Alcohol Abstinence Does Not Fully Reverse Abnormalities of Mucosal-Associated Invariant T Cells in the Blood of Patients With Alcoholic Hepatitis

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OBJECTIVES: Alcoholic hepatitis (AH) develops in approximately 30% of chronic heavy drinkers. The immune system of patients with AH is hyperactivated, yet ineffective against infectious diseases. Mucosal-associated invariant T (MAIT) cells are innate-like lymphocytes that are highly enriched in liver, mucosa, and peripheral blood and contribute to antimicrobial immunity. We aimed to determine whether MAIT cells were dysregulated in heavy drinkers with and without AH and the effects of alcohol abstinence on MAIT cell recovery.

METHODS: MR1 tetramers loaded with a potent MAIT cell ligand 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil were used in multiparameter flow cytometry to analyze peripheral blood MAIT cells in 59 healthy controls (HC), 56 patients with AH, and 45 heavy drinkers without overt liver disease (HDC) at baseline and 6- and 12-month follow-ups. Multiplex immunoassays were used to quantify plasma levels of cytokines related to MAIT cell activation. Kinetic Turbidimetric Limulus Amebocyte Lysate Assay and ELISA were performed to measure circulating levels of 2 surrogate markers for bacterial translocation (lipopolysaccharide and CD14), respectively.

RESULTS: At baseline, patients with AH had a significantly lower frequency of MAIT cells than HDC and HC. HDC also had less MAIT cells than HC (median 0.16% in AH, 0.56% in HDC, and 1.25% in HC). Further, the residual MAIT cells in patients with AH expressed higher levels of activation markers (CD69, CD38, and human leukocyte antigen [HLA]-DR), the effector molecule granzyme B, and the immune exhaustion molecule PD-1. Plasma levels of lipopolysaccharide and CD14 and several cytokines related to MAIT cell activation were elevated in patients with AH (interferon [IFN]-α, interleukin [IL]-7, IL-15, IL-17, IL-18, IL-23, IFN-γ, and tumor necrosis factor α). Decreased MAIT cell frequency and upregulated CD38, CD69, and HLA-DR correlated negatively and positively, respectively, with aspartate aminotransferase level. MAIT cell frequency negatively correlated with IL-18. HLA-DR and CD38 levels correlated with several cytokines. At follow-ups, abstinent patients with AH had increased MAIT cell frequency and decreased MAIT cell activation. However, MAIT cell frequency was not fully normalized in patients with AH (median 0.31%).

DISCUSSION: We showed that HDC had a reduction of blood MAIT cells despite showing little evidence of immune activation, whereas patients with AH had a severe depletion of blood MAIT cells and the residual cells were highly activated. Alcohol abstinence partially reversed those abnormalities.


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INTRODUCTION

Excessive alcohol consumption causes hepatocellular injury through direct cytotoxic effects and oxidative stress mediated by ethanol and its metabolites and induction of proinflammatory cascades. Up to a third of long-term heavy drinkers develop a spectrum of severe alcoholic liver disease (ALD), ranging from alcoholic hepatitis (AH), fibrosis/cirrhosis, to hepatocellular carcinoma. Approximately 10%–35% of the heavy drinking population develops AH, a severe and progressive acute-on-chronic liver inflammation disease with significant morbidity and mortality, for which there are limited treatment options (1–3). AH is clinically characterized by hyperbilirubinemia, coagulopathy, elevation of liver enzyme levels, and features of the systemic inflammatory response syndrome in heavy drinkers with a history of recent alcohol abuse. Although the exact trigger for development of AH is still not well-understood, alcohol-induced dysregulation of both innate and adaptive immune systems has been implicated in the pathogenesis of AH (4–7).

Excessive drinking leads to dysbiosis of gut microbiome (8–10), which contributes to alcohol-induced breakdown of gastrointestinal (GI) tract barrier. Increased intestinal permeability results in the translocation of microbes and microbial products from the GI tract into blood, which reach the liver through the portal vein, activating both innate and adaptive immune cells that have been sensitized by alcohol and its metabolites (11,12). The liver-resident macrophages, Kupffer cells, play a critical role in initiating and driving liver inflammation in AH by releasing proinflammatory effectors including cytokines and chemokines upon recognition of pathogen-associated molecular patterns, such as lipopolysaccharides (LPS), and danger-associated molecular patterns released from injured hepatocytes. Cytokines and chemokines secreted by Kupffer cells and other intrahepatic immune and nonimmune cells promote recruitment of polymorphonuclear neutrophils, monocytes, and T cells into the liver, leading to further liver injury and inflammation (13,14). Patients with AH have elevated circulating levels of a variety of proinflammatory factors, such as IL-8, tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemotractant protein (MCP)-1, and macrophage inflammatory protein (MIP)3, which serves as a positive feedback loop to further enhance hepatocellular damage, GI tract permeability, and immune dysregulation. In addition, both the innate and adaptive immune cells are functionally defective in patients with AH (5,15). Innate immune cells including neutrophils and monocytes exhibit defective antimicrobial responses including impaired phagocytosis and oxidative burst in patients with AH (16). T cells in patients with AH express high levels of activation markers and immune inhibitory/exhaustion molecules, such as PD-1 and TIM3 (3,16). Those primed T cells have defective IFN-γ production and are ineffective against pathogens, leading to an increased susceptibility to infection in patients with AH (15–17).

Increasing evidence has suggested that mucosal-associated invariant T (MAIT) cells, an innate-like T lymphocyte subset, play a protective role in antimicrobial immunity (18,19). MAIT cells express high levels of the C-type lectin receptor CD161 and are predominantly CD8-positive with only a small fraction of CD4-positive subset and some double negative (DN) (CD8−CD4−) cells. The T-cell receptor (TCR) expressed by MAIT cells is of limited diversity, consisting of a dominant invariant alpha chain (Vα7.2-Jα33) and a restricted number of beta chain (Vβ2 or Vβ13) (20). MAIT cells are activated by vitamin B2 biosynthesis precursor derivatives produced by a range of pathogenic and commensal microbes in the context of the highly evolutionarily conserved monomorphic MHC class-I-like molecule MR1 on antigen-presenting cells (21,22). In addition, MAIT cells express high levels of IL-18 receptor and can be activated by a TCR-independent but cytokine-dependent pathway, in which IL-18 synergizes with IL-12, IL-15, or IFN-α/β (23–26). IL-23 can also stimulate MAIT cells (27). Thus, this cytokine-dependent activation of MAIT cells broadens their role to viral infections and inflammatory/autoimmune diseases even in the absence of bacterial antigen recognition (19,28). Further, IL-7 and IL-15 cytokines enhance TCR-mediated MAIT cell activation (29–31). Upon activation, MAIT cells rapidly produce proinflammatory cytokines TNF-α, IFN-γ, and IL-17 and upregulate cytotoxic molecules granzyme B and perforin that contribute to inflammation and host defense.

MAIT cells are abundant in the mucosal tissues, such as the intestine (2%–10% of T cells) and the lungs (1%–10% of T cells), and the peripheral blood (0.1%–10% of T cells). Strikingly, MAIT cells are highly enriched in the human liver, accounting for 10%–40% of hepatic T cells (32). This dominance of MAIT cells in the liver suggests that they could play a major role in host defense and inflammation in various liver diseases, including ALD (19,32–34). It has been demonstrated that the frequency of MAIT cells in the blood and liver is significantly reduced in several chronic inflammatory and autoimmune liver diseases and ALD (35,36). In addition, a recent comprehensive study has revealed that CD8+ MAIT cells are markedly depleted from the blood of patients with severe AH and alcohol-related cirrhosis (ARC), and the remaining MAIT cells have hyperactivated phenotype yet are functionally defective in cytokine and cytotoxic responses (37). Furthermore, these MAIT cell alterations are not reversed after a short-term alcohol abstinence of 2–5 days (37). As it takes several weeks for GI tract barrier function to recover (10,38), the effect of long-term alcohol cessation on MAIT cell recovery remains unknown.

Currently, it is not well-understood why alcohol abuse causes AH in a subset of patients with excessive alcohol use and what determines the severity of AH. Most studies have compared patients with AH with healthy controls (HC) or patients with ARC to try to understand the immunological differences underlying the apparent individual susceptibility to AH. Here, we conducted a study to characterize MAIT cells in a large cohort of patients with AH, heavy drinkers without clinic evidence of liver disease (HDC), and HC. We also correlated MAIT cell phenotype with patients’ clinical parameters and plasma levels of several markers of bacterial translocation and cytokines related to MAIT cell activation and function. Finally, we performed cross-sectional and longitudinal analysis to determine the effect of long-term alcohol abstinence on reversing abnormalities in MAIT cell frequency and phenotype. We found that alcohol cessation improved but did not completely reverse MAIT cell abnormalities in patients with AH.

METHODS

Study subjects

This study was approved by the Institutional Review Boards at Indiana University School of Medicine, Mayo Clinic, and Virginia Commonwealth University. All participants provided a written informed consent form before blood was drawn. The study subjects (56 patients with AH and 45 HDC at baseline, 24...
Table 1. Comparison of characteristics of patients with AH and HDC in the TREAT study cohort

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 0 (baseline)</th>
<th>Day 180</th>
<th>Day 360</th>
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<tbody>
<tr>
<td></td>
<td>HC (n = 59)</td>
<td>AH (n = 56)</td>
<td>HDC (n = 45)</td>
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<tr>
<td>Age at enrollment (yr)</td>
<td>41 ± 14</td>
<td>45 ± 10</td>
<td>43 ± 10</td>
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<tr>
<td>Gender (% male)</td>
<td>53</td>
<td>61</td>
<td>64</td>
</tr>
<tr>
<td>Race (% white)</td>
<td>71</td>
<td>95</td>
<td>78</td>
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<tr>
<td>Total drinks in the last 30 d</td>
<td>237 ± 221</td>
<td>412 ± 409</td>
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<tr>
<td>Total drinking d in the last 30 d</td>
<td>22 ± 10</td>
<td>25 ± 7</td>
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<tr>
<td>MELD score</td>
<td>25 ± 7</td>
<td>7 ± 2</td>
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<tr>
<td>Creatinine (mg/dL)</td>
<td>0.9 ± 0.2a</td>
<td>1 ± 0.7</td>
<td>0.9 ± 0.3</td>
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<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.5 ± 0.2a</td>
<td>18.1 ± 12.8###</td>
<td>0.6 ± 0.3</td>
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<tr>
<td>AST (IU/L)</td>
<td>18 ± 6a</td>
<td>123 ± 55###</td>
<td>26 ± 9§§</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>14 ± 7a</td>
<td>50 ± 27###</td>
<td>25 ± 10§§</td>
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<tr>
<td>Prothrombin time (INR)</td>
<td>1.9 ± 0.5</td>
<td>1 ± 0.1</td>
<td>***</td>
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</table>

Data are represented as mean ± SD. Kruskal-Wallis test with Dunn’s correction for pairwise comparisons of continuous variables among HC, patients with AH, and HDC at enrollment (day 0). Mann-Whitney test comparing patients with AH vs HDC at 180-day and 360-day follow-up. Chi-square test for analysis of categorical variables.

AH, alcoholic hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HC, healthy controls; HDC, heavy drinking controls; INR, international normalized ratio; MELD, model for end-stage liver disease; ns, not significant.

aData from 44 HC.

### P < 0.001 for comparison between patients with AH and HC at day 0; * P < 0.05; ** P < 0.01; *** P < 0.001 for comparison between patients with AH and HDC; §§ P < 0.01; §§§ P < 0.001 for comparison between HDC and HC at day 0.
patients with AH and 25 HDC at 180-day follow-up, and 15 patients with AH and 22 HDC at 360-day follow-up) were part of the multicenter prospective Translational Research and Evolving Alcoholic Hepatitis Treatment 001 study (TREAT 001, NCT02172898). Demographic and clinical characterizations as well as drinking patterns of the study subjects are shown in Table 1. Detailed definitions of AH and HDC and the inclusion and exclusion criteria were previously described (39). HDC were individuals with a comparable history of alcohol consumption but had no clinical evidence of liver disease (aspartate aminotransferase [AST] ≤ 50 U/L, alanine aminotransferase [ALT] ≤ 50 U/L, and total bilirubin within normal limits) and were matched for age, sex, and race. Individuals with liver diseases of other etiologies and clinically overt and active infection were excluded from this study.

Blood samples
Peripheral blood was collected in heparin-coated tubes (BD Biosciences, Franklin Lakes, NJ) and separated into plasma and peripheral blood mononuclear cells (PBMCs). Both plasma and PBMCs were stored at −80°C until use. PBMC and plasma samples from 59 age-, sex-, and race-matched healthy volunteers were included as HC.

Flow cytometry
PBMCs were subjected to cell surface staining and intracellular staining to determine leukocyte frequency and phenotype. For cell surface staining, PBMCs were incubated with fixable viability dye (ThermoFisher Scientific, Waltham, MA) to discriminate live and dead cells, followed by staining with fluorochrome-conjugated antibodies against human CD3, CD4, CD8, CD161, CD38, CD56, CD69, HLA-DR, gamma delta T (γδ T) cells, invariant natural killer T (iNKT) cells (all from BioLegend, San Diego, CA), and human MR1 tetramers loaded with a potent MAIT cell ligand 5-OP-RU (5-(2-oxopropylideneamino)-6-d-ribitylaminouracil). Appropriate isotype controls were used at the same protein concentration as the test antibodies for control staining, and human MR1 tetramers loaded with 6-EP (6-formylpterin) were used as a negative control for MAIT cell staining. Human MR1 tetramers loaded with 5-OP-RU or 6-EP were produced by the NIH Tetramer Core Facility as permitted to be distributed by the University of Melbourne. Cells stained with surface markers were fixed and permeabilized with the Cytofix/Cytoperm reagents (BD Biosciences, San Jose, CA). For intracellular staining, fixed cells were permeabilized with the Cytofix/Cytoperm reagents (BD Biosciences), followed by staining with antibodies against granzyme B (BioLegend) and active form of caspase 3 (BD Biosciences). Cells were subsequently acquired using a BD LSRFortessa flow cytometer (BD Biosciences). Flow data were analyzed using FlowJo v10 software (Tree Star, San Carlos, CA).

Plasma endotoxin and soluble CD14
Plasma levels of the endotoxin LPS from Gram-negative bacteria and soluble CD14 (sCD14) were used as surrogate markers for bacterial translocation from gut to circulation. LPS amounts were determined using the PYROGENT-5000 Kinetic Turbidimetric Limulus Amebocyte Lysate Assay (Lonza, Walkersville, MD). Plasma samples were diluted 5-folds in endotoxin-free water and heated at 70°C for 20 minutes before being subjected to the assay. Plasma sCD14 levels were quantified using the Human CD14 Quantikine kit (R&D Systems, Minneapolis, MN).

Multiplex immunoassays
The Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex Panel 1 (ThermoFisher Scientific) was used to simultaneously measure plasma concentrations of 45 proteins (BDNF, Eotaxin/CCL11, EGF, FGF-2, GM-CSF, GROα/CXCL1, HGF, NGF-β, LIF, IFN-α, IFN-γ, IL-1β, IL-1α, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, regulated upon activation normal T cell expressed and secreted [RANTES]/CCL5, stromal cell-derived factor [SDF]-1α/CXCL12, TNF-α, TNF-β/LTA, platelet-derived growth factor [PDGF]-BB, placenta Growth Factor [PLGF], stem-cell factor [SCF], vascular endothelial growth factor [VEGF]-A, and VEGF-D). Multiplex assay was conducted as previously described (17). The concentrations of cytokines/chemokines were calculated using the Bio-Plex Manager v6.1 software (Bio-Rad, Hercules, CA). For statistical analyses, values below the detection limit of the assay were replaced with the minimal detectable concentrations for each analyte as provided by the manufacturer.

Statistical analysis
Differences in cross-sectional analysis for continuous variables between 2 groups were calculated using Mann-Whitney test and Kruskal-Wallis test with Dunn’s corrections for comparisons among 3 groups. Chi-square test was used for comparison between groups for categorical variables. The linear relationship between 2 variables was analyzed using the Spearman correlation test. Differences in longitudinal analysis were calculated using Friedman rank sum test with Dunn’s corrections. P < 0.05 was considered statistically significant.

RESULTS
Characteristics of the study cohort
Table 1 summarizes the demographic and clinical characteristics of 56 patients with AH, 45 HDC, and 59 HC at enrollment; 24 patients with AH and 25 HDC at 6-month follow-up; and 15 patients with AH and 22 HDC at 12-month follow-up. There were no differences in age, gender, and race distributions and creatinine levels among the 3 groups at baseline and between patients with AH and HDC at follow-up. Although the amounts of baseline total bilirubin were similar between HDC and HC, both AST and ALT levels were modestly and significantly higher in HDC than HC. The baseline model for end-stage liver disease (MELD) score or liver biochemistries (AST, ALT, and total bilirubin) were highly elevated in patients with AH as compared to HDC and HC. Prothrombin time was also significantly longer for the patients with AH than HDC. Interestingly, the HDC had significantly more drinks than patients with AH before the enrollment. At the follow-up, both patients with AH and HDC drank much less and the liver biochemistries greatly improved in AH patients and remained unchanged for HDC. However, patients with AH still had a significantly higher MELD score due to elevated levels of liver biochemistries and prolonged prothrombin time. The complete abstinence rate was 79% and 87% for patients with AH and 68% and 59% for HDC at 6 and 12 months, respectively. The MELD score, total bilirubin, AST, and prothrombin time remained higher in abstinent patients with AH at 6 and 12 months compared to abstinent HDC (Supplementary Table 1, Supplementary Digital Content 3, http://links.lww.com/CTG/A54). However, ALT levels were similar between patients with AH and HDC at 12-month follow-up (Supplementary Table 1, Supplementary
Circulating MAIT cells were severely depleted in patients with AH. To analyze the impact of chronic heavy drinking on peripheral MAIT cells, we first analyzed the frequency of MAIT cells in peripheral blood in patients with AH and HDC as compared to HC. MAIT cells were identified as CD3+ lymphocytes that express high level of CD161 and are stained by the 5-OP-RU-loaded MR1 tetramers (40). The staining specificity was verified by the negative control MR1-6-FP tetramers. At baseline, MAIT cells were significantly depleted in patients with AH as compared to HC and HDC (Figure 1a,b). HDC also had significantly less MAIT cells than HC (Figure 1a,b). Specifically, MAIT cells accounted for a median of 0.16% (range: 0.03%–1.9%; interquartile range [IQR]: 0.09%–0.34%), 0.56% (range: 0.03%–7.22%; IQR: 0.23%–1.41%), and 1.25% (range: 0.05%–5.17%; IQR: 0.63%–2.32%) T cells in patients with AH, HDC, and HC, respectively. This decrease of peripheral MAIT cells was not due to enhanced cell death, because there was no increase in active form of caspase 3, a marker for apoptosis (Figure 1c) or down-regulation of CD161 for MAIT cells in patients with AH or HDC (Figure 1a). As a comparison, the frequency of CD161-expressing non-MAIT T cells, which contain highly proinflammatory CD4+ and CD8+ cells (41), was not decreased, but significantly increased in patients with AH compared to HC and HDC (Figure S1A, Supplementary Digital Content 1, http://links.lww.com/CTG/A52).
In homeostasis, the majority of MAIT cells are CD8-positive with a small percentage being CD4-positive, or DN for CD4 and CD8 (42). There was a significant decrease of CD8-expressing MAIT cells, and a corresponding increase in CD4-expressing MAIT cells in patients with AH as compared to HDC and HC (Figure S1B/S1C, Supplementary Digital Content 1, http://links.lww.com/CTG/A52). The frequencies of DN cells were similar among patients with AH, HDC, and HC (Figure S1D, Supplementary Digital Content 1, http://links.lww.com/CTG/A52). There were no significant differences in the frequency of several other types of innate T cells, including NKT, iNKT, and γδ T cells, among patients with AH, HDC, and HC (Figure S2, Supplementary Digital Content 2, http://links.lww.com/CTG/A53).

Patients with AH tended to have lower frequency of MAIT cells than HDC at 6-month follow-up (P = 0.08) and at 12-month follow-up (P = 0.10) (Figure 1b). Furthermore, frequency of MAIT cells at 12-month follow-up in both patients with AH (median: of 0.31%; range: 0.04%–1.45%; IQR: 0.17%–0.46%) and
Table 2. Comparison of levels of inflammatory factors related to MAIT cell activation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day 0 (baseline)</th>
<th>Day 180</th>
<th>Day 360</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HC (n = 43–47)</td>
<td>AH (n = 51–55)</td>
<td>HDC (n = 40–43)</td>
</tr>
<tr>
<td>Stimulation related factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS (EU/mL)</td>
<td>0.01 ± 0.01###</td>
<td>2.78 ± 10.34***</td>
<td>0.02 ± 0.04</td>
</tr>
<tr>
<td>sCD14 (ng/mL)</td>
<td>1.391 ± 530###</td>
<td>2.489 ± 1.110***</td>
<td>1.619 ± 462</td>
</tr>
<tr>
<td>IFN-α (pg/mL)</td>
<td>1.1 ± 1.6</td>
<td>3.4 ± 4.7**</td>
<td>1.3 ± 3</td>
</tr>
<tr>
<td>IL-7 (pg/mL)</td>
<td>0.5 ± 1##</td>
<td>2.1 ± 3.3**</td>
<td>0.5 ± 1.3</td>
</tr>
<tr>
<td>IL-12 (pg/mL)</td>
<td>1.3 ± 0.5###</td>
<td>2.1 ± 1.8</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>IL-15 (pg/mL)</td>
<td>18.2 ± 37</td>
<td>41.8 ± 73.2*</td>
<td>22.4 ± 59.4</td>
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<td>IL-18 (pg/mL)</td>
<td>15.4 ± 15.9###</td>
<td>87.7 ± 91***</td>
<td>26.5 ± 37.7</td>
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<tr>
<td>IL-23 (pg/mL)</td>
<td>35.6 ± 35###</td>
<td>115.7 ± 204***</td>
<td>25.3 ± 14.9</td>
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<tr>
<td>IFN-γ (pg/mL)</td>
<td>4.9 ± 5.1###</td>
<td>32.1 ± 33.8***</td>
<td>9.2 ± 10.6</td>
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<tr>
<td>IL-17 (pg/mL)</td>
<td>0.8 ± 1.8##</td>
<td>15.3 ± 32**</td>
<td>0.7 ± 1.6</td>
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<tr>
<td>TNF-α (pg/mL)</td>
<td>6.5 ± 6.1###</td>
<td>14.8 ± 18.1**</td>
<td>8.3 ± 10.2</td>
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</table>

Data are represented as mean ± SD. Kruskal-Wallis test with Dunn’s correction for pairwise comparisons among HC, patients with AH, and HDC at baseline (day 0). Mann-Whitney test comparing patients with AH vs HDC at 180-day and 360-day follow-up. AH, alcoholic hepatitis; HC, healthy controls; HDC, heavy drinking controls; IFN, interferon; IL, interleukin; LPS, lipopolysaccharides; MAIT, mucosal-associated invariant T cell; sCD14, soluble CD14; TNF, tumor necrosis factor. ###p < 0.01; ##p < 0.001 for comparison between patients with AH and HC at day 0; *P < 0.05; **P < 0.01; ***P < 0.001 for comparison between AH patients and HDC.
HDC (median: 0.61%; range: 0.03%–4.05%; IQR: 0.17%–1.37%) was still significantly lower than that in HC (Figure 1b). However, the percentage of CD8\(^+\), CD4\(^+\), DN, and caspase 3\(^+\) MAIT cells and CD161\(^+\) non-MAIT cells was not different between the AH and HDC groups at the follow-up. Thus, at baseline, MAIT cells were selectively depleted in patients with AH, and the remaining MAIT cells had altered CD4 and CD8 T cell coreceptor expression. There was a partial, but not complete, recovery of MAIT cells at follow-up in patients with AH.

Residual MAIT cells in patients with AH expressed higher levels of activation markers

We have recently reported that the CD4 and CD8 T cells from patients with AH are highly dysregulated, expressing higher activation markers such as CD69 and CD38 than T cells from HDC (17). Here, we examined whether MAIT cells in patients with AH were also hyperactivated. Compared with HDC and HC, significantly higher percentages of MAIT cells from patients with AH expressed CD69 and CD38, whereas those markers were not significantly elevated in MAIT cells from HDC relative to HC (Figure 2a,b). MAIT cells from patients with AH also expressed significantly more HLA-DR (another T-cell activation marker) than HC, whereas the frequencies of CD38 and HLA-DR double-positive cells (often used as an indicator of T-cell activation in chronic inflammation) were not different among the heavy drinkers and HC (Figure 2c,d). Consistent with expressing higher levels of surface activation markers, MAIT cells from patients with AH also contained significantly more granzyme B than HC, but not more than HDC (Figure 2e).

Soluble factors related to MAIT cell activation were elevated in patients with AH

MAIT cells recognize and are activated by MR1 receptors complexed with vitamin B2 biosynthesis precursor derivatives from

<table>
<thead>
<tr>
<th>Variable</th>
<th>% MAIT</th>
<th>% CD69(^+)</th>
<th>% CD38(^+)</th>
<th>% HLA-DR(^+)</th>
<th>% GRB(^+)</th>
<th>% PD-1(^+)</th>
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<tr>
<td>Clinical parameters</td>
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<tr>
<td>MELD</td>
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<tr>
<td>Creatinine</td>
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<tr>
<td>AST</td>
<td>$-0.36^{**}$</td>
<td>0.35**</td>
<td>0.28*</td>
<td>0.3*</td>
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<td>INR</td>
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<td>Stimulation-related factors</td>
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<td>LPS</td>
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<td>sCD14</td>
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<td>IFN-(\alpha)</td>
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<td>0.46***</td>
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<td>IL-7</td>
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<td>0.35*</td>
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<td>IL-15</td>
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<td>IL-18</td>
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<td>IL-23</td>
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<td>0.54***</td>
<td>0.56***</td>
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The numbers represent Spearman’s coefficients. Negative numbers represent negative correlations.

AH, alcoholic hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GRB, granzyme B; IFN, interferon; IL, interleukin; INR, international normalized ratio for prothrombin time; LPS, lipopolysaccharides; MELD, model for end-stage liver disease; MAIT, mucosal-associated invariant T cell; sCD14, soluble CD14; TNF, tumor necrosis factor.

\( *P < 0.05; **P < 0.01; ***P < 0.001. \)
a wide range of Gram-negative and Gram-positive bacteria. In AH, impaired integrity of the GI tract leads to translocation of bacterial components to circulation. We therefore measured circulating levels of LPS derived from Gram-negative bacteria and sCD14, a receptor for LPS that is secreted by activated macrophages and monocytes, as a marker for bacterial translocation. As shown in Table 2, the baseline LPS and sCD14 levels were elevated in patients with AH in comparison to HDC and HC, whereas levels of those factors were not significantly upregulated in HDC compared to HC. At 6- and 12-month follow-ups, the amounts of both factors were similar between the AH and HDC groups.

MAIT cells can also be activated in a TCR-independent manner by cytokines, such as IL-18, IL-12, IL-15, IFN-α, IL-23, IL-7, to produce effector cytokines (IFN-γ, IL-17, and TNF-α), which might further enhance expression of MAIT cell activation markers. As shown in Table 2, the baseline LPS and sCD14 levels were elevated in patients with AH in comparison to HDC and HC, whereas levels of those factors were not significantly upregulated in HDC compared to HC. At 6- and 12-month follow-ups, the amounts of both factors were similar between the AH and HDC groups.

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At 6-month follow-up, all upregulated cytokines except IFN-α remained significantly higher in patients with AH than in HDC. At 12-month follow-up, IL-18, IL-23, and IFN-γ were still elevated in the AH group. Taken together, our data indicated that patients with AH had a higher circulatory level of factors associated with MAIT cells activation, which could contribute to the hyperactivated status of MAIT cells at baseline. Reduced cytokine production in patients with AH at follow-up was reflected by partial recovery of MAIT cell frequency and phenotype.

**Association of MAIT cell frequency and phenotype with clinical profiles and cytokine levels in patients with AH**

To explore whether MAIT cells might be related to pathogenesis of AH, we determined whether MAIT cell frequency and the activation markers correlated with disease severity represented by clinical scores (MELD score) and biochemical measurements (creatinine, total bilirubin, AST, ALT) and prothrombin time in patients with AH at baseline. Although MAIT cell frequency and phenotype did not correlate with the MELD score, the frequencies of MAIT cells and MAIT cell activation markers CD69, CD38, and HLA-DR correlated negatively and positively with level of the liver enzyme AST, respectively, suggesting dysregulated MAIT cells might be
involved in liver damage. The percentage of PD-1-positive MAIT cells correlated with the creatinine level (Table 3).

Next, we analyzed the correlations between MAIT cell phenotype and factors that are linked to MAIT cell activation. The significant correlations were listed in Table 3. Circulatory levels of LPS did not correlate with MAIT frequency or activation phenotype, whereas sCD14 level correlated with percentages of CD69^+ and granzyme B^+ MAIT cells. IL-18 was negatively associated with MAIT frequency and positively with CD38^+ (%) and HLA-DR^+ (%) MAIT cells. Other MAIT-stimulatory cytokines (IL-7, IL-12, IL-15, and IL-23) also correlated with CD38^+ (%) and HLA-DR^+ (%) MAIT cells. Effector cytokines IL-17, IFN-γ, and TNF-α also showed significant correlations with the frequency of MAIT activation markers CD38 and HLA-DR. Interestingly, the frequency of PD-1^+ MAIT cells in the patients with AH inversely correlated with the percentage of MAIT cells (r = 0.41, P = 0.003) as well as CD8^+ MAIT cells (r = 0.35, P = 0.01). These results suggested that stimulation by bacterial products and multiple cytokines could contribute to MAIT cell hyperactivation and that immune exhaustion might play a role in MAIT cell depletion.

Loss of MAIT cells was not fully reversed in patients with AH by alcohol abstinence

To determine whether the partial recovery of MAIT abnormalities at follow-up was linked to alcohol abstinence in patients with AH, we performed cross-sectional analysis on follow-up samples between abstinent patients with AH and HDC. As shown in Figure 3a,b, the frequencies of MAIT cells and CD38^+ MAIT cells were still significantly lower and higher, respectively, in the AH group at 6-month follow-up. Other MAIT cell activation markers, including CD69, HLA-DR, and

Figure 4. Longitudinal analysis of dysregulated immunological factors in abstinent subjects. Longitudinal analysis of the effects of alcohol abstinence on expression levels of MAIT cell markers in patients with AH (a) and HDC (b). Friedman rank sum test with Dunn’s correction for comparing surface marker expression at day 0 (D0) with 180-day (D180) follow-up or 360-day (D360) follow-up (n = 6–7). *P < 0.05, **P < 0.01. AH, alcoholic hepatitis; HDC, heavy drinking controls; MAIT, mucosal-associated invariant T cell; ns: not significant.
Peripheral MAIT Cells in Patients with Alcoholic Hepatitis

Granulocytes, not completely, with alcohol cessation. MAIT activation markers in patients with AH recovered greatly, data indicated that the dysregulated MAIT cell frequency and changes throughout the study (Figure 4b). Taken together, our activation markers in abstinent HDC did not have significant reductions in CD69 and CD38 expression at 12-month follow-up and in CD38 expression at 12-month (Figure 4a). In contrast, frequencies of MAIT cells and those activation markers in abstinent AH did not have significant changes throughout the study (Figure 4b). Taken together, our data indicated that the dysregulated MAIT cell frequency and MAIT activation markers in patients with AH recovered greatly, but not completely, with alcohol cessation.

**DISCUSSION**

In the present study, we performed cross-sectional and longitudinal analysis to compare frequency, phenotype, and function of peripheral blood MAIT cells from a large cohort of patients with AH, matched heavy drinkers, and HC (Table 1). The design of our study allowed us to identify changes in MAIT cells related to heavy drinking alone, during AH developments, and immune system recovery following alcohol abstinence. We demonstrated that patients with AH had a severe reduction of MAIT cells and the residual MAIT cells exhibited a highly activated and exhausted phenotype (Figures 1 and 2). On the other hand, HDC only had a slight decrease in MAIT cell frequency and the remaining MAIT cells did not exhibit significant upregulation of activation markers compared to HC. Baseline plasma levels of markers of bacterial translocation to circulation (LPS and sCD14) and several cytokines linked to MAIT cell activation (IFN-α, IL-7, IL-12, IL-15, IL-18, IL-23, IFN-γ, IL-17, and TNF-α) were highly elevated in patients with AH (Table 2). Baseline MAIT cell frequency and levels of MAIT activation markers CD69, CD38, and HLA-DR correlated negatively and positively with AST in patients with AH, respectively. MAIT cell frequency was also negatively associated with IL-18, whereas up-regulated CD38 and HLA-DR showed positive correlations with many of the cytokines implicated in MAIT cell activation (Table 3). Analysis of follow-up and longitudinal samples indicated MAIT cell abnormalities in patients with AH drastically improved but were not completely reversed with alcohol cessation (Figures 3 and 4), which was reflected by changes in their clinical characteristics.

![Gut microbiome](image-url) **Figure 5.** Model of MAIT cell activation and depletion in patients with AH. Chronic heavy drinking induces dysbiosis of gut microbiome and breakdown of GI tract barrier, which leads to translocation of gut bacteria and their products to the portal and systemic circulation in patients with AH. Bacterial products, such as LPS, activate APC and other cells to release MAIT cell-activating cytokines, including IL-18, IL-7, IL-15, and IL-23, and IFN-α. In addition, bacterial-derived vitamin B2 metabolites presented by the major histocompatibility complex class I-related protein 1 (MR1) on APC activate MAIT cells through the invariant Vα7.2-Jα33 T-cell receptor. Both the cytokine- and antigen-dependent signaling pathways of MAIT cell activation contribute to upregulation of T-cell activation markers (CD69, CD38, and HLA-DR), the T-cell exhaustion marker PD-1, and production of cytotoxic effector proteins (granulyme B and perforin), and effector cytokines (IFN-γ, IL-17, and TNF-α, which can serve as a positive feedback to further activate MAIT cells). These hyperactivated and exhausted MAIT cells are prone to activation-induced cell death, leading to depletion of hepatic and circulating MAIT cells and impaired antimicrobial cytokine and cytotoxic responses for the residual MAIT cells in patients with AH. AH, alcoholic hepatitis; APC, antigen-presenting cells; GI, gastrointestinal; LPS, lipopolysaccharides; MAIT, mucosal-associated invariant T cell; TNF, tumor necrosis factor.
Peripheral blood MAIT cells were markedly depleted in patients with AH at enrollment as quantified by MR1-5-OP-RU tetramer staining. This result confirmed a previous finding that blood MAIT cells are profoundly reduced in patients with severe AH using CD161<sup>+</sup>-TCR-Vα7.2<sup>+</sup> phenotyping to identify MAIT cells (37). On the other hand, we did not find major changes in several other innate T-cell populations, such as NKT, iNKT, and γδ T cells, indicating that MAIT cells were specifically depleted in AH. CD8<sup>+</sup> MAIT cells have been identified as the dominant subset of MAIT cells. We and others have shown that patients with AH have reduced frequency of total CD8 T cells in circulation (5,17,19). In line with this general reduction of CD8 T cells in peripheral blood, we found that the frequency of CD8<sup>+</sup> MAIT cells was reduced with a corresponding increase in CD4<sup>+</sup> MAIT cells. Similar decrease in CD8<sup>+</sup> MAIT cells and increase in CD4<sup>+</sup> MAIT cells has been observed in several chronic liver diseases, including ALD (35). In addition to overt cell exhaustion, the loss of peripheral MAIT cells could be partly due to redistribution to the liver, although the existing data are contradictory. One study has reported that CD161<sup>+</sup>-TCR-Vα7.2<sup>+</sup> MAIT cells dramatically depleted in the blood, but the TCR-Vα7.2<sup>+</sup> cells (also include non-MAIT cells) were preserved in the liver of patients with ARC (37). However, 2 other studies have demonstrated that frequency of CD161<sup>+</sup>-TCR-Vα7.2<sup>+</sup> MAIT cells are drastically reduced in both the blood and liver of patients with end-stage ALD (35,43). Thus, further study with MR1 tetramers to quantify MAIT cells in liver biopsies from patients with AH is required to assess whether blood MAIT cell depletion results from MAIT cell recruitment into the liver or dysregulation of cell homoeostasis and survival.

MAIT cell depletion from the periphery, which is a common occurrence in many infectious and inflammatory diseases, is concurrent with upregulation of activation and exhaustion markers (19,32,34). Hyperactivation often leads to activation-induced cell death by apoptosis. Human MAIT cells are known to have a proapoptotic phenotype in response to cell activation (44). MAIT cells in this large cohort of patients with AH expressed high levels of activation markers CD69, CD38, HLA-DR, and the immune inhibitory/exhaustion marker PD-1. This is in line with findings showing that hyper-activated MAIT cells in other diseases also express higher levels of inhibitory molecules PD-1 and TIM3 (19). However, a recent study found no increase in expression levels of 3 immune checkpoint inhibitors (PD-1, TIM3, and LAG3) in 9 patients with AH and ARC (37). In our study, we did not observe an increase in apoptotic MAIT cells or non-MAIT T cells in patients with AH or HDC. It is possible that apoptotic MAIT cells were rapidly cleared from the circulation before sample collection or the cell death was independent of apoptosis.

In TCR-dependent activation, MAIT cells are activated by MR-1 complexed with bacterial-derived vitamin B2 metabolites. In AH, bacterial components and metabolites are released from gut to circulation due to increased intestinal permeability (11,45). We used LPS and sCD14 as surrogate markers for gut bacterial translocation. Both markers were significantly higher in patients with AH than HDC and HC, which is consistent with the results recently reported (9). Interestingly, fecal extracts containing gut bacteria and bacterial antigens and metabolites (FEB) from patients with severe AH and ARC, but not FEB from healthy donors or fixed laboratory strain of Escherichia coli, can induce depletion and dysfunction of MAIT cells from PBMCs of healthy donors (37). Although the underlying mechanism for this observation is unknown, dysbiosis of gut microbiome likely plays a role. Recently, dysbiosis of the circulating microbiome in patients with AH as well as in HDC has been shown as compared to nondrinking controls (9). The relationship between dysbiosis of gut and blood microbiome and MAIT cell dysregulation requires further investigation.

Of the several MAIT cell activation-related cytokines that were upregulated in patients with AH, we found that multiple inflammatory cytokines correlated with expression of the T-cell activation markers CD38 and HLA-DR, which likely reflected hyperinflammation in AH. Interestingly, we found that IL-18 level negatively correlated to MAIT frequency, suggesting that IL-18 likely plays a role in MAIT cell depletion in patients with AH. Currently, the relative contribution of TCR- and cytokine-dependent pathways to MAIT cell alterations in AH is unknown. Ex vivo studies showed that neither ALD plasma, which contained higher levels of bacterial products and inflammatory cytokines, nor alcohol treatment could induce MAIT cell depletion from PBMCs obtained from healthy donors (37). We also confirmed that plasma from neither patients with AH nor HDC could induce MAIT cell depletion from PBMCs of healthy donors. Based on our results and published studies (35,37), we propose that both antigen-dependent and cytokine-dependent pathways play a role in driving MAIT cell hyperactivation and depletion in patients with AH (Figure 5). Future study on the potential role of alcohol or alcohol metabolites, bacterial metabolites, and inflammatory cytokines in MAIT cell depletion in patients with AH is warranted.

HDC in our study were heavy drinkers without obvious signs of clinical liver disease, although their AST and ALT levels were slightly higher than HC. Our research groups have shown that those subjects have higher serum levels of LPS and markers of monocyte/macrophage activation (sCD14 and sCD163) compared to nonexcessive drinkers in terms of bacterial translocation (38). We showed here that HDC had a significant decrease of MAIT cells in the peripheral blood compared to HC. HDC had slightly increased levels of the bacterial translocation marker sCD14 and MAIT activation-associated cytokines IL-18 and IL-12. This increase was significant when 2-group comparison between HDC and HC was performed. However, this difference did not reach significance due to lower statistical power for 3-group comparison (HC, AH, and HDC). Our study indicates that chronic excessive drinking leads to slight immune abnormalities in HDC (17). Interestingly, MAIT cell frequency did not differ between HDC who stopped drinking and those who continued drinking at 12-month follow-up (data not shown). This MAIT cell stability is also reflected by the nonprogression of clinical disease severity. We have recently reported that the circulating microbiome in HDC is also altered compared to non-drinking controls (9), which might contribute to this MAIT cell abnormality in HDC.

Complete alcohol abstinence is the key to immune system recovery from AH, but it does not lead to full recovery in all patients (46). Consistent with this, clinical scores and liver function in our abstinent patients with AH greatly improved but were still abnormal during the follow-up (Supplementary Table 1, Supplementary Digital Content 3, http://links.lww.com/CTG/A54). We have recently reported that levels of several proinflammatory cytokines, such as TNF-α and IL-8, are still elevated in insentient patients with AH than HDC at the end of
12-month follow-up (17). Here, we found levels of 2 MAIT cell-activating cytokines IL-18 and IL-23 remained higher in patients with AH patients after alcohol withdrawal. Consistent with a possible role of IL-18 in depletion of peripheral blood MAIT cells, MAIT cell frequency in abstinent patients with AH did not reach levels found in healthy donors. This pattern of MAIT cell recovery is reminiscent of previous studies showing that severe depletion and functional impairment of MAIT cells in the periphery of patients with HIV and chronic HCV are not recovered after successful treatment with antiretroviral therapies and direct-acting antivirals, respectively (33,47,48). We previously showed that alcohol abstinence restored abnormalities in CD4 and CD8 T cells, B cells, and monocytes, suggesting alcohol might have a longer-lasting impact on MAIT cell compartment. It is possible that MAIT cells have a long proliferation rate (49), and a longer abstinence might be required for a full recovery of MAIT cells. MAIT cells might also be more sensitive to bacterial products/cytokines, as ARC had defect in MAIT cells, but not in adaptive T cells (16,37).

In conclusion, we found that HDC had a reduction of MAIT cells despite showing little evidence of immune activation, whereas patients with AH had a severe depletion of MAIT cells and the residual cells had highly dysregulated expression of multiple T-cell activation markers in the background of a higher level of bacterial translocation and immune activation. MAIT cell dysfunction might be a contributing factor to the weakened immunity against microbial infection in patients with AH. Complete abstinence from alcohol consumption greatly but not completely reversed MAIT abnormalities. Current medical therapies for AH are limited. Thus, development of therapies to target dysregulated immune systems, including MAIT cells, may represent a promising strategy for restoring immune homeostasis and host defense against infections in AH.

CONFLICTS OF INTEREST
Guarantor of the article: Qigui Yu, MD, PhD.
Specific author contributions: W.L.: designed and performed all the experiments, performed statistical analysis and data analysis, interpreted data, and wrote the manuscript. E.L.L. and S.C.: designed data analysis. J.L.: did experiments and artwork in Figure 5. S.R.: conducted experiments. S.I., P.P., P.S.K., A.J.S., Y.H.S., S.R., D.W.C., and N.C.: provided clinical samples, funding and feedback for the project, and critically reviewed the manuscript. Q.Y.: conceptualized the study, obtained funding for the study, critically reviewed, and finalized the manuscript.
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Potential competing interests: None.

ACKNOWLEDGEMENTS
We thank all volunteers for donating their time and blood for this multicenter prospective observational cohort study. We thank Kayla Peterson for coordinating the study.

Study Highlights

WHAT IS KNOWN
 ✓ MAIT cells are markedly depleted from the blood of patients with severe AH, and the remaining MAIT cells have hyperactivated phenotype yet are functionally defective in cytokine and cytotoxic responses.
 ✓ MAIT cell alterations are not reversed after a short-term alcohol abstinence of 2–5 days.

WHAT IS NEW HERE
 ✓ We studied circulating MAIT cells in a large cohort of patients with AH and suitable controls.
 ✓ Both cross-sectional and longitudinal studies were performed.
 ✓ Correlation of MAIT cell phenotype and function with clinical parameters was analyzed.
 ✓ HDC had a reduction of MAIT cells despite showing little evidence of immune activation.
 ✓ Patients with AH had a severe depletion of MAIT cells and the residual cells had highly dysregulated expression of T-cell activation/exhaustion markers.
 ✓ Long-term complete abstinence from alcohol consumption greatly but not completely reversed MAIT abnormalities.

TRANSLATIONAL IMPACT
 ✓ MAIT cell dysfunction likely contributes to compromised immunity against microbial infection in patients with AH.
 ✓ Therapies targeting dysregulated MAIT cells may represent a promising strategy to restore host defense against infections in AH.

REFERENCES


