MAIT cells are chronically activated in patients with autoimmune liver disease and promote pro-fibrogenic hepatic stellate cell activation

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List of abbreviations:

AIH, autoimmune hepatitis; **AILD**, autoimmune liver disease; **ECM**, extracellular matrix; EOMES, eomesodermin; **HSC**, hepatic stellate cell; **hHSC**, primary human hepatic stellate cell; **MAIT cell**, mucosal-associated invariant T cell; **PBC**, primary biliary cholangitis; **NASH,** non-alcoholic steatohepatitis**; PMA**, phorbol 12-myristate 13-acetate; **PSC**, primary sclerosing cholangitis; **RORγT**, retinoic acid-related orphan receptor γT; **SD**, standard deviation; **SEM**, standard error of the mean; **STAT**, signal transducer and activator of transcription; **TCR**, T cell receptor

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Conflict of interest

The authors declare no conflict of interest with regards to the present manuscript.

Abstract

Autoimmune liver diseases (AILD) are chronic liver pathologies characterised by fibrosis and cirrhosis due to immune-mediated liver damage. In this study, we addressed the question whether mucosal-associated invariant T (MAIT) cells, innate-like T cells, are functionally altered in patients with AILD and whether MAIT cells can promote liver fibrosis through activation of hepatic stellate cells. We analysed the phenotype and function of MAIT cells from AILD patients and healthy controls by multi-colour flow cytometry and investigated the interaction between human MAIT cells and primary human hepatic stellate cells (hHSCs). We show that MAIT cells are significantly decreased in peripheral blood and liver tissue of patients with AILD. Notably, MAIT cell frequency tended to decrease with increasing fibrosis stage. MAIT cells from AILD patients showed signs of exhaustion, such as impaired IFNγ production and high *ex vivo* expression of the activation and exhaustion markers CD38, HLA-DR and CTLA-4. Mechanistically, this exhausted state could be induced by repetitive stimulation of MAIT cells with the cytokines IL-12 and IL-18, leading to decreased IFNγ and increased exhaustion marker expression. Of note, repetitive stimulation with IL-12 further resulted in expression of the pro-fibrogenic cytokine IL-17A by otherwise exhausted MAIT cells. Accordingly, MAIT cells from both healthy controls and AILD patients were able to induce an activated, pro-inflammatory and pro-fibrogenic phenotype in hHSCs *in vitro*, which was partly mediated by IL-17. **Conclusion:** Our data provide evidence that MAIT cells in AILD patients have evolved towards an exhausted, pro-fibrogenic phenotype and can contribute to the development of HSC-mediated liver fibrosis. These findings reveal a

cellular and molecular pathway for fibrosis development in AILD that could be exploited for anti-fibrotic therapy.

Introduction

Mucosal-associated invariant T (MAIT) cells are innate-like T cells characterised by an evolutionarily conserved semi-invariant T cell receptor (TCR) consisting of an invariant α chain (Vα7.2-Jα33/Ja20/Jα12 in humans) and varying β chains (1, 2), as well as high expression of the C-type lectin CD161 (3). Human MAIT cells are abundant in peripheral blood, but particularly enriched in liver tissue, representing up to 30% of hepatic $CD3^+$ T cells (3, 4). The ability of MAIT cells to recognize microbial-derived vitamin B metabolites presented on the major histocompatibility complex class I-related molecule MR1 (5) allows MAIT cells to detect various strains of bacteria and yeasts *in vitro* and *in vivo* (6, 7). In line with their innate-like nature, MAIT cells are also activated by the pro-inflammatory cytokines IL-12 and IL-18 in a TCR independent manner (8). Once activated, MAIT cells exert cytotoxic properties, and secrete pro-inflammatory cytokines such as IL-17 and IFNγ (3, 6). Besides their role in mediating anti-microbial defence, MAIT cells have been implicated in the development of non-infectious diseases, including autoimmune diseases such as multiple sclerosis (9, 10) and inflammatory bowel diseases (11), suggesting that MAIT cells can orchestrate inflammatory responses in absence of infection. AILD, i.e. primary sclerosing cholangitis (PSC), primary biliary cholangitis (PBC) and autoimmune hepatitis (AIH) are characterized by chronic hepatic inflammation, eventually leading to the development of liver fibrosis and cirrhosis, associated with a high risk of morbidity and mortality (12). The pathogenesis of AILD is poorly understood, although it is becoming increasingly clear that

environmental triggers and genetic aberrations of immunoregulatory pathways contribute to chronic inflammation and T cell mediated damage in the liver (12). Genome-wide association studies recently demonstrated that variations in the *IL-12* pathway are associated with the development of PBC (13). A potential role of IL-12 in AILD development is further supported by murine studies, in which deletion of the IL-12 subunit p40 ameliorated the development of autoimmune cholangitis (14), and overexpression of IL-12 resulted in development of an AIH-resembling liver inflammation (15). Central to the development of liver fibrosis by inflammation is the activation of hepatic stellate cells (HSCs), non-parenchymal liver cells that transform into extracellular matrix (ECM) secreting myofibroblasts following activation (16). HSC activation and the subsequent development of liver fibrosis are mediated by IL-17 in murine models, evident by ameliorated liver fibrosis in IL-17RA$^{-/-}$ mice *in vivo*, as well as increased collagen secretion and expression of α-SMA, IL-6 and TGF-β by IL-17 stimulated HSCs *in vitro* (17, 18).

However, whether MAIT cell derived IL-17A can mediate HSC activation in humans remains unclear. Here, we investigated the role of human MAIT cells in fibrosis development and HSC activation in AILD. Our data show that MAIT cells are severely reduced in peripheral blood and liver tissue from AILD patients, and the remaining MAIT cells show features of chronic activation and functional exhaustion, which could be induced by long-term exposure to the pro-inflammatory cytokines IL-12 and IL-18. Despite their exhausted state, MAIT cells produce large amounts of IL-17A in response to repetitive stimulation with IL-12. MAIT cells are further able to activate HSCs, leading to HSC proliferation and the expression of pro-fibrogenic and pro-inflammatory genes in HSCs in an IL-17 and cell-cell contact dependent manner. Our data reveal a crucial role of MAIT cells in liver fibrosis development and

provide important mechanistic insights that will help to design novel therapeutic approaches targeting HSC activation in fibrosis.

Material and Methods

A complete description of materials and methods can be found in the online supplementary material.

Ethics statement

This study was fully approved by the Royal Free Hospital ethical board and all participants gave written informed consent before sample collection.

Primary Cell Isolation

All tissue samples were obtained from patients or healthy volunteers at the Royal Free Hospital. See table S1 for patient information. *PBMCs* were isolated using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) gradient centrifugation. *Liver associated lymphocytes* were isolated from cirrhotic liver tissue derived from explanted livers of patients with AILD, from wedge sections of patients undergoing liver surgery in the absence of background liver disease, or from healthy liver explants considered unsuitable for transplantation (enabled by NHSBT). *Primary human HSCs (hHSC)* were isolated from wedge sections of human liver tissue as described before (19).

Flow cytometric analysis

Cells were stained for surface markers and intracellular markers (see supplementary information for a full list of antibodies). Dead cells were excluded using live/dead fixable UV dead stain kit (Invitrogen, Carlsbad, CA). For staining of intracellular markers, cells were fixed and permeabilized using Intracellular Fixation and Permeabilisation Buffer (ebioscience, San Diego, CA), or human FOXP3 buffer set (BD Biosciences, San Jose, CA). CountBright Absolute Counting Beads (Molecular Probes, Eugene, OR) were used to determine absolute numbers of MAIT cells in peripheral blood. Samples were acquired with LSRFortessa (BD Biosciences, San Jose, CA) and analysed with FlowJo 10.0 (Treestar, Ashland, OR).

Transient Elastography

Transient elastography was performed to measure liver stiffness using FibroScan® (Echosens). Fibrosis stage was defined using previously described cut-offs with F0-1: stiffness <7.1 kilopascal (kPa), F2: 7.1-9.4 kPa, F3: 9.5-12.4 kPa, F4: >12.5 kPa (20).

***In vitro* stimulation assays**

PBMCs or purified Vα7.2$^+$ cells, enriched by magnetic cell separation (see supplementary information) were stimulated *in vitro* with 50ng/ml phorbol 12-myristate 13-acetate (PMA) and 1μM Ionomycin (both Sigma Aldrich, St. Louis, MO), or Dynabead Human T-activator CD3/CD28 (Invitrogen, Carlsbad, CA) for 16 hours. PMBCs or purified Vα7.2$^+$ cells were stimulated in *vitro* with 50ng/ml IL-1β, IL-18 (R&D systems, Minneapolis, MN), or IL-12 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 24 hours (short-term stimulation) or every 24 hours for 72 hours (long-term, repetitive stimulation). Brefeldin A and Monensin

(both ebioscience, San Diego, CA) were added for the last 4 hours of culture when intracellular cytokine expression was analysed by flow cytometry.

***In vitro* co-culture of MAIT cells and HSCs**

Vα7.2$^+$ cells were purified from PBMCs by magnetic cell separation and added to cultured primary human HSCs for 48 hours. Dynabead Human T-activator CD3/CD28 (Invitrogen, Carlsbad, CA), or 2μg/ml IL-17RA/IL-17R antibody (R&D systems, Minneapolis, MN) was added where indicated. After extensive washing to remove MAIT cells, HSC gene expression was determined by RT-PCR and HSC proliferation was assessed using BrdU Cell proliferation ELISA (Roche, Basel, Switzerland).

Statistical analysis

Statistical analyses were performed using Microsoft Excel or Graph Pad Prism. Values are expressed as mean +/- standard deviation (SD), mean +/- standard error of the mean (SEM), or mean +/- 95% confidence interval (CI) as indicated in the figures. Statistical significance was analysed with the appropriate test as indicated in the figures.

Results

MAIT cells are significantly declined and phenotypically altered in AILD patients

Human MAIT cells are defined by expression of CD3, CD161 and Vα7.2 TCR (2, 3) (Supplementary figure 1a). To investigate the MAIT cell compartment in patients with PSC, PBC and AIH, we first determined the frequency of CD3$^+$ CD161^{++} Vα7.2$^+$ MAIT cells

(hereafter referred to as MAIT cells) by flow cytometry in peripheral blood and liver tissue of AILD patients and healthy controls. MAIT cells were severely reduced in frequency and absolute number in peripheral blood (Figure 1a) and liver tissue (Figure 1b) of AILD patients, regardless of the subtype of disease. Moreover, MAIT cell frequency was significantly decreased in peripheral blood of patients with non-alcoholic steatohepatitis (NASH) (Figure 1c), suggesting that MAIT cells are decreased in various types of chronic liver disease. The significant decrease in frequency was specific to MAIT cells in AILD, since the frequency of other conventional and non-conventional lymphocyte populations was unchanged (Supplementary figure 1b). MAIT cells can be divided into subsets based on CD4 and CD8 expression, with the majority of MAIT cells being $CD8^+$ or $CD4/CD8^{-/-}$(2). In AILD, all subsets of MAIT cells were significantly reduced in peripheral blood (Supplementary figure 1c). $CD8^+$ MAIT cells represented the major subset in peripheral blood and liver tissue of healthy controls and AILD patients (Figure 1d-e), although a relative increase of $CD4^+$ MAIT cells was observed (Figure 1d-e) in AILD patients. Moreover, the frequency of MAIT cells in peripheral blood of AILD patients decreased with increasing fibrosis stage and liver tissue stiffness assessed by transient elastography (Figure 1f), suggesting that the frequency of MAIT cells declines with disease progression in AILD. To analyse the localisation of MAIT cells in the liver, Vα7.2 TCR was immunohistochemically detected in liver tissue of patients and controls. Whereas MAIT cells localised around the portal tracts and in the parenchyma in normal livers (Figure 2a), MAIT cells were observed in inflammatory infiltrates around portal tracts and fibrotic septae in diseased livers (Figure 2a). Quantification of Vα7.2$^+$ cells revealed a marked decrease of MAIT cell numbers in liver tissue of PSC and PBC patients compared to healthy controls, while MAIT cells were unchanged in highly inflamed AIH and

NASH (Figure 2b). Taken together, these data establish that the frequency of MAIT cells is severely reduced in peripheral blood and liver tissue of patients with AILD.

MAIT cells from AILD patients show features of chronic activation and functional exhaustion

As AILD are characterized by chronic inflammation (12), we next probed MAIT cells from patients with AILD for features of immune activation and exhaustion *ex vivo*. Whereas the surface expression of the inhibitory receptor PD-1 was comparable in MAIT cells from peripheral blood of patients and healthy controls (Supplementary figure 2a), levels of CTLA-4 and TIM-3 were higher in MAIT cells from patients with AILD. Importantly, MAIT cells from AILD patients showed higher expression of CD39, a marker of terminal and irreversible T cell exhaustion (21). In line with increased expression of exhaustion markers, MAIT cells from patients with AILD showed significantly higher levels of the activation marker CD38, and markedly increased expression of CD69 and HLA-DR (Figure 3a), indicating that MAIT cells in peripheral blood of AILD patients are chronically activated. Mirroring the findings in MAIT cells from peripheral blood, hepatic MAIT cells from AILD patients showed significantly higher levels of CTLA-4 compared to healthy controls (Figure 3b), while the levels of other activation and exhaustion markers were similar (Supplementary figure 2b). Of note, MAIT cells isolated from peripheral blood of NAFLD/NASH patients showed increased expression of the activation and exhaustion markers PD-1, CD39, CD38 and HLA-DR as well (Supplementary figure 2c), suggesting that MAIT cell activation also occurs in non-autoimmune liver disease. As T cell exhaustion is associated with a loss of effector function (22), we next assessed the ability of AILD MAIT to produce effector cytokines and cytolytic

proteins in response to stimulation. Compared to healthy controls, IFNγ production was significantly impaired in MAIT cells from patients with PBC, and markedly reduced in MAIT cells from patients with PSC and AIH following *in vitro* stimulation with PMA/Ionomycin (Figure 3c). Moreover, MAIT cells from AILD patients showed higher granzyme B (GrzB) levels at steady state, but failed to significantly upregulate GrzB expression upon CD3/CD28 bead stimulation, compared to healthy controls (Figure 3d). In contrast, IL-17A and TNFα production in response to such stimulation remained unchanged (Figure 3e and supplementary figure 2d). These data strongly suggest that MAIT cells in AILD are highly activated and show signs of early functional exhaustion, while maintaining their ability to express IL-17A and TNFα. Correlation analyses further revealed a negative correlation between MAIT cell frequency in AILD and expression of activation markers such as CD38 and HLA-DR as well as exhaustion markers such as CTLA4, CD39 and TIM-3 (Figure 3f and supplementary figure 2e). These data indicate that the low frequency of circulating MAIT cells in AILD patients may result from reduced survival of MAIT cells as a consequence of chronic activation and exhaustion *in vivo*.

Long-term stimulation with IL-12+IL-18 drives MAIT cell exhaustion and cell death

High expression of the cytokine receptors IL-12R and IL-18R by MAIT cells allows for TCR-independent stimulation of MAIT cells with IL-12 and IL-18 resulting in IFNγ expression (8). In line with this, we observed high levels of IL-12R and IL-18R expression on MAIT cells from peripheral blood and liver tissue of healthy controls and AILD patients (Supplementary figure 3 a-b). AILD are characterized by persistent liver inflammation and high serum levels of pro-inflammatory cytokines such as IL-1β, IL-12 and IL-18 (23, 24). We mimicked this

chronic inflammatory setting by repetitively stimulating MAIT cells with IL-12, IL-18 and IL-1β *in vitro*, and assessed their ability to express IFNγ. Both short-term (24 hours) and repetitive, long-term (72 hours) *in vitro* stimulation with a combination of IL-12 and IL-18, but not IL-12 or IL-18 alone, induced a robust IFNγ response in MAIT cells from healthy controls and AILD patients, which was not further exacerbated by additional IL-1β (Figure 4a), demonstrating that the response to cytokine mediated stimulation is maintained in MAIT cells from patients with AILD, despite their exhausted phenotype. To exclude effects from bystander immune cells in the stimulated PBMC pool on the induction of IFNγ expression in MAIT cells, purified Vα7.2$^+$ MAIT cells from healthy controls were stimulated with IL-1β, IL-12 and IL-18 next. Like in PBMCs, IL-12 + IL-18 specifically induced expression of IFNγ in purified MAIT cells (Figure 4b), indicating that IL-12 + IL-18 can directly stimulate MAIT cell activation. Interestingly, IFNγ expression of MAIT cells from healthy controls and AILD patients significantly decreased following repetitive, long-term stimulation with IL-12 + IL-18 *in vitro* (Figure 4c), despite constant levels of IL-12 receptor expression (Supplementary figure 4a). Since decreasing IFNγ expression is a hallmark of T cell exhaustion, we next tested the expression of inhibitory receptors in cytokine-stimulated MAIT cells. Following repetitive stimulation with IL-12 + IL-18, we observed increased PD-1 and TIM-3 expression, as well as significantly higher expression of CD39 in MAIT cells from healthy controls (Figure 4d) and AILD patients (Supplementary figure 4b). Moreover, the frequency of live MAIT cells declined in response to repetitive stimulation with IL-12 + IL-18, whereas the frequency of CD3$^+$ cells remained constant (Supplementary figure 4c), showing that MAIT cell loss was specific and not accompanied by general T cell death in culture. These data suggest that repetitive stimulation with the pro-inflammatory cytokines IL-12 + IL-18 can to drive MAIT cell exhaustion and cell death *in vitro*. In order to investigate

possible mechanisms of MAIT cell exhaustion in AILD *in vivo*, we analysed CD3 expression in hepatic MAIT cells as a surrogate marker for antigen exposure (25). Indeed, hepatic AILD MAIT cells showed significantly lower expression of CD3 compared to healthy controls (Figure 4e), suggesting that MAIT cells are chronically exposed to their cognate antigen in AILD livers *in vivo*. Of note, exposure to bacterial antigens has been shown to induce downregulation of the transcription factors eomesodermin (EOMES) and T-bet in MAIT cells, which has been linked to MAIT cell exhaustion (26). We therefore analysed T-bet and EOMES expression in MAIT cells *ex vivo*. Indeed, MAIT cells from peripheral blood of AILD patients expressed significantly lower levels of EOMES and lower levels of T-bet compared to healthy controls (Figure 4f). These data suggest that MAIT cell exhaustion in AILD might be induced by chronic exposure to both inflammatory cytokines and bacterial antigen and may be regulated by downregulation of T-bet and EOMES.

Repetitive IL-12 stimulation significantly induces the expression of the pro-fibrogenic cytokine IL-17A in MAIT cells

Previous reports have described the induction of IFNγ expression in MAIT cells in a PBMC pool by IL-12 + IL-18 (8, 27). However, to our knowledge, there is no evidence for cytokine-mediated stimulation of IL-17A expression, which has been shown to contribute to fibrosis development in animal models and to stimulate HSC activation *in vitro* (17, 18). Interestingly, no IL-17A expression was detected in MAIT cells after short-term (24 hours) cytokine stimulation (Figure 5a). In contrast, repetitive, long-term (72 hours) stimulation with IL-12 alone or in combination with other cytokines resulted in a significant increase in IL-17A$^+$ MAIT cells from healthy controls (Figure 5b). Similarly, repetitive stimulation of

purified MAIT cells with IL-12 significantly induced IL-17A expression in MAIT cells (Figure 5c), demonstrating that the observed IL-17A expression resulted from direct IL-12 signalling on MAIT cells. Along these lines, long-term stimulation with IL-12 induced IL-17A expression in MAIT cells from AILD patients (Figure 5d), which was indistinguishable from IL-17A expression in MAIT cells from healthy controls (Figure 5e). These data demonstrate that MAIT cells from AILD patients, despite their exhausted phenotype, produce IL-17A in response to repetitive stimulation with inflammatory cytokines. In order to elucidate a mechanism through which IL-12 stimulates IL-17A expression in MAIT cells, we analysed expression of the transcription factor retinoic acid-related orphan receptor γT (RORγt), which is required for the expression of IL-17 in T cells (28). We detected significantly increased levels of RORγt expression in MAIT cells from healthy controls (Figure 5f) and AILD patients (Supplementary figure 5a) following 24 hour IL-12 stimulation, which was sustained in MAIT cells stimulated for 72 hours (Figure 5f and supplementary figure 5a). These data indicate that IL-12 signalling induces robust upregulation of RORγt expression in MAIT cells, which may mechanistically enable IL-17A expression.

MAIT cells stimulate hHSC proliferation and induce an activated, pro-inflammatory HSC gene signature in a cell-cell contact and IL-17 dependent manner

Chronic hepatic inflammation in AILD results in the development of liver fibrosis (12) as a consequence of HSC activation (29), which can be mediated by IL-17A in murine models (17, 18). As we observed IL-17A expression by MAIT cells upon repetitive stimulation with IL-12, we hypothesised that human MAIT cells are able to activate HSCs *in vitro*, and analysed primary human HSC (hHSC) proliferation in the presence of MAIT cells. Co-culture of hHSCs

with MAIT at a ratio of 1:1 hHSCs:MAIT cells resulted in a significant increase in hHSC proliferation (Figure 6a). We next investigated gene expression in hHSCs co-cultured with MAIT cells. MAIT cells induced increased expression of the activation marker α-SMA (ACTA2) in hHSCs, as well as a significant increase in expression of the pro-fibrogenic genes collagen 1 (Col1A1), lysyl oxidase (LOX) and TIMP-1 (Figure 6b). Similarly, expression of pro-inflammatory genes, such as IL-1β, IL-6, IL-8 and CCL2 was significantly increased in hHSCs following 48h co-culture with MAIT cells (Figure 6b). We next investigated whether MAIT cells from AILD patients, although exhausted, were able to increase the expression of genes typical of HSC activation. Similar to MAIT cells from healthy controls, MAIT cells from AILD patients induced gene expression in HSCs regardless of the type of disease (Figure 6c) and stimulated hHSC proliferation (Supplementary figure 6a), indicating that MAIT cells from AILD patients retain their pro-fibrogenic potential. Of note, MAIT cells isolated from patients with different fibrosis stages showed no difference in their ability to induce hHSC proliferation and gene expression. Moreover, similar to AILD MAIT cells, NASH MAIT cells stimulated pro-inflammatory gene expression in HSCs (Figure 6d). These data indicate that MAIT cells are able to further promote the features of hHSC activation *in vitro*, a process that contributes to fibrosis development in the liver *in vivo* (29). In order to determine whether IL-17A mediated hHSC activation by MAIT cells, we tested the effect of IL-17RA blockade on hHSC gene expression in our co-culture system. As IL-17 did not induce expression of pro-fibrogenic genes in hHSCs *in vitro*, but was sufficient to induce expression of pro-inflammatory genes, such as IL-1β, IL-6, IL-8 and CCL2 in hHSCs (Supplementary figure 6b), we tested the effect of IL-17R blockade on pro-inflammatory gene expression. Indeed, blocking IL-17R resulted in a decrease of IL-1β, IL-8 and CCL2 expression in hHSCs co-cultured with MAIT cells (Figure 6e), indicating that hHSC activation by MAIT cells is partly

mediated by IL-17A. We next investigated whether hHSC activation by MAIT cells also requires cell-cell contact. MAIT cells and hHSCs were either co-cultured allowing for cell-cell contact, or in a transwell system, thereby preventing cellular contact but allowing the exchange of soluble mediators. Separation of MAIT cells and hHSCs in the transwell system partly reversed upregulation of IL-1β, IL-8 and CCL2 expression and abrogated upregulation of IL-6, LOX and TIMP-1 expression in HSCs (Figure 6f). Taken together, these data suggest that MAIT cells from healthy individuals, as well as from patients with chronic liver disease, can induce hHSC activation *in vitro* in an IL-17A and cell-cell contact dependent manner.

Discussion

The pathogenesis of AILD development is still incompletely understood. Yet, it has recently become clear that chronic inflammation observed in AILD results from inadequate immune activation and a breakdown of self-tolerance in the liver, culminating in T cell mediated liver damage and fibrosis development (12), which is characterized and driven by HSC activation (29). Here, we demonstrate a potential role of MAIT cells in fibrosis development in AILD. Irrespective of the type of disease, MAIT cells in AILD patients showed phenotypical and functional features of cellular exhaustion and were severely reduced in number. The severe decline of MAIT cells observed in both peripheral blood and liver tissue of AILD patients probably results from chronic activation and cellular exhaustion, which is commonly characterized by a hierarchical loss of effector function, followed by cell death (30). T cell exhaustion arises from chronic exposure to various stimuli, e.g. chronic infection with pathogens (31, 32), long-term exposure to viral antigen (22), antigen-release from tumours

(33), or long-term inflammation and chronic exposure to inflammatory cytokines (34). Here, we show that exhaustion of human MAIT cells can be driven by long-term stimulation with the pro-inflammatory, third-signal cytokines IL-12 + IL-18. As both IL-12 and IL-18 levels are elevated in serum of patients with AILD (23, 24), it seems plausible that MAIT cells are chronically exposed to IL-12 + IL-18 in AILD patients, providing a potential mechanistic explanation for the observed exhaustion of MAIT cell in AILD patients *in vivo*. Moreover, we show that MAIT cells from AILD patients express significantly lower levels of CD3 as well as of the transcription factor EOMES, both of which can be induced by antigen exposure (25, 26). Thus, it is likely that additional mechanisms such as long-term exposure to bacterial-derived antigen contribute to MAIT cell activation and exhaustion *in vivo*. This is of special importance considering that AILD cirrhosis is often characterized by bacterial translocation from the gut to the liver (35), which might provide a source of bacterial-derived riboflavin derivatives that specifically activate MAIT cells (36, 37).

The pro-inflammatory cytokine IL-17A is regarded as a key cytokine for the development of tissue-specific autoimmune disorders and has recently been shown to be involved in hepatic fibrosis development in both non-autoimmune mediated liver disease (18, 38, 39) and AILD (13, 38-41). PSC, PBC and AIH patients show elevated serum levels of IL-17 (42-44), along with infiltration of IL-17$^+$ cells into the liver parenchyma (42, 45). Furthermore, the number of liver infiltrating IL-17$^+$ cells correlates with hepatic inflammation and fibrosis in AIH (46). Although IL-17 can be secreted by conventional T helper 17 (Th17) cells at high levels (47), up to now it is unclear which cells contribute to IL-17 production during human liver inflammation, especially in AILD. Interestingly, MAIT cells represent more than 60% of IL-17$^+$ cells in the liver (4), suggesting that IL-17 in the liver *in vivo* might be predominantly derived from MAIT cells rather than Th17 cells. As MAIT cells from AILD patients, despite exhibiting

clear features of chronic exhaustion, retain their ability to express the cytokine IL-17A *ex vivo*, it seems likely that MAIT cells constantly produce IL-17A in the liver of AILD patients. Furthermore, we describe for the first time that chronic stimulation of MAIT cells by the innate, pro-inflammatory cytokine IL-12 stimulates IL-17A expression in MAIT cells. IL-12 predominantly signals through activation of the transcription factors signal transducer and activator of transcription (STAT) STAT1, STAT3, STAT5, and in particular STAT4 (48, 49), leading to IFNγ expression (50). However, STAT3 signalling can further induce the expression of the transcription factor RORγT, which regulates IL-17 expression (28) and is constitutively expressed in MAIT cells (3). We show that IL-12 stimulation induces significant upregulation of RORγt in MAIT cells. Therefore, the observed expression of IL-17 in MAIT cells following prolonged exposure to IL-12 might result from "non-classical" IL-12 signalling, mediated through STAT3-regulated RORγT induction, although further experiments are necessary to fully confirm this hypothesis.

Activation of HSCs is a key event in the development of liver fibrosis, as HSCs transform into hyper-proliferative, myofibroblast-like cells producing large amounts of ECM proteins upon activation (29), thereby leading to the formation of liver fibrosis and cirrhosis (29). In murine model of liver fibrosis, it has been demonstrated that fibrosis development depends on IL-17A *in vivo*, and that IL-17A induced HSC activation *in vitro* (18). However, whether IL-17 contributes to fibrosis development through activation of HSCs in human AILD has not been investigated so far. Our findings show for the first time that MAIT cells induce HSC proliferation and an activated, pro-fibrogenic and pro-inflammatory phenotype in primary human HSCs *in vitro*. Interestingly, despite an exhausted phenotype, MAIT cells from AILD patients, regardless of the donor's fibrosis stage, retain their ability to secrete IL-17A and are equally able to induce HSC activation. These data suggest that MAIT cells may contribute

to fibrosis development in AILD *in vivo* throughout disease progression. Moreover, we show that MAIT cells isolated from NASH patients are highly activated and able to induce HSC activation *in vitro* as well, suggesting a rather general pro-fibrogenic role of MAIT cells in chronic liver disease. Mechanistically, the pro-fibrogenic activation of HSCs by MAIT cells depends on IL-17A but also involves direct cell-cell contact between MAIT cells and HSCs, at least *in vitro*.

Taken together, our findings demonstrate a role for IL-17 producing MAIT cells as pro-fibrogenic cellular mediators in AILD, which could serve as a potential therapeutic target for novel anti-fibrotic approaches.

Author contributions

KB designed the study. KB and KR conducted experiments. KB, FS, DR, MR, ET and DT collected patient samples and clinical data. KB, AH and TL performed and analysed immunohistochemistry. KB, KR and MP analysed data. KB drafted and wrote the manuscript, KR and MP critically revised the manuscript and gave intellectual input for study design and experiments. All authors have seen and approved the final manuscript.

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Bibliography

Author names in bold designate shared co-first authorship

1. Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. The Journal of Experimental Medicine 1993;178:1-16.

2. Tilloy F, Treiner E, Park S-H, Garcia C, Lemonnier F, de la Salle H, Bendelac A, et al. An Invariant T Cell Receptor α Chain Defines a Novel TAP-independent Major Histocompatibility Complex Class Ib–restricted α/β T Cell Subpopulation in Mammals. The Journal of Experimental Medicine 1999;189:1907-1921.

3. **Dusseaux M, Martin E**, Serriari N, Peguillet I, Premel V, Louis D, Milder M, et al. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. Blood 2011;117:1250-1259.

4. **Tang XZ, Jo J, Tan AT, Sandalova E**, Chia A, Tan KC, Lee KH, et al. IL-7 licenses activation of human liver intrasinusoidal mucosal-associated invariant T cells. J Immunol 2013;190:3142-3152.

5. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, Bhati M, et al. MR1 presents microbial vitamin B metabolites to MAIT cells. Nature 2012;491:717-723.

6. **Le Bourhis L, Dusseaux M**, Bohineust A, Bessoles S, Martin E, Premel V, Core M, et al. MAIT cells detect and efficiently lyse bacterially-infected epithelial cells. PLoS Pathog 2013;9:e1003681.

7. Le Bourhis L, Martin E, Péguillet I, Guihot A, Froux N, Coré M, Lévy E, et al. Antimicrobial activity of mucosal-associated invariant T cells. Nature Immunology 2010;11:701-708.

8. **Ussher JE, Bilton M**, Attwod E, Shadwell J, Richardson R, de Lara C, Mettke E, et al. CD161 ++CD8 +T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner. European Journal of Immunology 2013;44:195-203.

9. **Annibali V, Ristori G, Angelini DF**, Serafini B, Mechelli R, Cannoni S, Romano S, et al. CD161highCD8+T cells bear pathogenetic potential in multiple sclerosis. Brain 2011;134:542-554.

10. **Salou M, Nicol B**, Garcia A, Baron D, Michel L, Elong-Ngono A, Hulin P, et al. Neuropathologic, phenotypic and functional analyses of Mucosal Associated Invariant T cells in Multiple Sclerosis. Clin Immunol 2016;166-167:1-11.

11. Serriari N-E, Eoche M, Lamotte L, Fumery M, Marcelo P, Chatelain D, Barre A, et al. Innate Mucosal-Associated Invariant T (MAIT) cells are activated in Inflammatory Bowel Diseases. Clinical & Experimental Immunology 2014:n/a-n/a.

12. Liaskou E, Hirschfield GM, Gershwin ME. Mechanisms of tissue injury in autoimmune liver diseases. Semin Immunopathol 2014;36:553-568.

13. Hirschfield GM, Siminovitch KA. Toward the molecular dissection of primary biliary cirrhosis. Hepatology 2009;50:1347-1350.

14. Yoshida K, Yang G-X, Zhang W, Tsuda M, Tsuneyama K, Moritoki Y, Ansari AA, et al. Deletion of IL-12p40 suppresses autoimmune cholangitis in dnTGFβRII mice. Hepatology (Baltimore, Md.) 2009;50:1494-1500.

15. **Gil-Farina I, Di Scala M**, Salido E, López-Franco E, Rodríguez-García E, Blasi M, Merino J, et al. Transient Expression of Transgenic IL-12 in Mouse Liver Triggers Unremitting Inflammation Mimicking Human Autoimmune Hepatitis. The Journal of Immunology 2016;197:2145-2156.

16. Seki E, Schwabe RF. Hepatic inflammation and fibrosis: functional links and key pathways. Hepatology 2015;61:1066-1079.

17. Meng F, Wang K, Aoyama T, Grivennikov SI, Paik Y, Scholten D, Cong M, et al. Interleukin-17 signaling in inflammatory, Kupffer cells, and hepatic stellate cells exacerbates liver fibrosis in mice. Gastroenterology 2012;143:765-776 e761-763.

18. **Tan Z, Qian X**, Jiang R, Liu Q, Wang Y, Chen C, Wang X, et al. IL-17A plays a critical role in the pathogenesis of liver fibrosis through hepatic stellate cell activation. J Immunol 2013;191:1835-1844.

19. Longato L, Andreola F, Davies SS, Roberts JL, Fusai G, Pinzani M, Moore K, et al. Reactive gamma-ketoaldehydes as novel activators of hepatic stellate cells in vitro. Free Radic Biol Med 2017;102:162-173.

20. Castéra L, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology 2005;128:343-350.

21. **Gupta PK, Godec J, Wolski D**, Adland E, Yates K, Pauken KE, Cosgrove C, et al. CD39 Expression Identifies Terminally Exhausted CD8+ T Cells. PLoS Pathog 2015;11:e1005177.

22. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, Ahmed R. Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med 1998;188:2205-2213.

23. Landi A, Weismuller TJ, Lankisch TO, Santer DM, Tyrrell DLJ, Manns MP, Houghton M. Differential Serum Levels of Eosinophilic Eotaxins in Primary Sclerosing Cholangitis, Primary Biliary Cirrhosis, and Autoimmune Hepatitis. Journal of Interferon & Cytokine Research 2014;34:204-214.

24. Yamano T, Higashi T, Nouso K, Nakatsukasa H, Kariyama K, Yumoto E, Kobayashi Y, et al. Serum interferon-gamma-inducing factor/IL-18 levels in primary biliary cirrhosis. Clinical and Experimental Immunology 2000;122:227-231.

25. Valitutti S, Müller S, Salio M, Lanzavecchia A. Degradation of T Cell Receptor (TCR)–CD3-ζ Complexes after Antigenic Stimulation. The Journal of Experimental Medicine 1997;185:1859-1864.

26. Leeansyah E, Svard J, Dias J, Buggert M, Nystrom J, Quigley MF, Moll M, et al. Arming of MAIT Cell Cytolytic Antimicrobial Activity Is Induced by IL-7 and Defective in HIV-1 Infection. PLoS Pathog 2015;11:e1005072.

27. **van Wilgenburg B, Scherwitzl I**, Hutchinson EC, Leng T, Kurioka A, Kulicke C, de Lara C, et al. MAIT cells are activated during human viral infections. Nat Commun 2016;7:11653.

28. **Ivanov II, McKenzie BS, Zhou L**, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, et al. The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17+ T Helper Cells. Cell 2006;126:1121-1133.

29. Pinzani M, Rombouts K. Liver fibrosis: from the bench to clinical targets. Digestive and Liver Disease 2004;36:231-242.

30. Wherry EJ. T cell exhaustion. Nature Immunology 2011;131:492-499.

31. Day CL, Abrahams DA, Lerumo L, Janse van Rensburg E, Stone L, O'rie T, Pienaar B, et al. Functional Capacity of Mycobacterium tuberculosis-Specific T Cell Responses in Humans Is Associated with Mycobacterial Load. The Journal of Immunology 2011;187:2222-2232.

32. Bhadra R, Gigley JP, Weiss LM, Khan IA. Control of Toxoplasma reactivation by rescue of dysfunctional CD8+ T-cell response via PD-1–PDL-1 blockade. Proceedings of the National Academy of Sciences 2011;108:9196-9201.

33. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, Rosenberg SA. Tumor antigen–specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. Blood 2009;114:1537-1544.

34. Stelekati E, Shin H, Doering Travis A, Dolfi Douglas V, Ziegler Carly G, Beiting Daniel P, Dawson L, et al. Bystander Chronic Infection Negatively Impacts Development of CD8+ T Cell Memory. Immunity 2014;40:801-813.

35. Lin R, Zhou L, Zhang J, Wang B. Abnormal intestinal permeability and microbiota in patients with autoimmune hepatitis. International Journal of Clinical and Experimental Pathology 2015;8:5153-5160.

36. **Corbett AJ, Eckle SBG, Birkinshaw RW**, Liu L, Patel O, Mahony J, Chen Z, et al. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. Nature 2014;509:361-365.

37. **Eckle SB, Birkinshaw RW**, Kostenko L, Corbett AJ, McWilliam HE, Reantragoon R, Chen Z, et al. A molecular basis underpinning the T cell receptor heterogeneity of mucosal-associated invariant T cells. J Exp Med 2014;211:1585-1600.

38. Lafdil F, Miller AM, Ki SH, Gao B. Th17 cells and their associated cytokines in liver diseases. Cell Mol Immunol 2010;7:250-254.

39. Hammerich L, Heymann F, Tacke F. Role of IL-17 and Th17 Cells in Liver Diseases. Clinical and Developmental Immunology 2011;2011:345803.

40. Trivedi PJ, Hirschfield GM. Treatment of autoimmune liver disease: current and future therapeutic options. Therapeutic Advances in Chronic Disease 2013;4:119-141.

41. Iwakura Y, Ishigame H. The IL-23/IL-17 axis in inflammation. Journal of Clinical Investigation 2006;116:1218-1222.

42. Harada K, Shimoda S, Sato Y, Isse K, Ikeda H, Nakanuma Y. Periductal interleukin-17 production in association with biliary innate immunity contributes to the pathogenesis of cholangiopathy in primary biliary cirrhosis. Clinical & Experimental Immunology 2009;157:261-270.

43. Yasumi Y, Takikawa Y, Endo R, Suzuki K. Interleukin-17 as a new marker of severity of acute hepatic injury. Hepatology Research 2007;37:248-254.

44. Zhang H, Bernuzzi F, Lleo A, Ma X, Invernizzi P. Therapeutic Potential of IL-17-Mediated Signaling Pathway in Autoimmune Liver Diseases. Mediators of Inflammation 2015;2015:436450.

45. Katt J, Schwinge D, Schoknecht T, Quaas A, Sobottka I, Burandt E, Becker C, et al. Increased T helper type 17 response to pathogen stimulation in patients with primary sclerosing cholangitis. Hepatology 2013;58:1084-1093.

46. **Zhao L, Tang Y**, You Z, Wang Q, Liang S, Han X, Qiu D, et al. Interleukin-17 Contributes to the Pathogenesis of Autoimmune Hepatitis through Inducing Hepatic Interleukin-6 Expression. PLoS ONE 2011;6:e18909.

47. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol 2009;27:485-517.

48. Watford WT, Hissong BD, Bream JH, Kanno Y, Muul L, O'Shea JJ. Signaling by IL-12 and IL-23 and the immunoregulatory roles of STAT4. Immunological Reviews 2004;202:139-156.

49. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 2003;3:133-146.

50. Lund RJ, Chen Z, Scheinin J, Lahesmaa R. Early Target Genes of IL-12 and STAT4 Signaling in Th Cells. The Journal of Immunology 2004;172:6775-6782.

Figure legends

Figure 1: MAIT cell frequency is significantly reduced in peripheral blood and liver tissue of patients with AILD.

MAIT cell frequency *(n=15 (HC), n=18 (PSC), n=14 (PBC), n=10 (AIH*)) and absolute number in peripheral blood (a, c) and liver tissue (b). Phenotype of MAIT cells in peripheral blood (d) and liver tissue (e). (f) Spearman correlation between MAIT cell frequency in peripheral blood of AILD patients and fibrosis stage or liver stiffness *(n=70)*. (a)-(e) Data represent mean +/- SD, pooled data from 4 independent experiments. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. healthy controls, assessed by One-way ANOVA with Tukey's multiple comparisons test (a, d) or Mann-Whitney test (b, c, e). HC = healthy control

Figure 2: MAIT cells localise around portal tracts and fibrotic septae in AILD livers

(a) Haematoxylin & Eosin (H&E), Sirius Red and immunohistochemistry of healthy and diseases liver tissue. (b) Number of Vα7.2^{+} MAIT cells/high power filed (HPF), each symbol represents one individual. Data represent mean +/- SD, *p< 0.05 vs. healthy control (HC), assessed by Kruskal-Wallis test.

Figure 3: AILD MAIT cells show signs of exhaustion and express IL-17A.

Ex vivo expression of surface markers in MAIT cells from (a) peripheral blood and (b) liver. Intracellular IFNγ (c), GrzB (d), IL-17A and TNF-α (d) expression in MAIT cells following *in vitro* PMA/Ionomycin (c, e) or CD3/CD28 bead (d) stimulation for 16h. (e, f) Spearman correlation between MAIT cell frequency in peripheral blood of AILD patients and expression of activation markers and inhibitory receptors. (a, c) Data represent mean +/-

SEM, (b, d-e) mean +/- SD, pooled data of at least 3 independent experiments. *p< 0.05 vs. healthy control, ****p<0.0001 vs. unstimulated control, assessed by Mann-Whitney test (a, b, e), or one-way ANOVA with Tukey's or Dunn's multiple comparisons test (c, d). HC = healthy control

Figure 4: Long-term exposure to pro-inflammatory cytokines drives MAIT cell exhaustion. Intracellular IFNγ expression of (a) MAIT cells in a PMCB pool *(n = 12)* or (b) purified Vα7.2+ cells *(n=3)* stimulated with IL-1β, IL-12 and IL-18 (24-72h). (c) Intracellular IFNγ expression of MAIT cells in a PBMC pool stimulated with IL-12+IL-18 (72h) *(n=12)*. (d) Surface expression of inhibitory receptors in peripheral blood MAIT cells from healthy controls stimulated with IL-12 + IL-18 (24-72h). (e) CD3 expression in hepatic MAIT cells. (f) EOMES and T-bet expression in peripheral blood MAIT cells. Data represent mean +/- SEM (a, c), or mean +/- SD (b, d-f), pooled data from at least 3 independent experiments.*p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. no stimulation, healthy control, or as indicated, assessed by 2-way ANOVA and Sidak's multiple comparisons test (a-d), or Mann-Whitney test (e-f).

Figure 5: Repetitive IL-12 stimulation induces IL-17A expression in MAIT cells

Intracellular IL-17A expression in MAIT cells in a PBMC pool from (a-b) healthy controls (HC) *(n=12)* and (d) AILD patients *(n=26)* stimulated with IL-1β, IL-12 and IL-18 for (a) 24h or (b, d) 24 -72h. (c) Intracellular IL-17A expression of purified Vα7.2^{+} MAIT cells following cytokine stimulation (24-72h) *(n=3)*. (e) Intracellular IL-17A expression of MAIT cells from AILD patients and HC following cytokine stimulation (72h) *(HC n=9, AILD n=26)*. (f) RORγt expression in MAIT cells following IL-12 stimulation (24-72h). Data represent mean +/- SEM (a-b, d-e) pooled data from 4 independent experiments. (c, f) Data represent mean +/- SD,

data from at least 2 independent experiments. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. no stimulation or as indicated, assessed by 2-way ANOVA and Sidak's multiple comparisons test (a-e) or paired t-test and Kruskal-Wallis test (f).

Figure 6: MAIT cells are able to stimulate HSC activation in a cell-cell contact and IL-17 dependent manner

(a) BrdU incorporation *(n=4)* and (b)-(d) gene expression of human HSCs after 48h co-culture with Vα7.2$^+$ MAIT cells from (a-b) healthy controls (HC), (c) AILD and (d) NASH patients. Gene expression of hHSCs after co-culture with Vα7.2$^+$ MAIT cells from healthy controls with IL-17R antibody (e) and in transwell system (f). (a, c-f) Data represent mean +/- SD *p < 0.05, ***p<0.001. ****p<0.0001 vs. unstimulated control, assessed by one-way ANOVA and Tukey's or Dunn's multiple comparisons test, representative data from 3 independent experiments. (b) data represent mean +/- 95% CI, *p = 0.05 vs. unstimulated control, pooled data from 3 independent experiments.

Supplementary information

Supplementary Methods

Antibodies for flow cytometry

Surface markers: CD3 FITC (HIT3a), CD4 PE (OKT4), CD8 APC (SK1) (ebioscience, San Diego, CA), CD4 PE-CF594 (RPA-T4), HLA-DR FITC (G46-6), CD152/CTLA-4 BV786 (BNI3), CD212/IL-12Rβ APC (2.4e6), CD16 APC-H7 (3G8), CD56 PE-Cy7 (B159), iNKT PE (6B11), Vδ2 FITC (B6), Granzyme B A700 (GB11) (BD Biosciences, San Jose, CA), CD161 BV605 (HP-3G10), Vα7.2 BV421 (3C10), Vα7.2 PE (3C10), CD279/PD-1 PE (EH12.2H7), CD69 PE dazzle 594 (FN50), CD38 PE dazzle 594 (HIT2), CD39 BV421 (A1), CD218/IL-18Rα PE (H44), CD3 BV605 (OKT3), Vα7.2 PE-Cy7 (3C10) (Biolegend, San Diego, CA), CD161 PE (191B8) (Miltenyi Biotec, Bergisch Gladbach, Germany) and TIM3 PE (344823) (R&D systems, Minneapolis, MN). Intracellular markers: IFNγ APC-Cy7 (4S.B3), IL-17A PerCP/Cy5.5 (BL168), TNFα PE-Cy7 (MAb11) (Biolegend, San Diego, CA), EOMES PE-eFlour610 (WD1928), T-bet eFlour660 (4B10) (ebioscience, San Diego, CA), RORγt BV421 (Q21-559) (BD Biosciences, San Jose, CA).

Isolation of Liver associated Lymphocytes

Briefly, liver associated lymphocytes were isolated from 20g cirrhotic liver tissue using collagenase type IV (Sigma Aldrich, St. Louis, MO) digestion (0.5μg/ml, 10 min, 37° C), or from 20g healthy liver tissue, and passed through a metal mesh. After incubation with DNase I (Sigma Aldrich, St. Louis, MO) (0.5μg/ml, 10 min, 37° C) cell suspension from either type of liver tissue was washed extensively and liver associated lymphocytes were enriched

using Ficoll-Paque Plus and 40% Percoll (both GE Healthcare, Uppsala, Sweden) gradient centrifugation (1).

Isolation of hHSCs

Briefly, 10g of liver tissue was digested with 0.01% Collagenase type IV, 0.05% Pronase and 0.001% DNase I (all Sigma Aldrich, St. Louis, MO). The homogenate was washed and Optiprep 11.5% (Sigma Aldrich, St. Louis, MO) gradient centrifugation was performed to enrich HSCs. The obtained HSC were cultured in Iscove's Modified DMEM (IMDM), supplemented with 20% foetal bovine serum (FBS), 2 mM Glutamine, 1X nonessential amino acids, 1.0 mM sodium pyruvate, 1X antibiotic-antimycotic (all from Life Technologies, Carlsbad, CA), referred to as complete hHSC medium (CM) hereinafter. Each hHSC preparation was maintained under standard conditions in a humidified incubator under 5% CO_2 at 37°C. Experiments described in this study were performed with hHSC cultured in CM of at least three cell preparations used between passage 2 and 9.

Transient elastography

The 5 MHz ultrasound transducer probe was placed perpendicularly to the skin between the ribs over the right lobe of the liver, the patient lying in dorsal decubitus with the right arm in maximal abduction. All patients underwent a fasting period of at least 3 hours prior to measurements. The M or XL probe was used according to patients' physical constitution. Liver stiffness was expressed in kilopascal (kPa) and a test was considered valid, when at least 10 successful measurements with a success rate of >60% and an interquartile range (IQR) <30% were obtained.

Magnetic cell separation

Vα7.2^{+} MIT cells were isolated using a Vα7.2 PE antibody (clone: 3C10) (biolegend, San Diego, CA). Cells were labelled with anti Vα7.2 PE 1:100 for 30 min on ice and incubated with anti-PE beads and purified using MS columns (both Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Magnetic beads purification achieved a purity of >80%, see supplementary figure 7.

Quantitative real time PCR (qPCR)

Prior to RNA isolation, co-cultures were extensively washed with PBS in order to remove MAIT cells. RNA was isolated from HSC cell lysates employing RNeasy mini Kit (Qiagen, Hilden, Germany), purity and RNA concentration were measured with Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized with MultiScribe reverse transcriptase, random primers, deoxyribose nucleoside triphosphate (dNTP) mix and RNase inhibitor (all Applied Biosystems, Foster City, CA) according to the following protocol, using Quanta Biotech Q Cycler II: 2 min 50°C, 10 min 95°C, followed by 40 cycles of 15 seconds 95°C and 60 seconds 60°C. Gene expression was measured via qPCR using Taqman gene assays (listed in table S2). To quantify gene expression, the comparative C_T method was used as described previously (2) using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control.

Immunohistochemistry and histochemical staining of liver tissue

Frozen, non-fixed, liver tissue samples were mounted in OCT compound, sectioned at 6 µm and fixed using cold acetone (5°C) for 5 minutes. Prior to staining, sections were washed in water to remove the OCT.

Histochemical stains: Tissue sections were stained with Harris's Haematoxylin and Eosin (Leica, Wetzlar, Germany) or Picro-Sirius Red (BDH Chemicals, Poole, UK).

Immunocytochemistry: Sections were stained for MAIT cells using an anti Vα7.2 TCR (clone 3C10) primary antibody (Biolegend, San Diego, CA) and visualised with the Novolink Max Polymer detection kit (Leica, Wetzlar, Germany). The slides were soaked in wash buffer (Tris buffered saline with 0.04% Tween-20) for 5 minutes, blocked with peroxidase blocking solution for 5 minutes and washed in wash buffer for 5 minutes. The slides were blocked for non-specific binding of the secondary layer using the kit's protein block. Primary antibody (dilution 1:150) was added and incubated for 1 hour at room temperature. The slides were then placed for 25 minutes in the post primary solution, 25 minutes in the polymer solution and developed with 3,3' di-amino-benzidine. Slides were counterstained with Mayer's Haematoxylin (Sigma Aldrich, Missouri, MO) for 3 minutes. All sections were dehydrated in graded industrial denatured alcohol, cleared in xylene and mounted with DPX (Leica, Wetzlar, Germany). The slides were observed using an Axioskop 40 microscope and images were captured with an Axiocam IcC5 using Axiovision (Zeiss, Oberkochen, Germany).

Supplementary figure legends

Supplementary figure 1: All MAIT cell subsets are significantly reduced in patients with AILD

(a) Gating strategy for MAIT cells, representative flow cytometry plots, gated on live cells. (b) Frequency of different lymphocyte populations in healthy controls and AILD patients. (c) Frequency of $CD8^+$, $CD4^-/CD8^-$, $CD4^+$ and $CD4^+/CD8^+$ MAIT cells in peripheral blood of AILD patients *(n=15 (HC), n=18 (PSC), n=14 (PBC), n=10 (AIH*)). Data represent mean +/- SD, pooled data from 4 independent experiments. *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. healthy controls, assessed by Kruskal-Wallis test (b), or one-way ANOVA with Tukey's multiple comparisons test (c). HC = healthy controls

Supplementary figure 2: Expression of activation and exhaustion markers negatively correlates with MAIT cell frequency in AILD patients

(a) PD-1 expression in peripheral blood MAIT cells. Surface activation and exhaustion marker expression in liver MAIT cells (b) and peripheral blood MAIT cells (c). (d) Intracellular IL-17A expression in MIAT cells following PMA/Ionokmycin stimulation (16h). (e) Spearman correlation between HLA-DR and TIM-3 expression and MAIT cell frequency in peripheral blood of patients with AILD. Data represent mean +/- SD, data from at least 3 independent experiments.

Supplementary figure 3: Expression of IL-12R and IL-18R in MAIT cells from AILD patients and controls

(a) IL-12R and IL-18R expression in MAIT cells from peripheral blood, *(n=7).* (c). IL-12R and IL-18R expression in MAIT cells from liver tissue. Data represent mean +/- SD, pooled data from at least 2 independent experiments. ns = not significant, assessed by One-way ANOVA with Tukey's multiple comparisons test (a) or Mann-Whitney test (b).

Supplementary figure 4: IL-12R expression is unchanged in MAIT cells following long-term cytokine stimulation

MAIT cells from peripheral blood of healthy controls and AILD patients were stimulated with IL-1β, IL-12 and IL-18 for 24-72h *in vitro:* (a) IL-12R and IL-18R MFI *(n=5),* (b) expression of surface exhaustion markers in MAIT cells, (c) MAIT cell and $CD3^+$ frequency *(n=70.* (a-c) Data represent mean +/- SD, pooled data from 3 independent experiments. ns = not significant, assessed by 2way ANOVA and Tukey's multiple comparisons test.

Supplementary Figure 5:

(a) RORγt expression in MAIT cells from AILD patients following IL-12 stimulation (24-72h). Data represent mean +/- SD, *p<0.05 vs. unstimulated control, assessed by paires t-test or Kruskal-Wallis test.

Supplementary figure 6: Expression of pro-inflammatory, but not pro-fibrogenic, genes is induced by IL-17A in primary human HSCs.

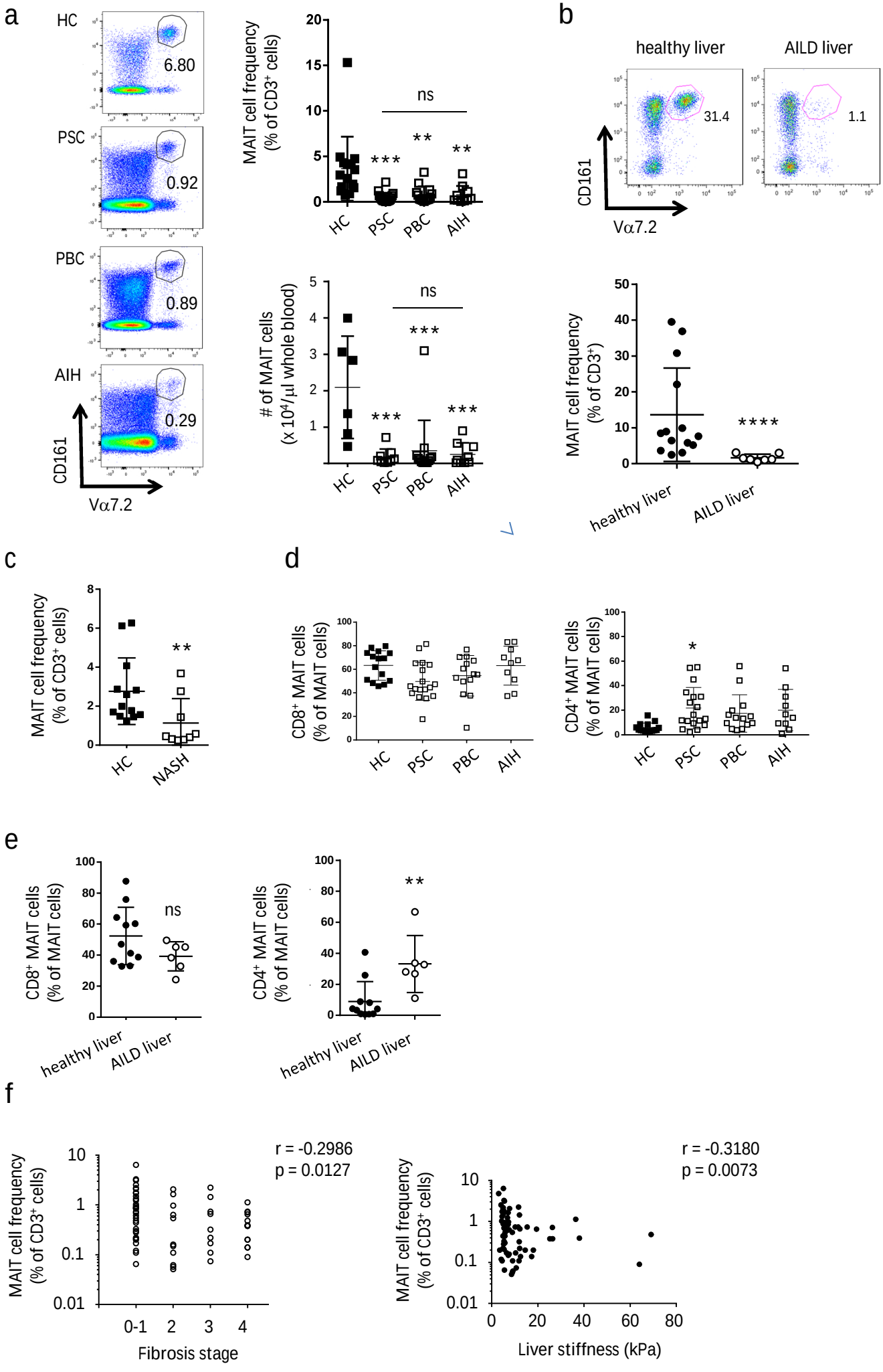
(a) BrdU incorporation in hHSCs *(n=4)* after 48h co-culture with purified $V\alpha7.2^+$ MAIT cells from AILD patients. (b) Relative gene expression in hHSCs following 48h treatment with IL-17A (1ng/ml) in *vitro*. Data represent mean +/- SD, **p<0.01, ****p<0.0001 vs. no stimulation, assessed by One-way ANOVA and Sidak's multiple comparisons test. Representative data of 3 independent experiments with up to 4 different HSC preparations.

Supplementary figure 7: Magnetic cell separation

(a) Vα7.2 expression before and after enrichment of $V\alpha7.2^+$ MAIT cells using magnetic bead separation, representative flow cytometry plots, gated on liver $CD3^+$ T cells.

Bibliography

1. Pallett LJ, Davies J, Colbeck EJ, Robertson F, Hansi N, Easom NJW, Burton AR, et al. IL-2high tissue-resident T cells in the human liver: Sentinels for hepatotropic infection. J Exp Med 2017;214:1567-1580.
2. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature Protocols 2008;3:1101-1108.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# of MAIT cells (x 10⁴/µl whole blood)
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CD161
Vα7.2
MAIT cell frequency (% of CD3+)
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AILD liver
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MAIT cell frequency (% of CD3+ cells)
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healthy liver
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healthy liver
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MAIT cell frequency (% of CD3+ cells)
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p = 0.0127
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Fibrosis stage
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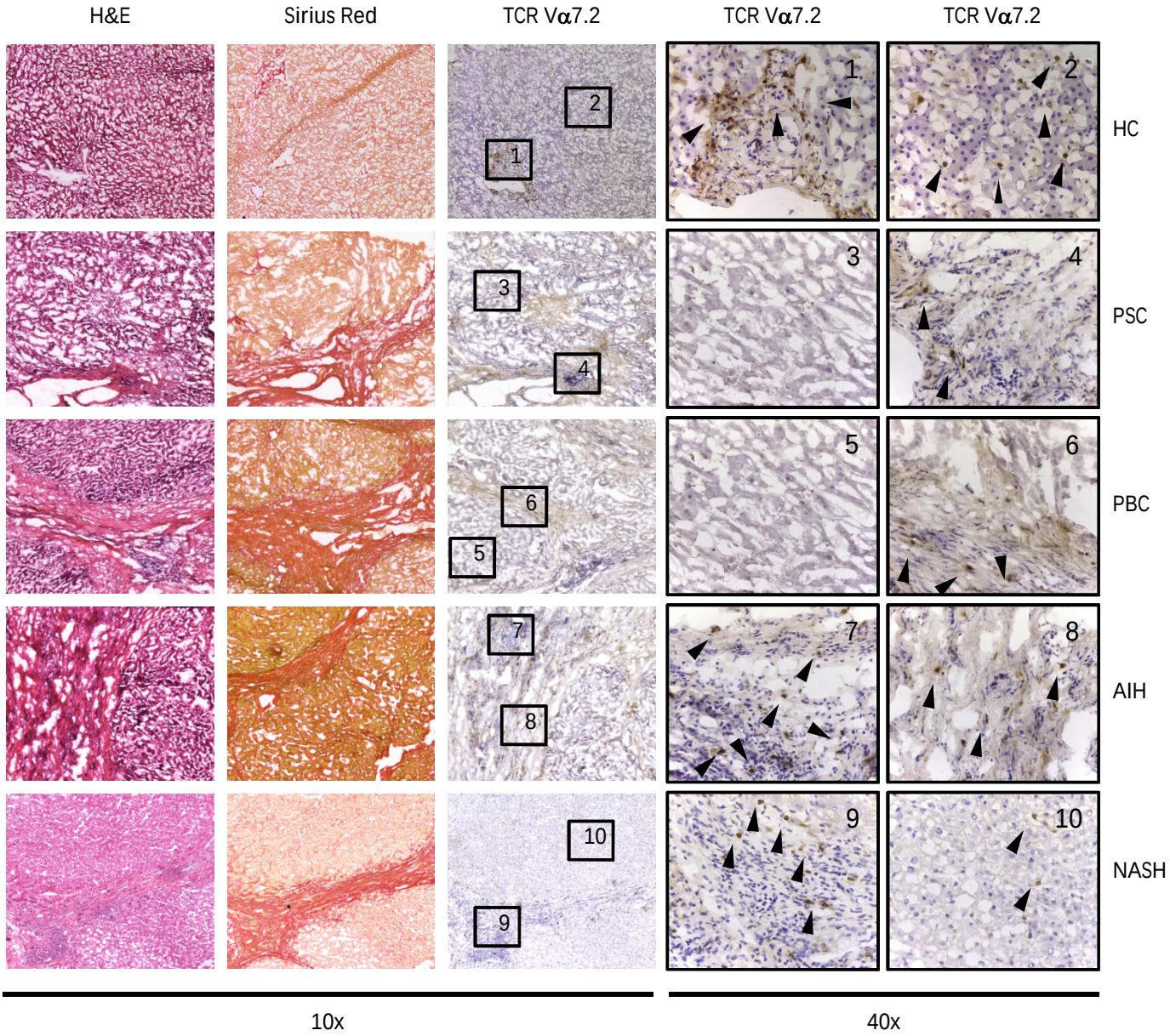
p = 0.0073
Liver stiffness (kPa)

a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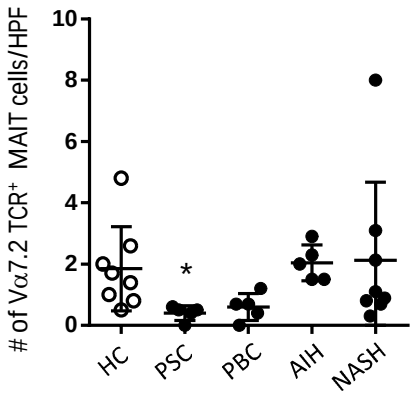
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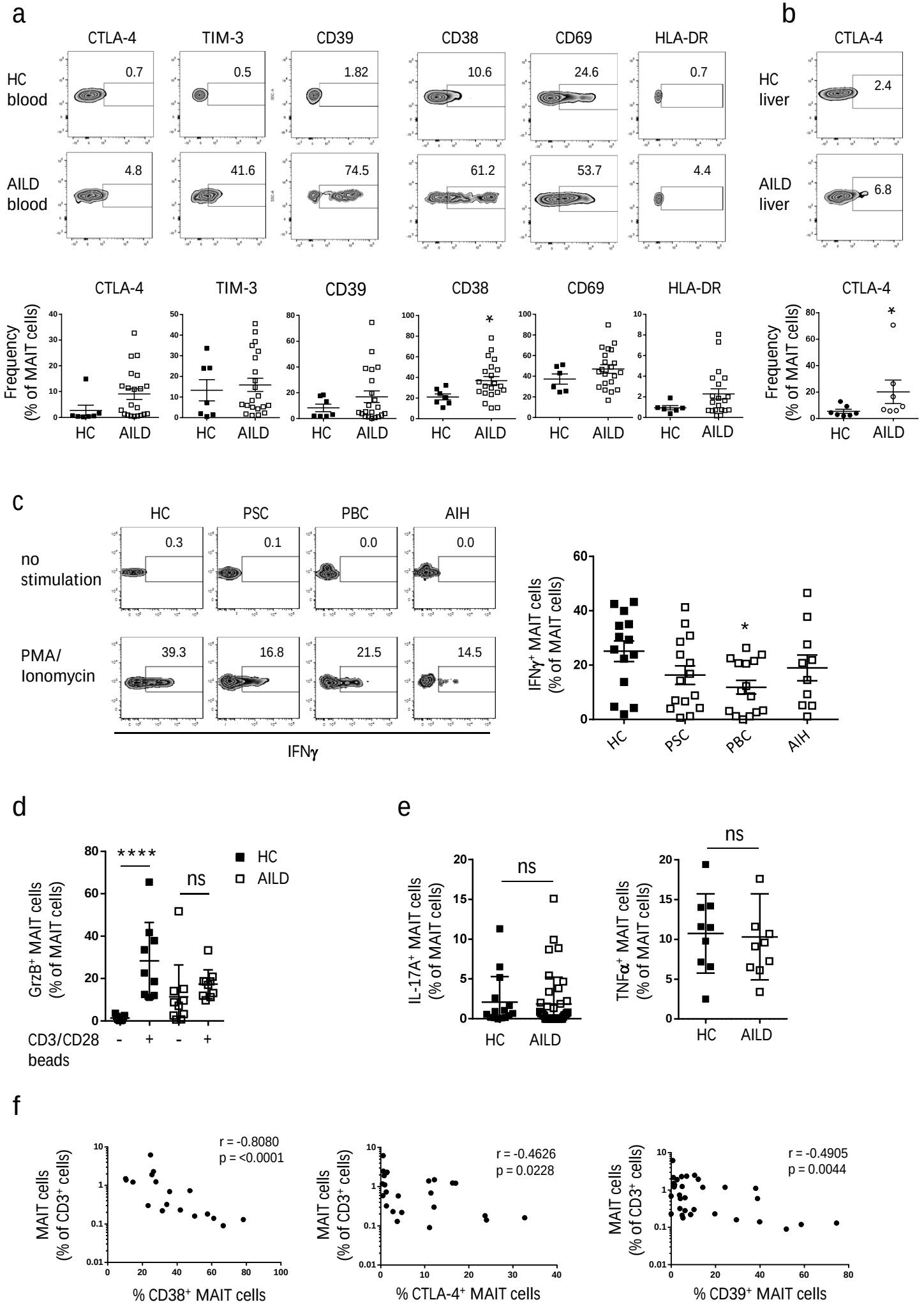
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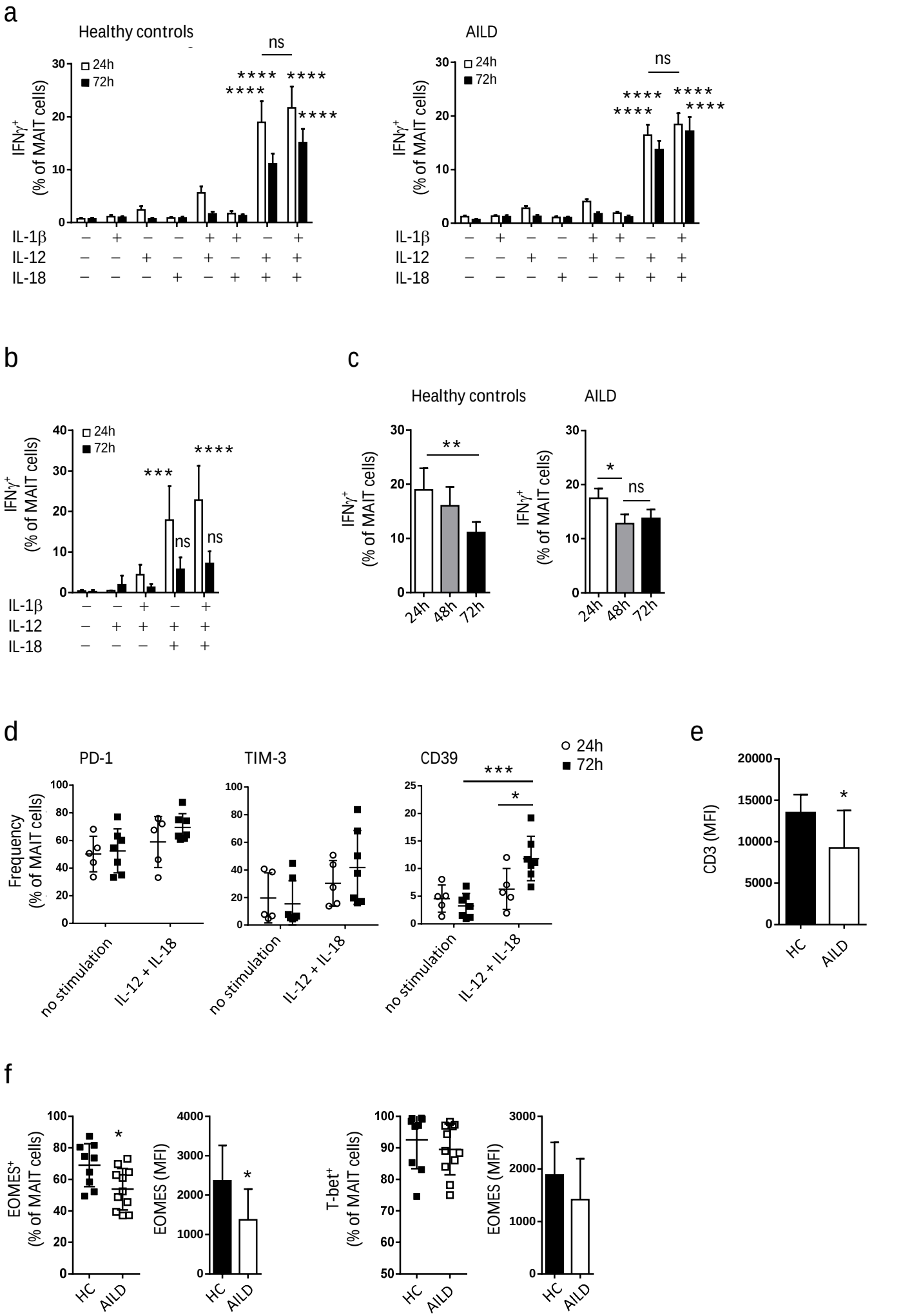
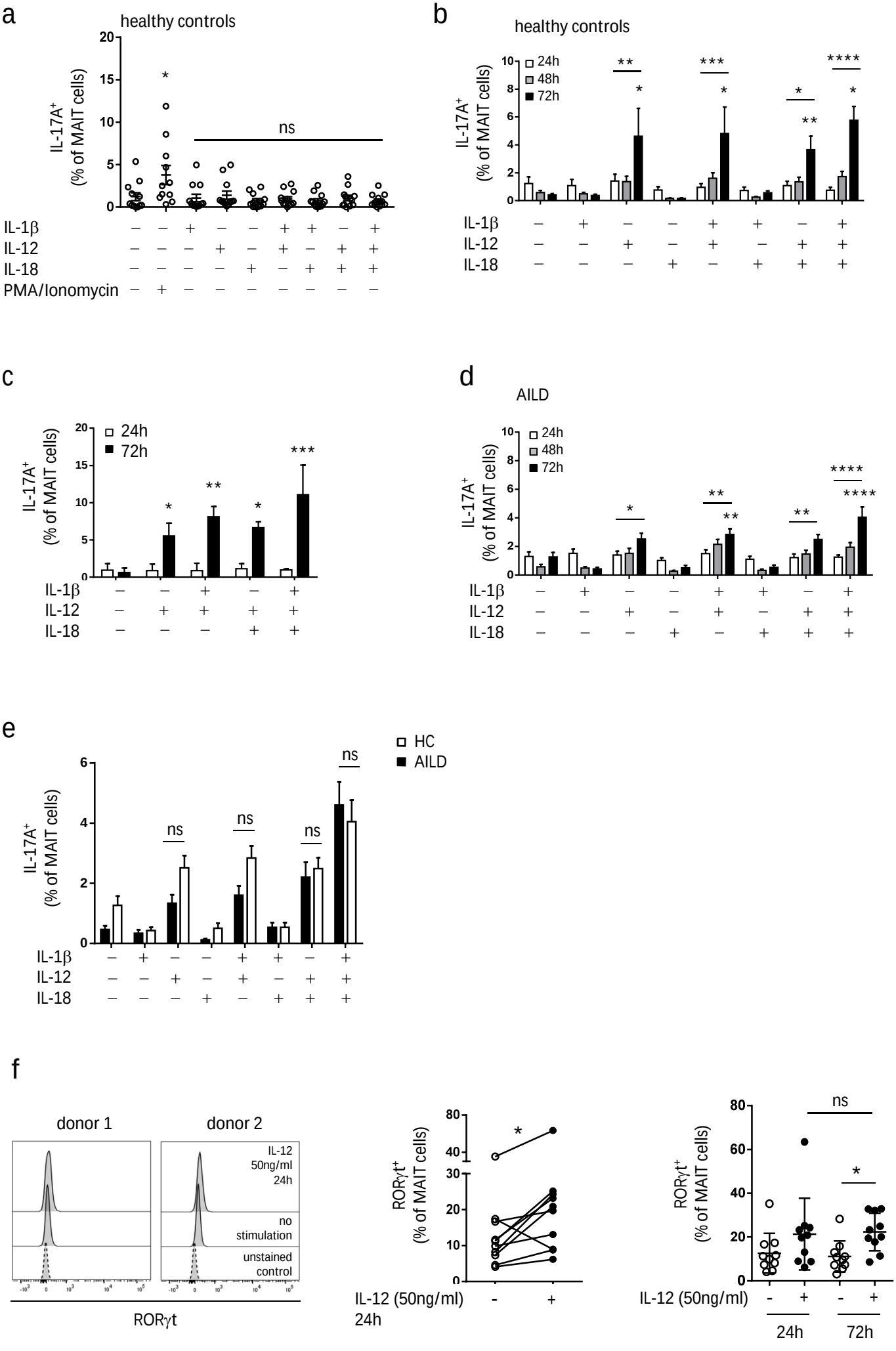
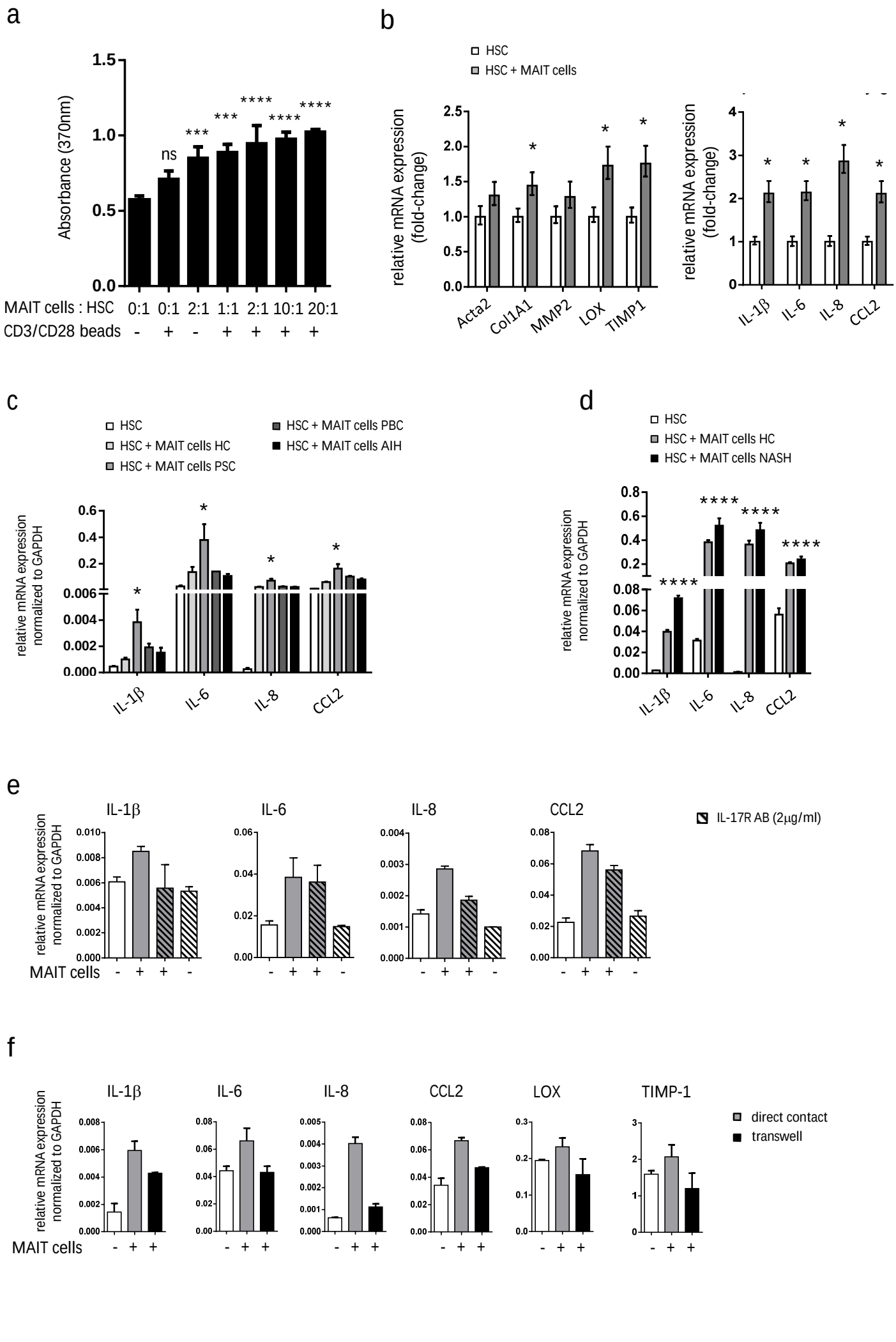
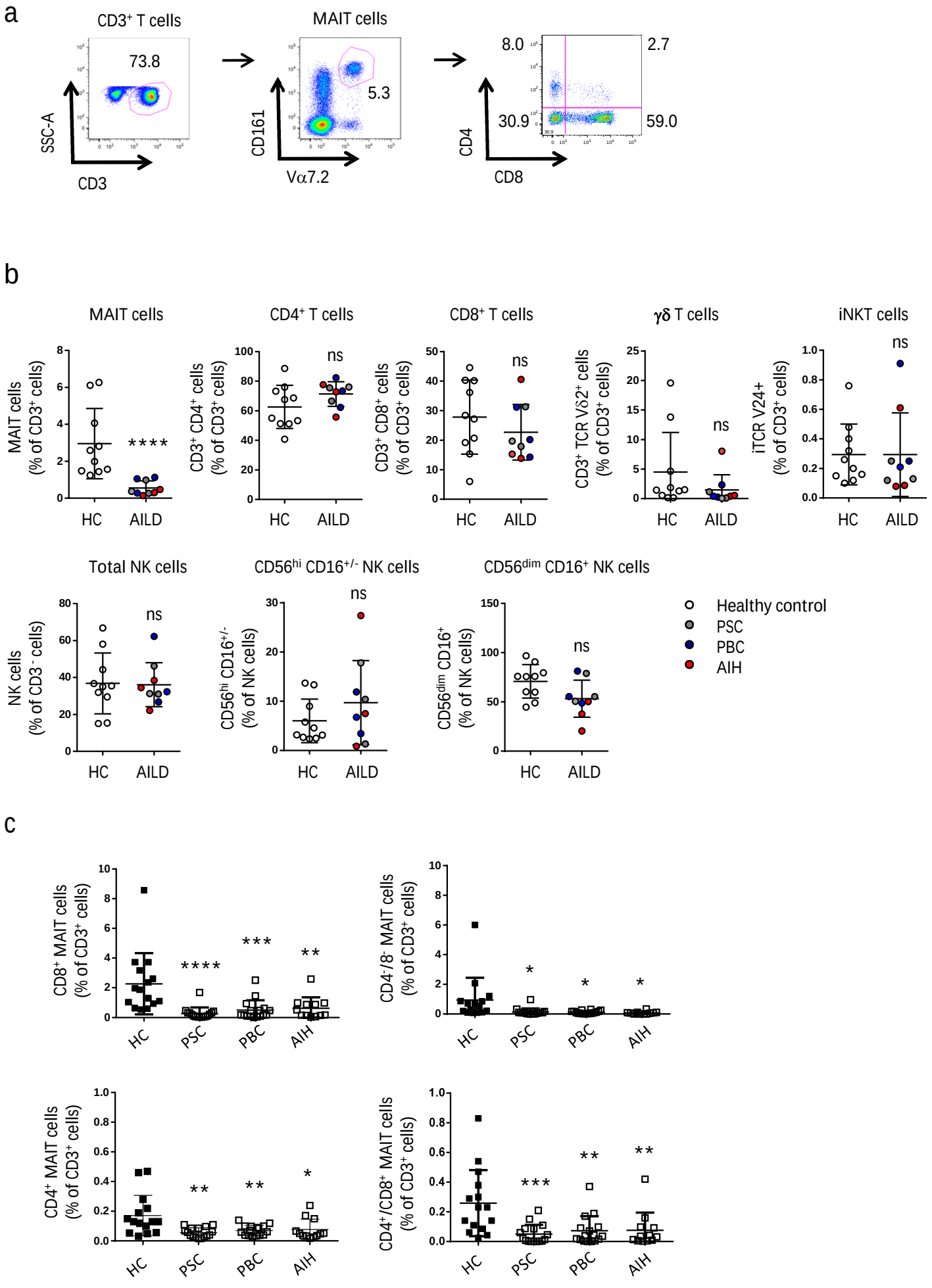
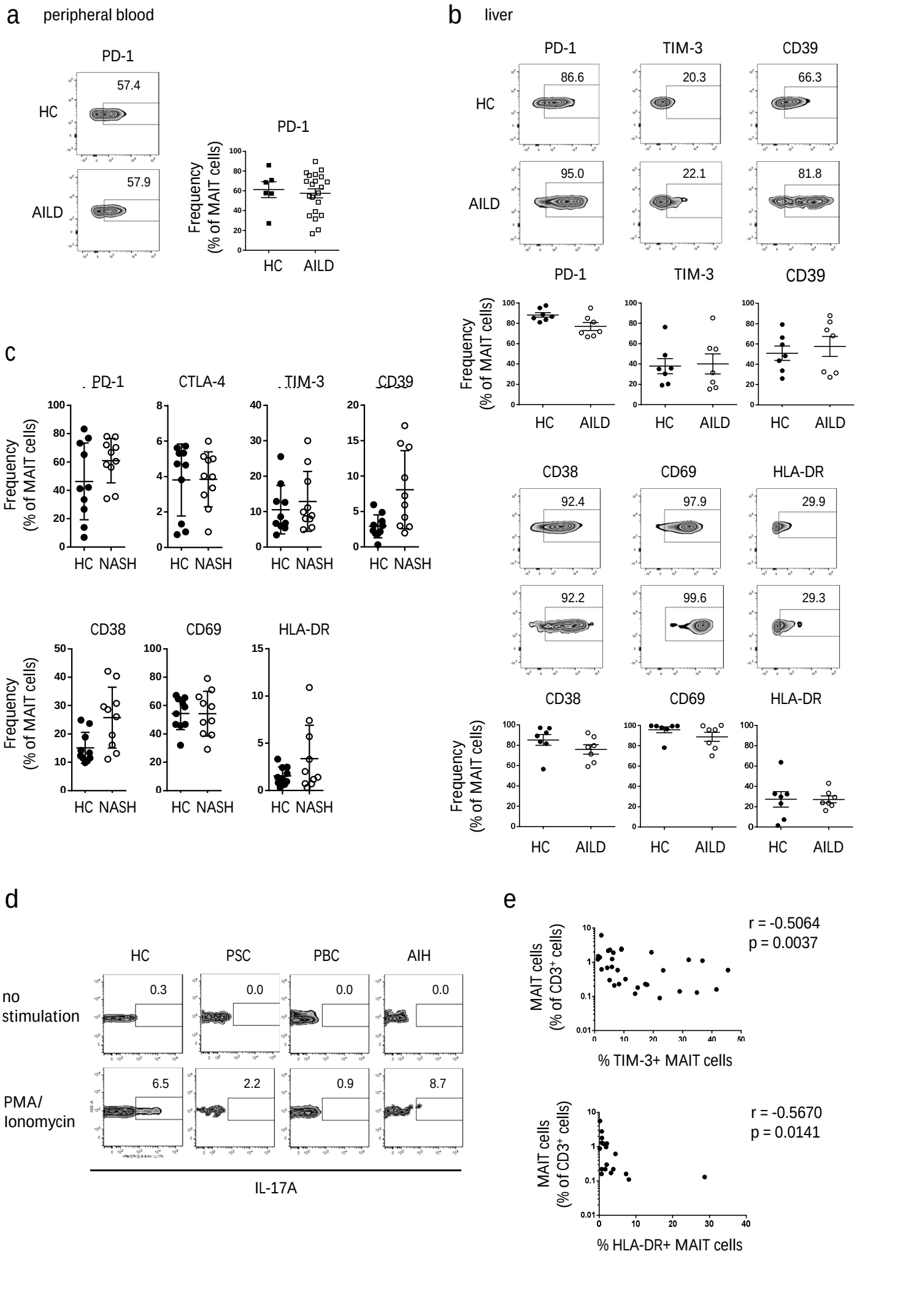
Frequency (% of MAIT cells)
HC
AILD
b
HC liver
AILD liver
2.4
6.8
c
HC
PSC
PBC
AIH
no stimulation
PMA/ Ionomycin
0.3
0.1
0.0
0.0
39.3
16.8
21.5
14.5
IFNγ
IFNγ+ MAIT cells (% of MAIT cells)
d
GrzB+ MAIT cells (% of MAIT cells)
****
ns
CD3/CD28 beads
e
IL-17A+ MAIT cells (% of MAIT cells)
TNFα+ MAIT cells (% of MAIT cells)
f
MAIT cells (% of CD3+ cells)
r = -0.8080
p = <0.0001
% CD38+ MAIT cells
r = -0.4626
p = 0.0228
% CTLA-4+ MAIT cells
r = -0.4905
p = 0.0044
% CD39+ MAIT cells


a
Healthy controls
AILD
24h
72h
IFNγ+ (% of MAIT cells)
ns
****
IL-1β
IL-12
IL-18
b
***
c
Healthy controls
AILD
**
*
24h
48h
72h
d
PD-1
TIM-3
CD39
Frequency (% of MAIT cells)
no stimulation
IL-12 + IL-18
e
CD3 (MFI)
HC
AILD
f
EOMES+ (% of MAIT cells)
EOMES (MFI)
T-bet+ (% of MAIT cells)

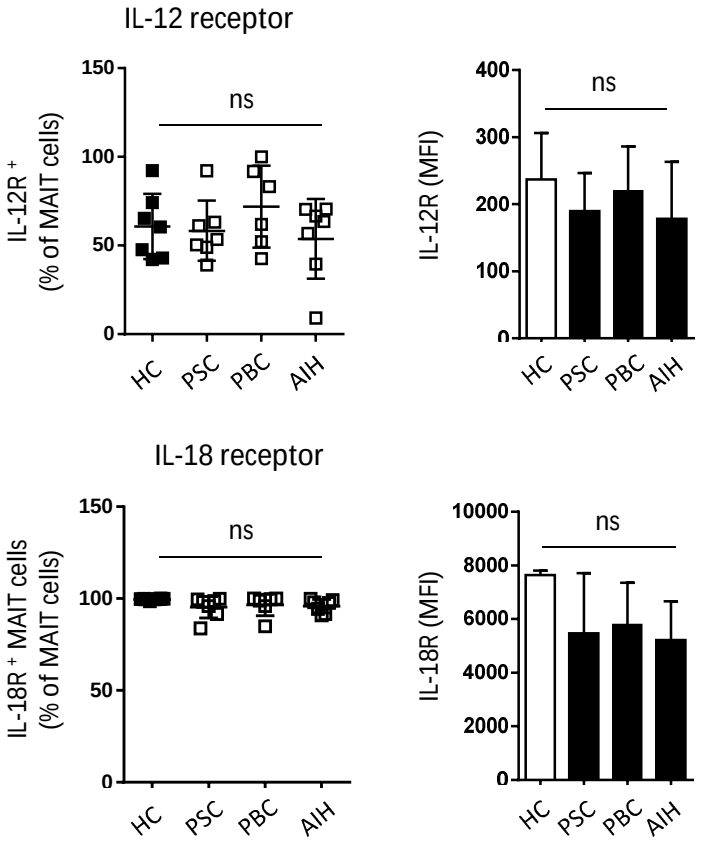

a
healthy controls
IL-17A+ (% of MAIT cells)
ns
IL-1β
IL-12
IL-18
PMA/Ionomycin
b
healthy controls
24h
48h
72h
IL-17A+ (% of MAIT cells)
IL-1β
IL-12
IL-18
c
24h
72h
IL-17A+ (% of MAIT cells)
IL-1β
IL-12
IL-18
d
AILD
24h
48h
72h
IL-17A+ (% of MAIT cells)
IL-1β
IL-12
IL-18
e
HC
AILD
IL-17A+ (% of MAIT cells)
ns
IL-1β
IL-12
IL-18
f
donor 1
donor 2
IL-12 50ng/ml 24h
no stimulation
unstained control
RORγt
RORγt+ (% of MAIT cells)
IL-12 (50ng/ml)
24h
ns
24h
72h


a
Absorbance (370nm)
ns
***
***
****
****
****
MAIT cells : HSC 0:1 0:1 2:1 1:1 2:1 10:1 20:1
CD3/CD28 beads - + - + + + +
b
HSC
HSC + MAIT cells
relative mRNA expression (fold-change)
Acta2
Col1A1
MMP2
LOX
TIMP1
IL-1β
IL-6
IL-8
CCL2
c
HSC
HSC + MAIT cells HC
HSC + MAIT cells PSC
HSC + MAIT cells PBC
HSC + MAIT cells AIH
relative mRNA expression normalized to GAPDH
d
HSC
HSC + MAIT cells HC
HSC + MAIT cells NASH
e
IL-1β
IL-6
IL-8
CCL2
IL-17R AB (2μg/ml)
MAIT cells - + + -
f
IL-1β
IL-6
IL-8
CCL2
LOX
TIMP-1
direct contact
transwell
MAIT cells - + +

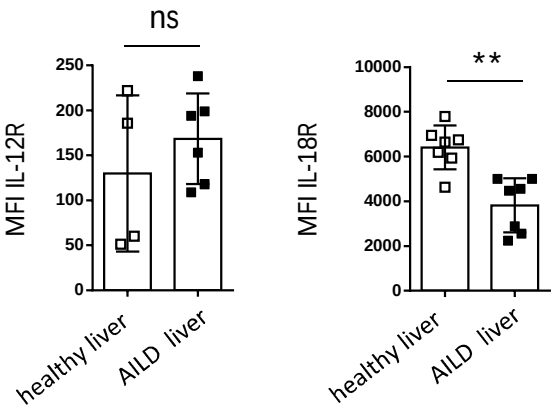

a
CD3+ T cells
73.8
SSC-A
CD3
MAIT cells
5.3
CD161
Vα7.2
8.0
2.7
30.9
59.0
CD4
CD8
b
MAIT cells
MAIT cells (% of CD3+ cells)
****
HC
AILD
CD4+ T cells
CD3+ CD4+ cells (% of CD3+ cells)
ns
CD8+ T cells
CD3+ CD8+ cells (% of CD3+ cells)
γδ T cells
CD3+ TCR Vδ2+ cells (% of CD3+ cells)
iNKT cells
iTCR V24+ (% of CD3+ cells)
Total NK cells
NK cells (% of CD3- cells)
CD56hi CD16+/- NK cells
CD56hi CD16+/- (% of NK cells)
CD56dim CD16+ NK cells
CD56dim CD16+ (% of NK cells)
Healthy control
PSC
PBC
AIH
c
CD8+ MAIT cells (% of CD3+ cells)
CD4-/8- MAIT cells (% of CD3+ cells)
CD4+ MAIT cells (% of CD3+ cells)
CD4+/CD8+ MAIT cells (% of CD3+ cells)
HC
PSC
PBC
AIH

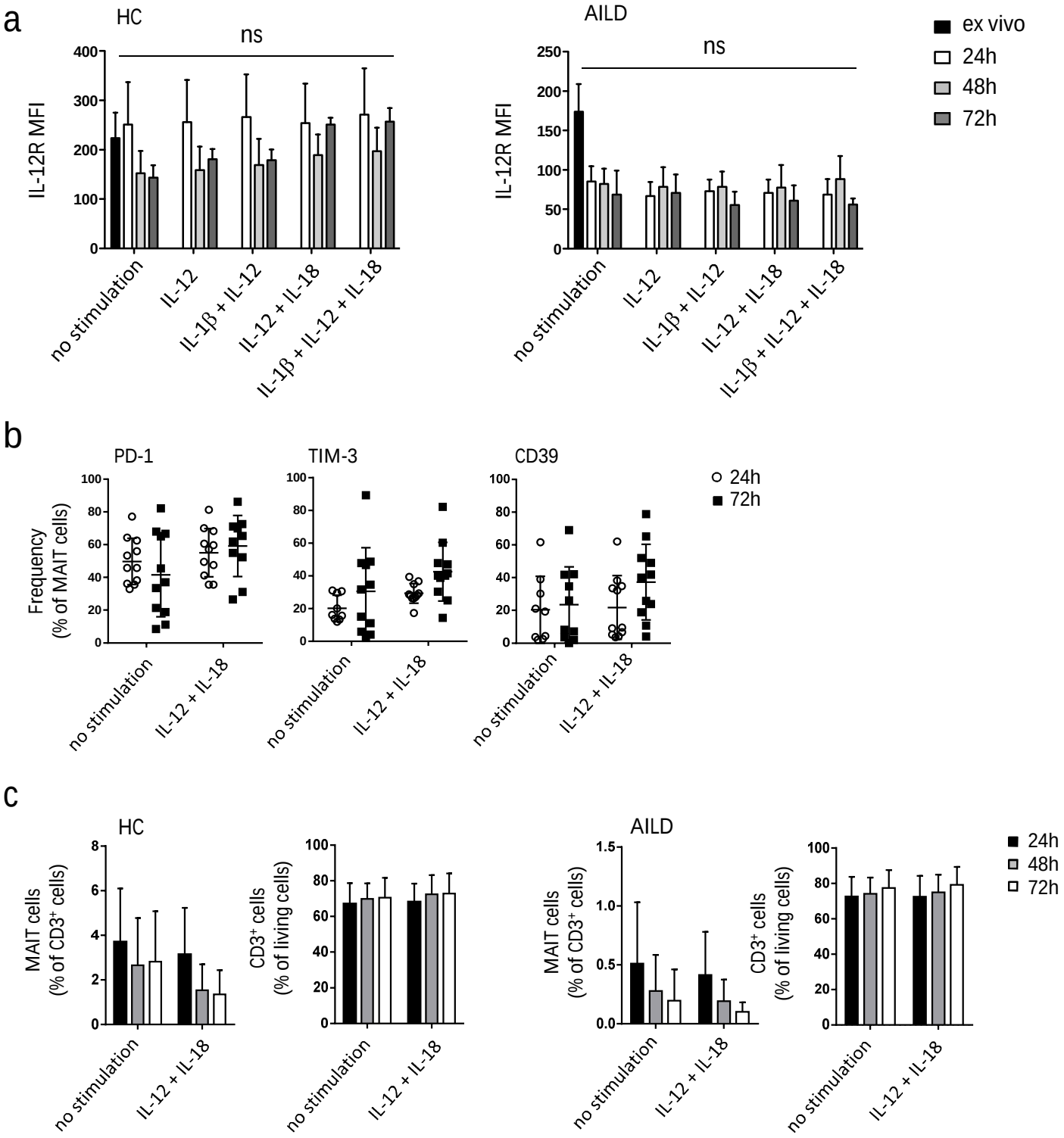

a
peripheral blood
PD-1
HC
AILD
57.4
57.9
PD-1
Frequency (% of MAIT cells)
HC
AILD
b
liver
PD-1
TIM-3
CD39
HC
AILD
86.6
20.3
66.3
95.0
22.1
81.8
Frequency (% of MAIT cells)
CD38
CD69
HLA-DR
92.4
97.9
29.9
92.2
99.6
29.3
c
PD-1
CTLA-4
TIM-3
CD39
CD38
CD69
HLA-DR
Frequency (% of MAIT cells)
HC
NASH
d
HC
PSC
PBC
AIH
no stimulation
PMA/ Ionomycin
0.3
0.0
0.0
0.0
6.5
2.2
0.9
8.7
IL-17A
e
MAIT cells (% of CD3+ cells)
% TIM-3+ MAIT cells
r = -0.5064
p = 0.0037
% HLA-DR+ MAIT cells
r = -0.5670
p = 0.0141

a

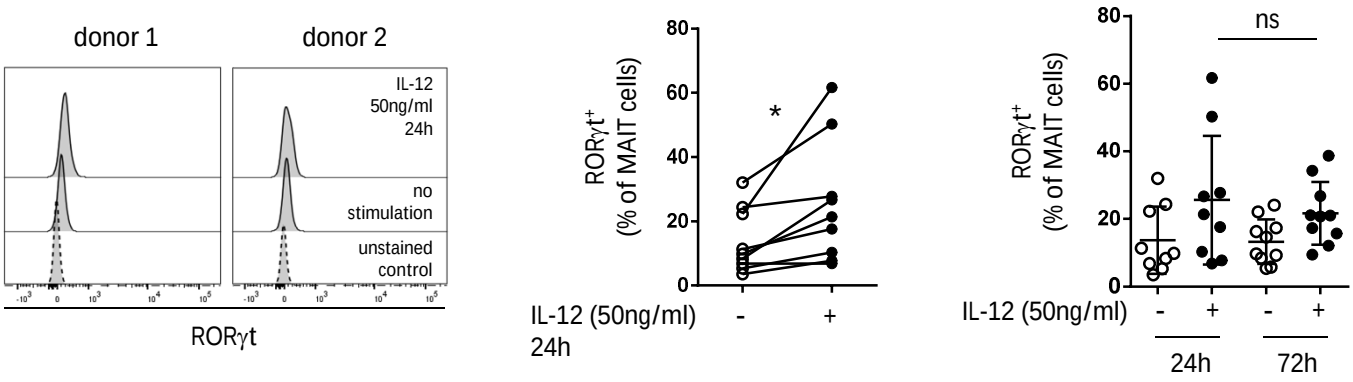

IL-12 receptor
IL-12R+ (% of MAIT cells)
150
100
50
0
ns
HC
PSC
PBC
AIH
IL-12R (MFI)
400
300
200
100
0
ns
HC
PSC
PBC
AIH
IL-18 receptor
IL-18R+ MAIT cells (% of MAIT cells)
150
100
50
0
ns
HC
PSC
PBC
AIH
IL-18R (MFI)
10000
8000
6000
4000
2000
0
ns
HC
PSC
PBC
AIH

b

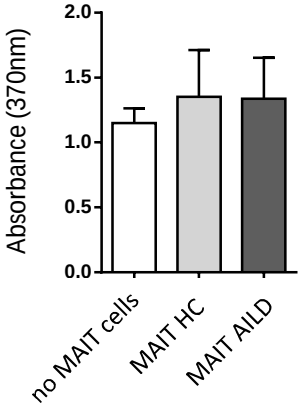

MFI IL-12R
250
200
150
100
50
0
ns
healthy liver
AILD liver
MFI IL-18R
10000
8000
6000
4000
2000
0
**
healthy liver
AILD liver


a
HC
ns
IL-12R MFI
400
300
200
100
0
no stimulation
IL-12
IL-1β + IL-12
IL-12 + IL-18
IL-1β + IL-12 + IL-18
AILD
ns
IL-12R MFI
250
200
150
100
50
0
no stimulation
IL-12
IL-1β + IL-12
IL-12 + IL-18
IL-1β + IL-12 + IL-18
ex vivo
24h
48h
72h
b
PD-1
TIM-3
CD39
Frequency (% of MAIT cells)
100
80
60
40
20
0
no stimulation
IL-12 + IL-18
24h
72h
c
HC
AILD
MAIT cells (% of CD3+ cells)
CD3+ cells (% of living cells)
no stimulation
IL-12 + IL-18
24h
48h
72h

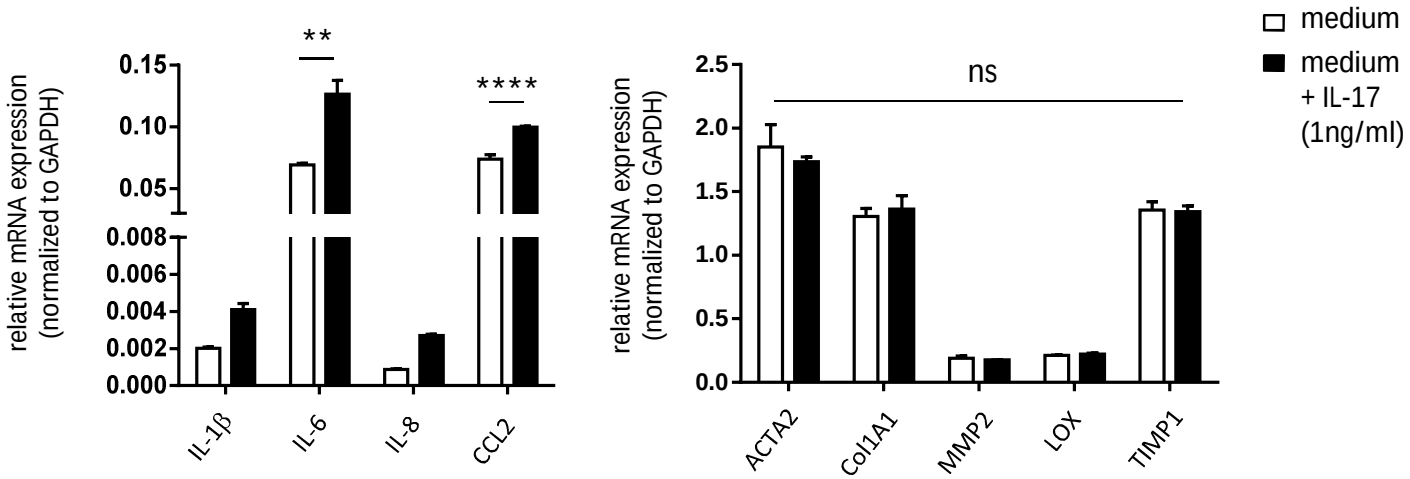
a


donor 1
donor 2
IL-12
50ng/ml
24h
no
stimulation
unstained
control
RORγt
RORγt+
(% of MAIT cells)
80
60
40
20
0
*
IL-12 (50ng/ml)
24h
-
+
ns
24h
72h

a


Absorbance (370nm)
2.0
1.5
1.0
0.5
0.0
no MAIT cells
MAIT HC
MAIT AILD

b


relative mRNA expression
(normalized to GAPDH)
**
****
0.15
0.10
0.05
0.008
0.006
0.004
0.002
0.000
IL-1β
IL-6
IL-8
CCL2
ns
2.5
2.0
1.5
1.0
0.5
0.0
ACTA2
Col1A1
MMP2
LOX
TIMP1
medium
medium
+ IL-17
(1ng/ml)

a

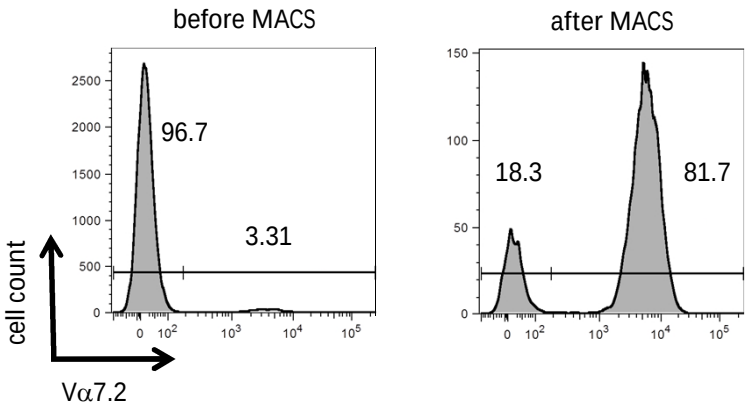


Table S1: Patient characteristics

		PSC	PBC	AIH	NASH	Healthy
Cases	number	46	41	21	11	21
Age (years)	median (range)	47.5 (24-82)	54 (30-78)	48 (24 - 71)	57 (28-72)	31 (25 - 46)
Male	number (%)	35 (76)	2 (5)	7 (33)	7 (63)	15 (71)
Female	number (%)	11 (24)	39 (95)	14 (67)	4 (37)	6 (29)
Fibroscan	number	45	35	20	5	0
Biopsy	number				7	
Fibrosis stage (F0-1)	number	16	17	9	3	n.a.
Fibrosis stage (F2)	number	10	3	3	2	n.a.
Fibrosis stage (F3)	number	7	6	5	3	n.a.
Fibrosis stage (F4)	number	12	9	3	2	n.a.
Liver stiffness	median (range)	8.9 (2.5 - 75)	7.6 (4.3 - 46)	7.6 (3.5 - 38)	10 (7.6-14)	n.a.

Table S2: Taqman gene assays (all Applied Biosystems)

ACTA2	Hs00426835_g1
TIMP-1	Hs00171558_m1
Col1A1	Hs00164004_m1
CCL2	Hs00234140_m1
LOX	Hs00942480_m1
MMP2	Hs01548727_m1
IL-1β	Hs01555410_m1
IL-8	Hs00174103_m1
IL-6	Hs00985639_m1
GAPDH	Hs02758991_m1