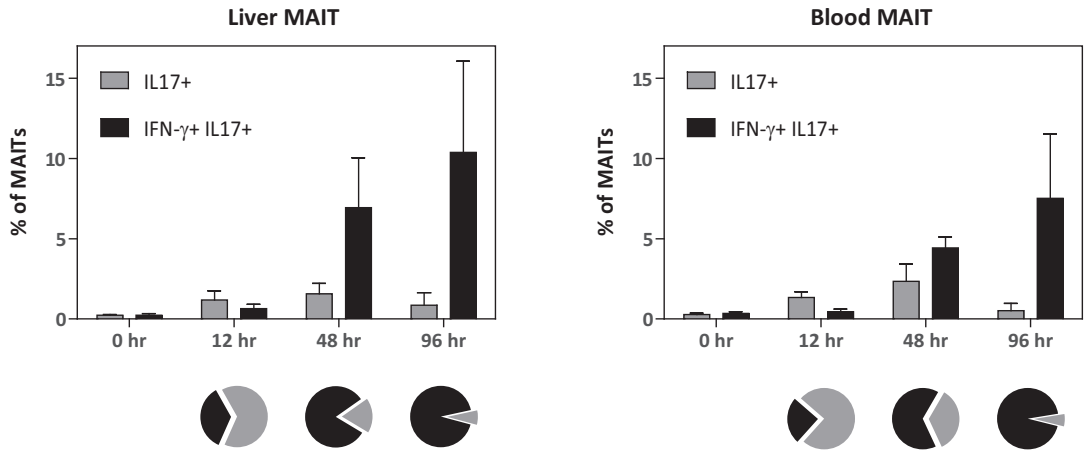
Supplementary Figure 2. Gating strategy for lymphocyte distribution pie chart in Figure 1A. Cells were gated based on forward and side scatter, following which doublets and dead cells were excluded. CD3 gates distinguish between T cells and other lymphocytes. From $CD3^-$ population, B cells and $CD56^{bright}$ and $CD56^{dim}$ NK cells were identified. From the $CD3^+$ population, γδ T cells were first identified before CD56- and CD161-expressing T cells were identified from non-γδ T cells. Gates for CD56 and CD161 were set based on FMO staining. Percentages in Figure 1A show proportion of these lymphocyte populations within the 'live' cell gate.



C

Differentially Expressed (2 fold)				
Gene Name	Liver MAIT (n=2)		Blood MAIT (n=2)	
CCL24	54.31	43.81	147.59	80.49
CD9	263.22	254.90	49.20	21.95
CFH	142.05	131.43	245.98	376.82
CXCL11	12.53	19.91	34.73	40.24
FCAR	25.07	47.79	14.47	21.95
GFI1	777.12	760.72	364.62	376.82
GNLY	162.94	250.92	257.55	1225.59
HLA-DQA1	33.42	51.78	20.26	10.98
HLA-DQB1	29.25	31.86	164.95	62.19
IFNG	179.66	127.45	26.04	36.58
IL1B	12.53	31.86	54.98	65.85
IL8	54.31	179.23	295.17	453.65
IRF4	359.31	665.13	150.48	270.73
ITGA6	79.38	79.66	243.08	190.24
ITGAM	33.42	31.86	66.56	109.75
KIR2DL1	12.53	3.98	28.94	25.61
PPARG	54.31	43.81	20.26	25.61
SELL	37.60	55.76	98.39	95.12

Supplementary Figure 3. MAIT cells have a unique profile of chemokine receptors. Nanostring gene expression analysis of chemokine receptors in sorted MAIT (n=4) and $CD8^+$ T cells (n=4) from the liver and blood compartments (2 donors for each compartment). (B) Representative histograms of CCR6, CXCR6, CCR4 and CCR10 staining on MAIT and non-MAIT $CD3^+$ cells from the liver and blood. (C) Normalized counts of genes that were differentially expressed by at least 2-fold between sorted Liv- and B-MAIT.



Supplementary Figure 4. IL-7 modulation of IFN-γ and IL-17A double producing MAIT cells. The mean frequencies of IFN-γ and IL-17A double-producing and IL-17A single-producing MAIT cells upon TCR stimulation of total blood (n=5) or liver lymphocytes (n=5) at various time points after IL-7 addition are shown as bars. Pie charts show the mean proportion of single and double producers at the respective time points.