

Persistent Deficiency of Mucosa-associated Invariant T (MAIT) cells During Alcohol-related Liver Disease

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Research

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Abstract

Background: Alcohol-related liver disease (ALD) is a major cause of chronic liver diseases, and the inflammatory response is a basic pathological feature of ALD. Immunity throughout different stages of ALD has not yet been elucidated. The mucosal-associated invariant T (MAIT) cells are a novel population of innate immune cells, which may be depleted in various inflammatory diseases. However, the mechanism of MAIT cell loss in ALD remains unclear.

Results: In this study, we found that MAIT cells were depleted in patients with alcoholic fatty liver disease, alcoholic cirrhosis, and mixed cirrhosis (alcoholic + viral). Furthermore, the reduction in circulating MAIT cells was correlated with liver function in patients with cirrhosis. We detected functional changes among circulating MAIT cells in patients with alcoholic cirrhosis, including increased production of IL-17A and perforin, and reduced production of TNF- α . Plasma cytokine and chemokine levels were quantified using multiple immunoassays and ELISA. Serum levels of chemokine IL-8 were correlated with MAIT cell frequency in patients with alcoholic cirrhosis. Moreover, no differences were observed in the expression of CCR6, CXCR6, and PD-1 in circulating MAIT cells of patients with alcoholic cirrhosis. The MAIT cells in patients with alcoholic cirrhosis were prone to apoptosis, which was promoted by IL-12, IL-18, and IL-8.

Conclusions: Our findings indicate persistent MAIT cell loss during alcohol-related liver disease, and suggest that MAIT cells can be promising indicator and therapeutic targets in ALD.

Background

Alcohol-related liver disease (ALD) is a major cause of chronic liver diseases worldwide. Its recent prevalence in China is similar to that in Europe and the United States (1). The term ALD can refer to alcoholic fatty liver disease, alcoholic hepatitis, alcoholic cirrhosis and hepatocellular cancer. According to data from the World Health Organization (WHO), alcohol is the main cause of cirrhosis. Excessive alcohol intake is the main co-factor of liver disease, which accelerates the progression of chronic liver diseases (2). Alcohol consumption can increase an individual's susceptibility to viral infection and promote viral replication in vivo, thus promoting the occurrence of liver cirrhosis and liver cancer (3, 4).

Moreover, binge drinking has gradually spread and become popular among young people (5). Notably, some healthy people with a similar history of drinking may not show signs of liver diseases. There are few studies on changes in immune function among patients with alcoholism and chronic liver diseases caused by multi-factor mixed drinking alcohol. The pathophysiology of ALD includes inflammation, gut dysbiosis, poor liver regeneration and immune dysfunction (6). Inflammatory responses play important roles in the pathophysiology of alcohol-related liver diseases. Immune cells, parenchymal cells, cytokines, and chemokines are involved in the inflammatory responses associated with ALD. Thus, it is imperative to better understand the pathogenesis of inflammatory responses in ALD to develop novel diagnostic and therapeutic targets.

Mucosal-associated invariant T (MAIT) cells are innate immune cells that are abundant in the liver (7). The MAIT cells can be activated by riboflavin metabolites derived from microorganisms, as well as inflammatory cytokines, such as IL-12, IL-18, IL-15 and IL-23. Upon activation, MAIT cells secrete various cytokines and regulate cytolytic capacity by secreting granzyme B and perforin. The MAIT cells reportedly have anti-bacterial potency (8), and profibrogenic function (9), tissue repair (10) and antitumor (11) function. In contrast, a recent study reported that MAIT cells may also display tumor promoting functions (12). Recently, an increasing number of studies have been focused on MAIT cells, including those on infectious diseases, metabolic diseases, immune diseases, and tumors.

The diagnostic and prognostic value of MAIT cells in Type 1 diabetes (13) and cancer (9,14) have been reported. MAIT cells are also associated with the progression of HBV infection (15). Furthermore, circulating IL-17-producing MAIT cells are associated with asthma symptoms in children (16). However, further exploration is required to identify specific biomarkers of MAIT cells in ALD. Most studies confirm a significant reduction in systemic levels of MAIT cells and ascribe

MAIT cell loss to diseased states, especially activation-induced cell death. Presently, there is no consensus on the exact mechanisms of MAIT cell death in ALD. Studies on metabolic diseases have described several factors that stimulate the apoptosis of circulating MAIT cells, such as glucose concentrations (17) and OX40 (18). Furthermore, MAIT cell frequency is correlated with cytokine and chemokine levels (17, 19). To validate this, we investigated the phenotype and functions of MAIT cells in binge drinkers, patients with alcoholic cirrhosis, patients with mixed (viral + alcohol) cirrhosis, heavy drinkers without clinical evidence of liver disease, and healthy controls (HCs). We further determined whether MAIT cells could be used to identify patients with ALD at high risk for liver injury. We also explored the potential mechanism of MAIT cell loss associated with inflammatory cytokines and chemokines.

Results

Characteristics of patients

The clinical characteristics of patients are summarized in **Table 1**. Patients with alcoholic cirrhosis (53.6 ± 15.62 years of age) and those with mixed cirrhosis (52.21 ± 6.30 years of age) tended to be elderly individuals, while binge drinkers were mainly among the younger age group (28.34 ± 9.59 years). Moreover, male patients were more likely to drink than female patients. Liver biochemistry analyses, including alanine aminotransferase (ALT), aspartate transaminase (AST), total bilirubin (TBIL), γ -glutamyl transferase (GGT), alkaline phosphatase (ALP), and albumin (ALB), showed alterations in patients with cirrhosis. Interestingly, the proportion of neutrophils in binge drinkers was significantly increased.

Circulating CD4⁺, and CD8⁺ MAIT cells are depleted in patients with alcoholic cirrhosis and mixed cirrhosis

Human MAIT cells were gated on CD3⁺CD161⁺V α 7.2⁺ lymphocytes (Figure 1A). The frequency of MAIT cells was significantly depleted in patients with alcoholic fatty liver diseases ($p < 0.0001$), alcoholic cirrhosis ($p = 0.003$) and mixed cirrhosis ($p = 0.002$), compared with HCs (Figure 1B). However, no obvious reductions in the frequency of MAIT cells were observed among binge drinkers ($p = 1$) and heavy drinkers without overt liver diseases ($p = 0.982$). The frequency of MAIT cells were comparable in patients with alcoholic cirrhosis and mixed cirrhosis ($p = 0.903$). Furthermore, we found a significant reduction in the frequency of MAIT cells in patients with chronic HBV infection compared with healthy controls ($p = 0.029$). No significant differences were observed in patients with chronic HBV infection, alcoholic cirrhosis, or mixed cirrhosis (Supplementary Figure 1). We also used the 5-OP-RU tetramer and TCR β to confirm our gating strategy and found no significant differences between the two gating strategies ($p = 0.065$) (Figure 1C).

The MAIT cells are divided into subsets, based on CD4 and CD8 expression²⁰. Our further analysis indicated that the percentage of CD8⁺, CD4⁺, and CD4⁻CD8⁻ (double negative, DN) MAIT cells were comparable among patients with alcoholic cirrhosis, mixed cirrhosis, and HCs (Figure 1D). In patients with cirrhosis, CD8⁺ and CD4⁺ MAIT cells among the T cells were both lower compared with HCs. In addition, no significant associations were noted between gender ($p = 0.656$) or age ($p = 0.504$) and MAIT cell frequency in HCs (Supplementary Figure 2). We also divided patients with alcoholic fatty liver disease and alcoholic cirrhosis into 2 groups based on alcohol cessation (Figure 1E). However, MAIT cells in patients with ALD who had abstained from alcohol showed no significant changes, compared with patients who had abstained from alcohol ($p = 0.076$). Thus, MAIT cells are depleted in chronic liver diseases.

Correlation between MAIT cell frequency and liver function in patients with cirrhosis

To assess the clinical significance of reduced MAIT cells in patients with alcoholic cirrhosis and mixed cirrhosis, we performed Spearman's correlation analysis. Based on changes in clinical parameters presented in **Table 1**, we gained further insights regarding AST, GGT, TBIL, ALP, and albumin levels. Notably, a negative correlation was found between MAIT cell frequency and AST ($p = 0.02$), GGT ($p < 0.001$), and TBIL ($p = 0.004$) levels in patients with alcoholic cirrhosis, while no correlation was noted between ALB levels ($p = 0.747$) (Figure 2A). In addition, a negative correlation was noted between MAIT cell frequency and AST and TBIL among all patients with cirrhosis. The correlation between MAIT cell frequency and GGT was not as evident as that among patients with alcoholic cirrhosis (Figure 2B).

To assess the diagnostic ability of MAIT cell frequency as it relates to validated markers of liver function, we constructed a receiver operating characteristic curve. We evaluated the diagnostic value of MAIT cells for cirrhosis, and determined the area under the curve, sensitivity, specificity, and cut-off values to be 0.703, 49.4%, 83.1%, and 2.805%, respectively (Figure 2C). In order to discriminate between patients with cirrhosis and Child-Pugh A or Child-Pugh B or C, we constructed a ROC characteristic, which yielded an area under the curve, sensitivity, specificity and cut-off values of 0.687, 65.2%, 70.8%, and 1.515%, respectively (Figure 2D). Taken together, our data reveal a negative correlation between circulating MAIT cell frequency and liver function, and support the notion that MAIT can be a biomarker to evaluate liver injury in ALD.

Activated MAIT cells display an altered cytokine profile and cytotoxic ability in patients with alcoholic cirrhosis

We investigated the phenotype and effector functions of circulating MAIT cells in HCs and patients with ALD. We determined that MAIT cells in the blood were activated in patients with cirrhosis by the elevation of CD69 levels (Figure 3A). As T cell activation is associated with a change in effector function, we analyzed the ability of MAIT cells to produce cytokines and cytolytic proteins after PMA-ionomycin stimulation. Compared with HCs, IL-17 production was elevated in MAIT cells of patients with alcoholic cirrhosis ($p = 0.02$). The MAIT cells of patients with alcoholic cirrhosis produced markedly lower levels of TNF- α ($p = 0.033$). Moreover, MAIT cells from patients with ALD showed higher perforin levels ($p = 0.046$). In contrast, IFN- γ and granzyme B production were comparable among MAIT cells in the blood samples of all subjects (Figure 3B, 3C). Taken together, MAIT cells in patients with alcoholic cirrhosis may be highly activated, which results in an altered cytokine profile and cytotoxic ability.

Elevated IL-8 levels combined with elevated IL-12 and IL-18 levels promote apoptosis of MAIT cells in vivo and in vitro

We tried to determine the underlying cause for the reduced circulating MAIT cell frequency in patients with ALD. Previous studies have demonstrated that MAIT cells may be recruited to the liver in chronic liver diseases, which can be identified by elevated levels of CCR6 and CXCR6 (20,21). However, we observed no increase in levels of CCR6 and CXCR6 on circulating MAIT cells in patients with alcoholic and mixed cirrhosis (Figure 4A). Furthermore, previous studies have shown that apoptosis (22) or exhaustion (19,23) may lead to the reduction in MAIT cell frequency. The expression of the immune checkpoint inhibitory molecule PD-1, often indicates T cell exhaustion (20,23). However, PD-1 expression on circulating MAIT cells showed no significant differences between patients with cirrhosis and HCs (Figure 4B). As shown in Figure 4C, MAIT cells were prone to apoptosis in patients with alcoholic cirrhosis ($p = 0.034$).

Cytokine-dependent pathways may contribute to MAIT loss (17,19). We further quantified cytokines and chemokines in HCs, and patients with alcoholic cirrhosis, and mixed cirrhosis, as well as binge drinkers (Table 2). As shown in **Table 2**, IL-12 and IL-23, which activate MAIT cells, were significantly increased in patients with cirrhosis. The concentration of IL-18, which is associated with the activation and exhaustion of MAIT cells (24,25), was considerably elevated in all groups. In patients with alcoholic cirrhosis, levels of the proinflammatory chemokine, IL-8, were significantly increased compared with

those in HCs (Figure 4D). Moreover, MAIT cell frequency was inversely correlated with the levels of circulating IL-8 in patients with alcoholic cirrhosis (Figure 4E). The PBMCs of healthy subjects were exposed to increasing doses of IL-8 and subjected to 24 h of culture. We found that different levels of IL-8 did not promote apoptosis in MAIT cells (Supplementary Figure 3). Furthermore, we cultured PBMCs from HCs with IL-12, IL-18, and IL-8. Our results indicated that IL-12 and IL-18, combined with IL-8 can significantly enhance apoptosis of MAIT cells (Figure 4F). Collectively, these data suggest that the inflammatory environment in alcoholic cirrhosis could promote apoptosis in MAIT cells.

Discussion

The MAIT cells protect against inflammation and infection in ALD by producing cytokines and cytotoxic effector molecules. Persistent and systemic inflammation may contribute to apoptosis of MAIT cells. To our knowledge, few research studies have been focused on feasibility of MAIT cells as biomarkers for the severity of liver injury in ALD. These findings show the potential role of MAIT cells in alcohol-related liver diseases and reveal a possible apoptosis-related mechanism of MAIT cell loss.

The activation of innate immunity and adaptive immunity contributes to liver inflammation and injury in ALD. The MAIT cells are important lymphocyte subsets, which are considered to be an important bridge between innate and adaptive immunity. In the present study, we found no significant changes in the MAIT cells of binge drinkers or heavy drinkers without liver diseases. Rapid and excessive alcohol consumption had no effect on MAIT cell frequency. Previous studies have confirmed that short-term alcohol abstinence does not restore the frequency or function of MAIT cells, while long-term alcohol abstinence only partially reverses abnormalities (8, 19).

In the present study, alcohol abstinence had no effect on MAIT cells in patients with ALD. In addition, we found no evidence that the dual factors of a virus and alcohol lead to more severe conditions than alcohol alone on MAIT cell frequency. The changes in MAIT cells may have been related to persistent pathological changes associated with various diseased states. As a result, a significant reduction in MAIT cells was observed in patients with alcoholic fatty liver diseases, alcoholic cirrhosis and mixed cirrhosis. Furthermore, the frequency of MAIT cells was negatively correlated with AST and TBIL levels in patients with cirrhosis. Specifically, a negative correlation was noted between MAIT cells and GGT in patients with alcoholic cirrhosis. Although the sensitivity of MAIT cells as an initial screening tool is relatively low based on our ROC analysis, the circulating MAIT cell frequency can distinguish HCs from patients with cirrhosis. Moreover, MAIT cells could be used as indicators of the severity of liver disease. Further research will be required to establish the clinical utility of MAIT cells as markers for advanced liver diseases.

Activated MAIT cells led to altered functions in patients with ALD. Many studies have confirmed that drug therapy could affect MAIT cell frequency and phenotype (9, 20). Although the changes in circulating MAIT cells were not as informative as those in the liver, they did reflect the state of these cells in inflammatory diseases. The present study demonstrated that MAIT cells produce more IL-17A, and less TNF- α in patients with alcoholic cirrhosis. The elevation of IL-17 levels in MAIT cells may be associated with the control of bacterial dissemination (26). The reduction in TNF- α may protect against liver inflammation. Furthermore, increased perforin levels may indicate an increased antibacterial potency of MAIT cells in patients with alcoholic cirrhosis. In addition, the trends toward reducing levels of IFN- γ and increasing levels of Granzyme B may play a protective role in ALD. Altogether, we postulated that MAIT cells may promote anti-inflammatory and anti-bacterial responses in ALD.

Several factors and mechanisms may be responsible for MAIT cell depletion, including age (27), gender (20), redistribution (21), cell exhaustion and activation-induced cell death. However, we found no significant differences in gender and age among the MAIT cells of HCs. Neither did we observe any elevated expression of CCR6 or CXCR6 on circulating MAIT cells in patients with alcoholic cirrhosis or mixed cirrhosis. Owing to a lack of liver samples, we could not determine whether or not MAIT cells had migrated to the liver. Recent research has confirmed the migration of MAIT cells to ascitic fluid in

patients with decompensated liver cirrhosis (28). However, the most recent research has reported that MAIT cells could largely proliferate in situ in the infection organ but do not require CXCR6 for accumulation (29). Further study of the tissue-resident phenotype of MAIT cells in cross-talk between different organs in patients with ALD may yield promising findings. Furthermore, MAIT cells in this cohort of patients with alcoholic cirrhosis and mixed cirrhosis showed no expression of the immune exhaustion marker, PD-1. We also found that apoptosis was the driving cause of MAIT cell loss in patients with alcoholic cirrhosis, possibly through activation-induced cell death. In chronic HBV-infected patients, conjugated bilirubin not only promoted MAIT cell activation and apoptosis, but also impaired TCR-induced proliferation (15).

A wide variety of inflammatory cells and cytokines participate in inflammation associated with ALD. Poly ADP ribose polymerase (PARP) inhibition can ameliorate the signs of ALD, and thereby remarkably attenuate pro-inflammatory responses (30). Long-term systemic inflammation may contribute to the apoptosis of MAIT cells. We found that the MAIT cell activation-associated cytokines IL-12 and IL-23, were considerably increased in alcoholic cirrhosis. Li et al (19) reported that IL-18 is negatively correlated with MAIT frequency in alcoholic hepatitis.

In the present study, we observed high expression of IL-18 among all groups. We also observed that levels of the proinflammatory chemokine, IL-8, were elevated in patients with alcoholic cirrhosis and negatively correlated with MAIT cell frequency. We found that various concentrations of IL-8 had no effect on the apoptosis of MAIT cells. Considering the fact that MAIT cells become altered after activation, a lack of IL-8 receptors on activated MAIT cell is plausible. We cultured PBMCs from HCs with IL-12, IL-18, and IL-8. We further confirmed that IL-12, IL-18, and a combination with IL-8 could induce apoptosis. The findings of the present study suggest that a cytokine-rich inflammatory environment promotes MAIT cell loss in patients with ALD. Nonetheless, persistent inflammation may be associated with a variety of cytokines and chemokines. It is also possible that additional cytokines besides those under investigation in the present study, are able to affect MAIT cells. We have only confirmed the effects of a few.

Conclusions

Reduced MAIT cell frequency is negatively correlated with liver injury in patients with cirrhosis. The MAIT cells can be used as a new indicator for liver injury in ALD. The current data show that activated MAIT cells have anti-inflammatory and antibacterial effects. Increased levels of inflammatory cytokines and chemokines may promote the apoptosis of MAIT cells. Our data indicate the diagnostic and therapeutic potential of MAIT cells in ALD. Further studies are required to focus on the mechanisms of upregulation of MAIT cell frequency and the restoration of their function.

Materials And Methods

Study subjects

This study was approved by the Ethics Committee of Clinical Research of Anhui Medical University. All participants gave informed consent. All blood samples were collected at The First Affiliated Hospital of Anhui Medical University. The study subjects included patients with alcoholic fatty liver disease (n = 4), alcoholic cirrhosis (n = 48), mixed cirrhosis (n = 33), as well as those who were binge drinkers (n = 34), heavy drinkers without liver disease (n = 11), and those with chronic HBV infection (n = 7) and HCs (n = 116). The diagnosis of patients was based on previously described criteria (31,33). In addition to the criteria for alcoholic cirrhosis, patients with mixed cirrhosis also had HBV infection. All patients with HBV infection were HBV DNA-negative. Non-inclusion criteria included recent gastrointestinal bleeding, current bacterial infections, and immunomodulatory or antibiotic treatments. All participants with metabolic diseases, immune diseases, or cancer were excluded. Clinical characteristics of the subjects are presented in **Table 1**.

Blood samples

Peripheral blood samples were collected in EDTA tubes and separated into plasma and peripheral blood mononuclear cells (PBMCs). Plasma samples were stored at -80 ° C until further use. The PBMCs were immediately used for Flow cytometric analysis.

PBMC isolation and culture

The PBMCs were isolated by density gradient centrifugation, using the Ficoll-Hypaque technique (Solarbio, China), and freshly used for surface and intracellular staining to analyze frequency, phenotype, and function. To detect cytokine production and cytotoxicity markers, PBMCs were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, and stimulated with phorbol 12-myristate-13-acetate(PMA) /ionomycin and Brefeldin A/Monensin (Multi sciences, China) for 4 -6 h at 37 °C. Stimulated PBMCs were fixed and permeabilized using a FIX & PERM Kit (Multi sciences, China) to stain the intracellular markers. The PBMCs were further stimulated with IL-8, IL-12, or IL-18 (Novoprotein Scientific, China) at 50ng/mL for 24 h.

Flow cytometric analysis

Flow cytometry was performed as described using the following antibodies. The anti-CD3-FITC, anti-CD161-APC, and anti-TCR V α 7.2-Brilliant Violet 421(Biolegend, USA) were used to identify MAIT cells. Anti-CD4-PE/Cy7, anti-CD8-Perp/Cy5.5, anti-CCR6-PE/Cy7, anti-CXCR6-PE, anti-IL-17A-PE, anti-Granzyme B-PE/Cy7, and anti-Perforin-PE were obtained from Biolegend (USA). Anti-CD69-PE/Cy7, anti-PD-1-PE, anti-IFN- γ -PE/Cy7, anti-TNF- α -PE were obtained from BD (USA). Appropriate isotype control antibodies were used for each staining combination. We used 7-aminoactinomycin D(7-AAD) (BD Biosciences) staining to identify dead cells. Human MR1 tetramers loaded with a potent MAIT cell ligand-5-OP-RU or 6-FP were gifted from professor Li Bai. Apoptosis was allowed to progress and two channels were used to detect annexin V-FITC and 7-AAD (BD Biosciences) to determine the proportions of apoptotic cells. Data acquisition was achieved on BD FACS Verse system (BD Biosciences), and results were analyzed using FlowJo7.6 analysis software.

Multiplex immunoassays

Human cytokines and chemokines were identified in the plasma of patients with alcoholic cirrhosis, mixed cirrhosis, as well as binge drinkers and HCs. The cytokines and chemokines (GM-CSF, IFN- α 2, IL-1 α , IL-1 β , IL-11, IL-12p40, IL-12p70, IL-15, IL-18, IL-23, IL-27, IL-33, TSLP, CCL2/MCP-1, CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, CCL11/Eotaxin, CCL17/TARC, CCL20/MIP-3 α , CXCL1/GRO α , CXCL5/ENA-78, CXCL8/IL-8, CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC) were analyzed by flow cytometry bead-based immunoassay (LEGENDplex™ Human Cytokine Panel2/ Human Proinflammatory Chemokine Panel, BioLegend), according to the manufacturer's instructions. Samples acquisition was achieved on a FACS Verse system equipped with the FACSuite software (BD Biosciences), and samples were analyzed with the LEGENDplex™ Data Analysis Software v8.0.

Enzyme-linked immunosorbent assay (ELISA)

Circulating levels of IL-7 and IL-26 were not available in the above panel. Therefore, these cytokines in the plasma were quantified using enzyme-linked immunosorbent assay (ELISA; Jianglai Biotech, China).

Statistical analysis

All data were presented as the mean \pm SEM. The parametric Student's *t*-test for paired or unpaired samples as appropriate, or the Mann–Whitney U test, one-way analysis of variance (ANOVA) least significant difference (LSD), Welch's ANOVA test (Games–Howell test), or Spearman's correlation were used to calculate statistical significance. The diagnostic performance of MAIT cells in identifying the severity of disease was assessed by analyzing receiver operating characteristic (ROC) curves. Statistical calculations were performed using the GraphPad Prism 8.0 and SPSS17.0 software. In all tests, $p < 0.05$ was considered statistically significant.

List Of Abbreviations

ALD alcohol-related liver disease;

HC healthy control;

HDC heavy drinker without clinical evidence of liver disease;

MAIT mucosal-associated invariant T cells;

PBMC peripheral blood mononuclear cells;

ALT alanine aminotransferase;

AST aspartate transaminase;

TBIL total bilirubin

GGT γ -glutamyl transferase;

ALP alkaline phosphatase;

ALB albumin.

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of the First Hospital of Anhui Medical University of Anhui Medical University and the methods were carried out in accordance with the approved guidelines.

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Experiments were designed by Derun Kong and Hua Wang, and carried out by Yujue Zhang, Yuanyuan Fan and Wei He. Samples were collected with the help of Yujue Zhang, Yi Han, Huarui Bao, Renjun Yang, and Bingbing Wang. The manuscript was written by Yujue Zhang, and revised by Derun Kong and Hua Wang. All authors have read and approved the final version of manuscript.

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Authors' information (optional)

Not applicable.

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Tables

Table 1 Clinical characteristics of patients

| Parameter | Healthy control | Alcoholic fatty liver disease | Alcoholic cirrhosis | Mixed cirrhosis | Binge drinker | HDC |
|-------------------------------------|-----------------|-------------------------------|---------------------|-----------------|---------------|-------------|
| Number | 116 | 4 | 48 | 33 | 34 | 11 |
| Gender Male (%) | 93(80.2%) | 3(75%) | 47(97.9%) | 33(100%) | 19(55.9%) | 11(100%) |
| Age(years) | 48.89±11.21 | 40.25±3.30* | 53.6±15.62 | 52.21±6.30 | 28.34±9.59** | 51.36±10.62 |
| ALT(U/L) | 26.3±30.54 | 71.0±28.57 | 40.4±52.42 | 32.25±17.73 | 24.4±5.77 | 51.73±89.31 |
| AST(U/L) | 21.08±11.43 | 49.0±15.28 | 59.29±64.85** | 39.88±19.14** | 23.6±5.18 | 24.0±10.68 |
| TBIL (µmol/L) | 13.76±5.89 | 21.63±15.26 | 44.34±49.55** | 21.54±19.63 | 8.94±4.68 | 16.56±14.56 |
| ALB(g/L) | 41.75±3.38 | 37.08±9.40 | 31.63±4.86** | 32.76±6.45** | 46.82±4.03 | 39.24±4.98 |
| ALP(U/L) | 78.10±20.21 | 86.25±13.57 | 140.16±54.89** | 124.63±49.86** | 66.75±15.59 | 78.73±17.64 |
| GGT(U/L) | 31.71±47.43 | 143.75±108.69 | 149.78±170.36** | 70.16±59.74* | 23.75±10.99 | 49.64±58.96 |
| PT(s) | 11.70±1.02 | 13.1±0.95 | 15.80±3.21 | 15.39±3.16 | - | 11.95±0.7 |
| WBC (×10 ⁹ /L) | 5.8±1.59 | 7.87±2.42 | 4.83±2.22 | 4.0±4.04 | 9.8±3.64** | 6.1±0.73 |
| NEUT (%) | 54.59±7.57 | 71.45±10.58 | 61.37±15.98 | 58.73±11.27 | 71.37±12.45** | 58.6±8.64 |
| Neutrophil (×10 ⁹ /L) | 3.22±1.21 | 5.75±2.56 | 3.05±1.91 | 2.47±2.98 | 7.22±3.52** | 3.63±0.86 |
| Lymphocyte (×10 ⁹ /L) | 2.37±4.39 | 1.44±0.51 | 1.18±0.85 | 0.92±0.81* | 2.11±0.99 | 1.87±0.41 |

All values presented as the mean ± SEM; p-values were determined by the one-way analysis of variance (ANOVA) least significant difference (LSD), or Welch's ANOVA test (Games–Howell test).

*p < 0.05, **p < 0.01 (compared with the blood samples from healthy individuals).

HDC, heavy drinking controls; ALT, alanine aminotransferase; AST, aspartate transaminase; TBIL, total bilirubin; ALB, albumin; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; PT, prothrombin time; WBC, white blood cells.

Table 2 Cytokine and chemokine concentrations

| Parameter | HC (n=18-24) | Alcoholic cirrhosis (n=16) | Mixed cirrhosis (n=16-20) | Binge drinking (n=16-20) |
|-----------------|-----------------|-------------------------------|------------------------------|-----------------------------|
| TSLP (pg/ml) | 5.77±3.48 | 5.64±4.89 | 2.11±0.69** | 1.79±0.78** |
| IL-23 (pg/ml) | 4.88±2.49 | 9.47±12.34* | 9.62±6.27* | 4.27±1.51 |
| IL-12p40(pg/ml) | 50.21±24.02 | 139.10±151.39* | 178.98±166.71* | 42.46±14.87 |
| IL-12p70(pg/ml) | 1.90±0.42 | 2.15±0.96* | - | - |
| IL-15(pg/ml) | 17.37±23.80 | 59.09±109.97 | 121.43±139.51* | 10.70±42.48 |
| IL-18(pg/ml) | 201.0±85.4 | 267.37±201.57 | 304.48±177.26 | 283.61±303.28 |
| IL-8(pg/ml) | 3.22±3.74 | 27.30±34.52* | 17.13±24.24 | 3.28±5.83 |
| CXCL10(pg/ml) | 58.26±29.22 | 126.24±96.36* | 77.40±37.89 | 13.57±9.64** |
| CXCL5(pg/ml) | 111.18±168.80 | 15.74±21.58* | 16.45±25.73* | 82.79±123.30 |
| CXCL11(pg/ml) | 50.51±27.56 | 59.16±33.70* | 31.58±13.08 | 18.48±6.87** |
| IL-7(pg/ml) | 222.01±46.40 | 247.52±43.39 | 119.58±21.01** | 175.22±42.59* |
| IL-26 pg/ml | 324.42±122.10 | 240.47±115.20 | 609.6±177.29** | 485.3±160.26** |
| CCL11(pg/ml) | 50.51±27.56 | 59.16±33.70 | 31.58±13.08 | 18.48±6.87* |
| CCL2(pg/ml) | 294.88±245.48 | 260.48±169.80 | 152.33±71.46* | 99.41±109.09* |
| CXCL9(pg/ml) | 26.89±30 | 42.51±55.05 | 29.70±29.51 | 6.14±9.83* |

All values presented as the mean ± SEM. p-values were determined by the one-way analysis of variance (ANOVA) least significant difference (LSD), or Welch's ANOVA test (Games–Howell test). *p < 0.05, **p < 0.01, (compared with blood samples from healthy controls).

HC, healthy controls; TSLP, thymic stromal lymphopoietin; IL, interleukin; CXCL, chemokine (C-X-C motif) ligand; CCL, chemokine (C-C motif) ligand.

Figures

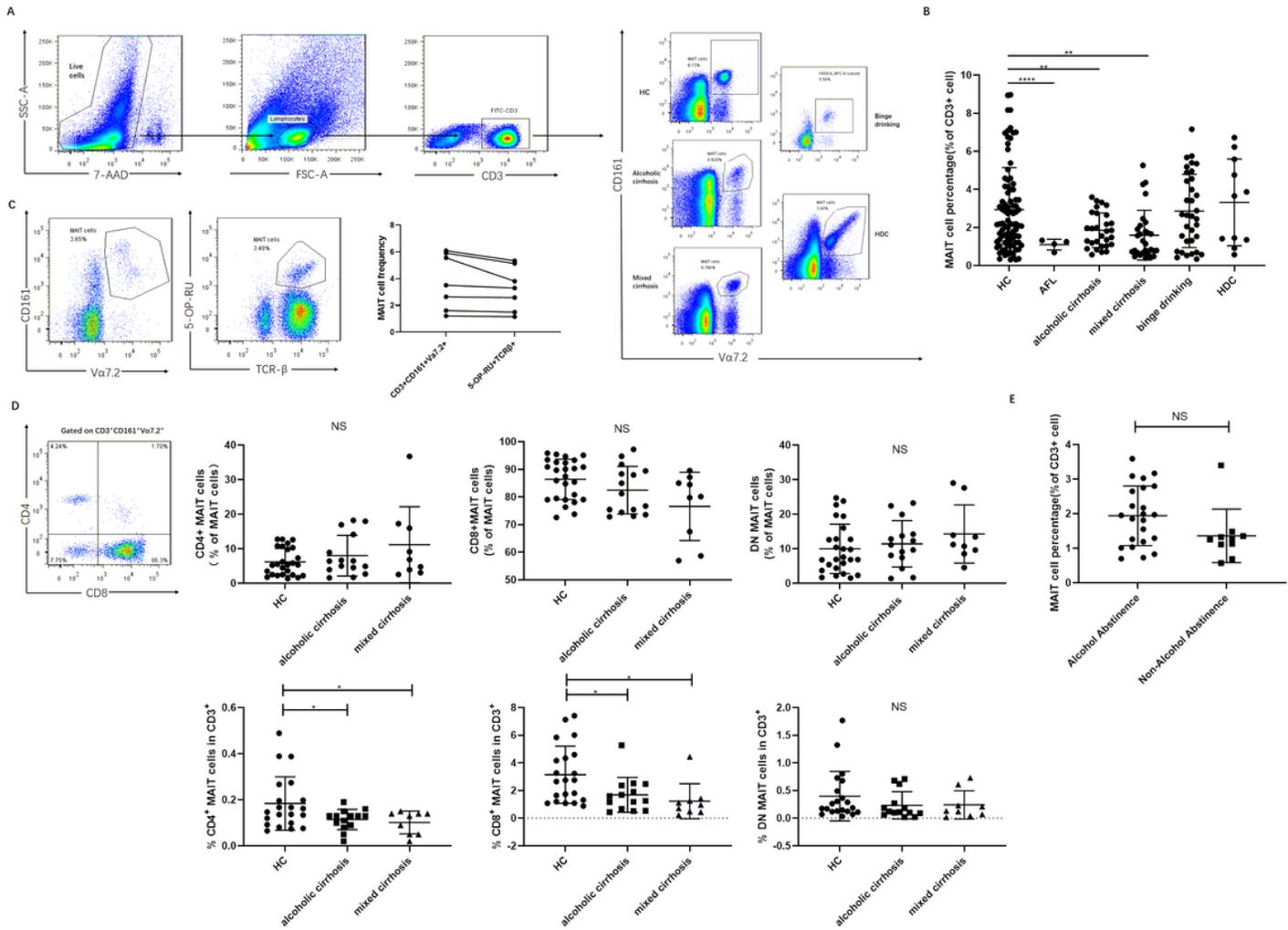


Figure 1

Circulating MAIT cell frequency was reduced in patients with ALD (A) Gating strategy to identify mucosal-associated invariant T (MAIT) cells. Flow cytometry analysis of MAIT cells in human blood using CD3+CD161+Va7.2+. (B) MAIT cells were depleted in patients with alcoholic fatty liver disease (n = 4), alcoholic cirrhosis (n = 29), mixed cirrhosis (n = 29), and in those who were binge drinker (n = 34) and heavy drinkers without overt liver diseases (n = 11), compared with HCs (n = 88). (C) MAIT cells were stained with the MR1 tetramer and T-cell receptor beta (TCR-β). No significant differences were observed between the two strategies (p = 0.065). (D) Percentage and constituent ratio of CD8+, CD4+, and double negative (DN) MAIT cells in patients with alcoholic cirrhosis, mixed cirrhosis, and HCs. (E) No significant differences were observed in MAIT cell frequency in patients with ALD, who either did (n = 23) or did not (n = 10) abstain from alcohol consumption. Data are presented as the Mean ± SEM and analyzed by the paired-sample t-test, two-sample t-test, Welch's analysis of variance (ANOVA) test, ANOVA test, LSD test, and Games-Howell test. HC, healthy control; HDC, heavy drinking controls (*p<0.05; **p<0.01; NS. p>0.05).

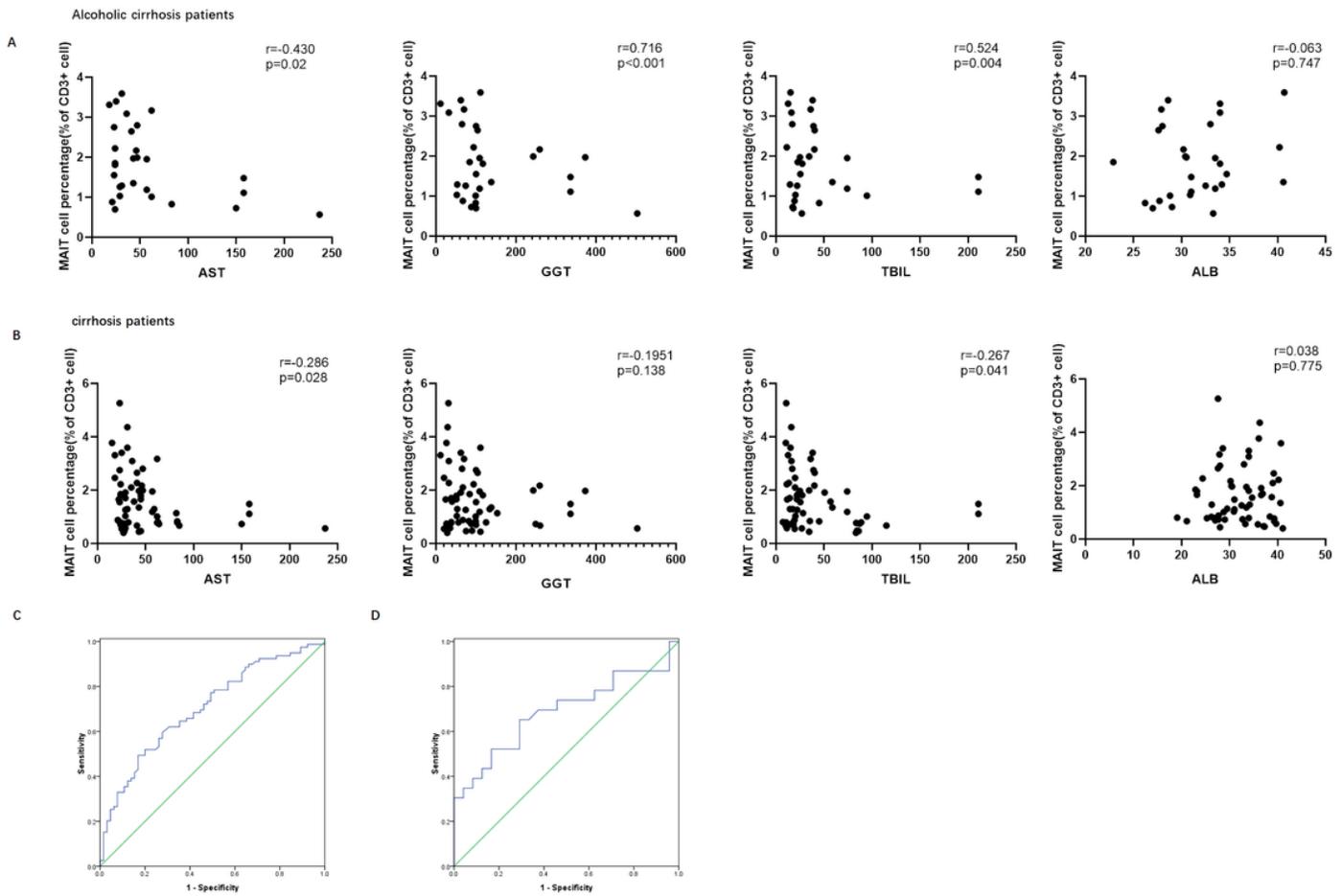


Figure 2

Circulating MAIT cell frequency correlated with liver function (A) Spearman correlation between mucosal-associated invariant T (MAIT) cell percentages among CD3+T cells in patients with alcoholic cirrhosis, and levels of aspartate transaminase (AST), γ -glutamyl transferase (GGT), total bilirubin (TBIL), and albumin (ALB) (n = 29); (B) Spearman correlation between MAIT percentages among CD3+T cells in patients with alcoholic cirrhosis and those with mixed cirrhosis, and levels of AST, GGT, TBIL, and ALB (n = 60); (C) Receiver operating characteristic curve analysis of the diagnostic value of MAIT cell frequency in cirrhosis (AUC=0.703, CI=0.619-0.788, P<0.001); (D) Receiver operating characteristic curve analysis of the diagnostic value of MAIT cell frequency for the severity of cirrhosis(Child-Pugh B/C)(AUC=0.687,CI=0.53-0.845,P=0.028).

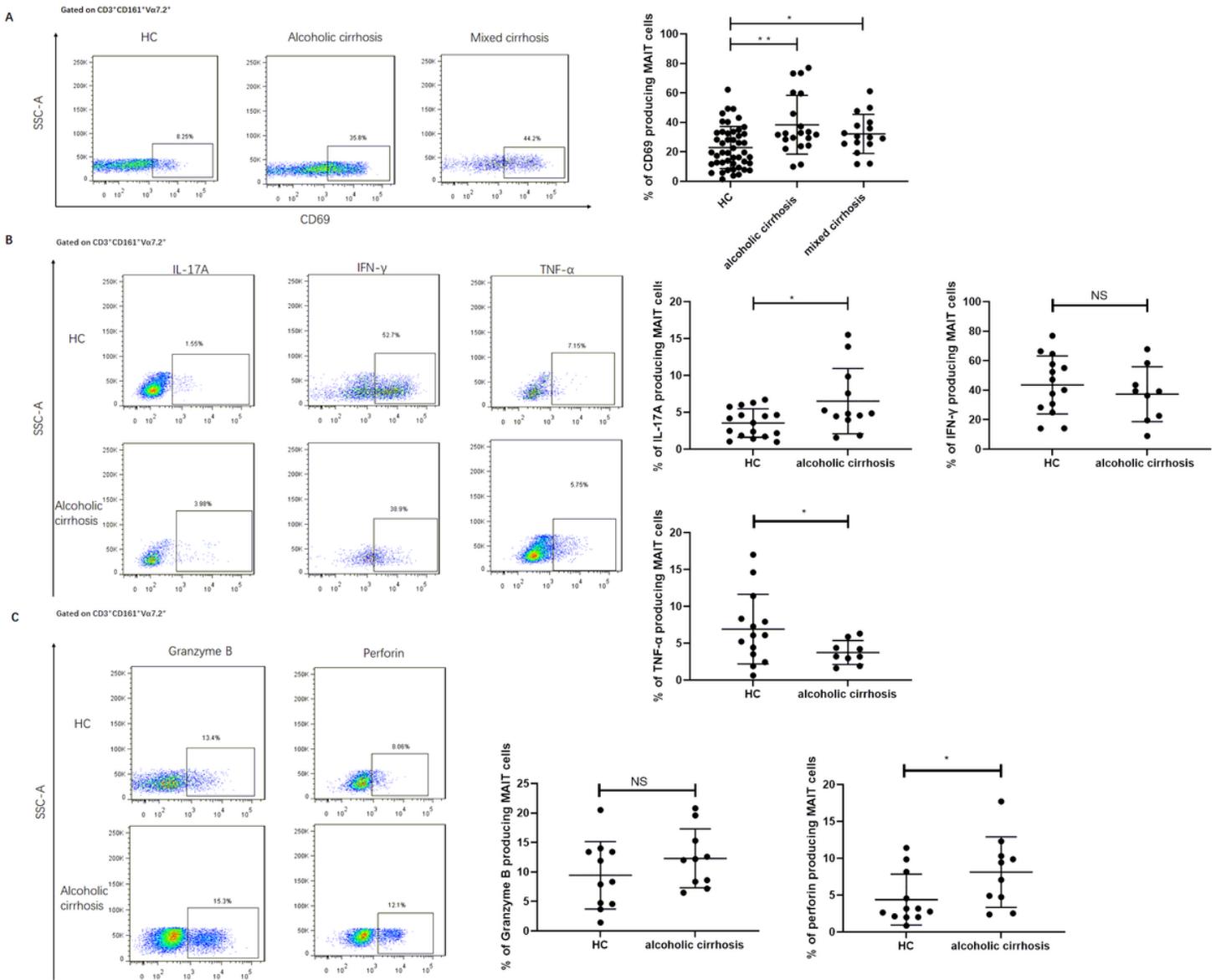


Figure 3

Phenotype and function of circulating MAIT cells in patients with alcoholic and mixed cirrhosis compared with healthy controls (A) Representative dot plot of CD69. Comparison of CD69 on peripheral mucosal-associated invariant T (MAIT) cells in alcoholic liver disease (n = 20), mixed cirrhosis (n = 16), and healthy control (n = 47) (F = 7.418, p = 0.001). (B) Representative intracellular cytokine staining of MAIT cells from healthy control (HCs) and patients with alcoholic cirrhosis. Comparison of IL-17A, IFN-γ, TNF-α levels between HC (n = 14-17) and patients with alcoholic cirrhosis (n = 9-12). (C) Cytolytic capacity profile of MAIT cells in the blood from HCs and patients with alcoholic cirrhosis. Comparison of Granzyme B and perforin between HC (n = 11-12) and alcoholic cirrhosis (n = 10-11). Data were analyzed using the analysis of variance (ANOVA) test, LSD test, and two-sample t-test. (*p<0.05; **p<0.01; NS. p>0.05)

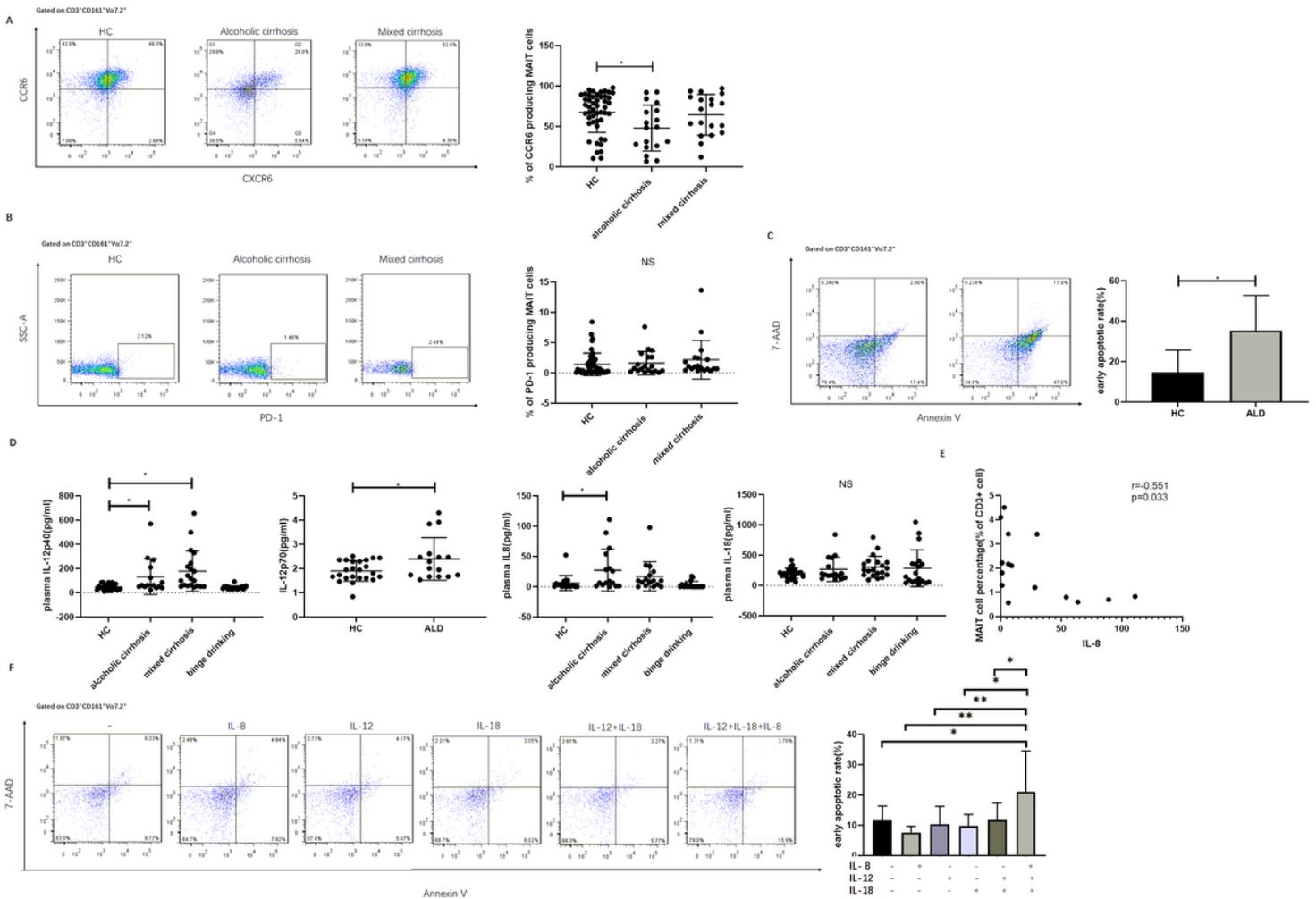


Figure 4

Inflammatory cytokines and chemokines promoted MAIT cell apoptosis (A) Representative dot plot of CCR6 and CXCR6. Comparison of CCR6 and CXCR6 on peripheral mucosal-associated invariant T (MAIT) cells in alcoholic liver disease (n = 18), mixed cirrhosis (n = 19) and healthy control (n = 48). (B) Representative dot plot of PD-1. Comparison of PD-1 on peripheral MAIT cells in alcoholic liver disease (n = 19), mixed cirrhosis (n = 19) and healthy control (n = 48) (F = 0.816, p = 0.446). (C) Peripheral blood mononuclear cells (PBMCs) from patients with alcoholic cirrhosis (n = 5) and healthy controls (n = 6) were gated on MAIT cells and then stained with 7-aminoactinomycin D (7-AAD) and Annexin V. Percentage of early apoptotic (7-AAD-Annexin V+) cells were measured in healthy controls and patients with alcoholic cirrhosis. (D) Levels of IL-12p40, IL-12p70, and IL-8 were elevated in patients with alcoholic cirrhosis (n = 16) compared with healthy controls (n = 18-24). IL-18 was highly expressed in all groups. (E) Spearman correlation between MAIT percentage among CD3+T cells in patients with alcoholic cirrhosis with IL-8 levels (n = 15); (F) Representation of gating strategy showing 7-AAD and Annexin V after gating on MAIT cells in PBMCs from healthy humans, which were cultured with 50ng/ml IL-12 or/ and IL-18 or/and IL-8 for 24h. Early apoptosis was evidenced by the percentages of 7-AAD-Annexin V+ cells. Induced apoptosis in MAIT cells after exposure to IL-12, IL-18, and IL-8 stimulation for 24 h (n = 5). Data were analyzed by using the analysis of variance (ANOVA) test, two-sample t-test. (*p<0.05; **p<0.01; NS. p>0.05)

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