Received: 22 January 2021 | Revised: 16 September 2021 | Accepted: 17 September 2021

DOI: 10.1002/hep.32193

ORIGINAL ARTICLE

Aberrant hepatic trafficking of gut-derived T cells is not specific to primary sclerosing cholangitis

Jonathon J. Graham[1,2] | **Sujit Mukherjee**[3] | **Muhammad Yuksel**[1,4] | **Rebeca Sanabria Mateos**[1] | **Tengfei Si**[1] | **Zhenlin Huang**[1] | **Xiahong Huang**[1] | **Hadil Abu Arqoub**[5] | **Vishal Patel**[1,6] | **Mark McPhail**[1] | **Yoh Zen**[5] | **Nigel Heaton**[1] | **Maria Serena Longhi**[2] | **Michael A. Heneghan**[1] | **Rodrigo Liberal**[1] | **Diego Vergani**[1] | **Giorgina Mieli-Vergani**[7] | **Yun Ma**[1] | **Bu'Hussain Hayee**[8]

[1]Institute of Liver Studies, School of Immunology & Microbial Sciences, Faculty of Life Sciences & Medicine, King's College London, King's College Hospital, London, UK

[2]Department of Anesthesia, Critical Care & Pain Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA

[3]Section of Hepatology, Department of Metabolism Digestion and Reproduction, Faculty of Medicine, Imperial College London, London, UK

[4]Koç University Research Centre for Translational Medicine, Istanbul, Turkey

[5]Liver Histopathology Laboratory, Institute of Liver Studies, King's College London, London, UK

[6]The Roger Williams Institute of Hepatology London, Foundation for Liver Research, London, UK

[7]Paediatric Liver, GI and Nutrition Centre, Mowat Labs, King's College London Faculty of Life Sciences & Medicine at King's College Hospital, London, UK

[8]Department of Gastroenterology, School of Immunology & Microbial Sciences, Faculty of Life Sciences & Medicine, King's College London, King's College Hospital, London, UK

Correspondence
Jonathon J. Graham, Institute of Liver Studies, King's College Hospital, School of Immunology & Microbial Sciences, Faculty of Life Sciences & Medicine, King's College Hospital, Denmark Hill, London SE5 9RS, UK.
Email: Jonathon.graham@kcl.ac.uk

Funding information
This work has been supported by the King's College Hospital Alex Mowat Charity and PSC Partners

Abstract

Background and Aims: The "gut homing" hypothesis suggests the pathogenesis of primary sclerosing cholangitis (PSC) is driven by aberrant hepatic expression of gut adhesion molecules and subsequent recruitment of gut-derived T cells to the liver. However, inconsistencies lie within this theory including an absence of investigations and comparisons with other chronic liver diseases (CLD). Here, we examine "the gut homing theory" in patients with PSC with associated inflammatory bowel disease (PSC-IBD) and across multiple inflammatory liver diseases.

Approach and Results: Expression of MAdCAM-1, CCL25, and E-Cadherin were assessed histologically and using RT-PCR on explanted liver tissue from patients with CLD undergoing OLT and in normal liver. Liver mononuclear cells were isolated from explanted tissue samples and the expression of gut homing integrins and cytokines on hepatic infiltrating gut-derived T cells was assessed using flow cytometry. Hepatic expression of MAdCAM-1, CCL25 and E-Cadherin was up-regulated in all CLDs compared with normal liver. There were no differences between disease groups. Frequencies of $\alpha4\beta7$, $\alpha E\beta7$, CCR9, and GPR15 expressing hepatic T cells was increased in PSC-IBD, but also in CLD controls, compared with normal liver. $\beta7$ expressing hepatic T cells displayed an increased inflammatory phenotype compared with $\beta7$ negative cells, although this inflammatory cytokine profile was present in both the inflamed and normal liver.

Conclusions: These findings refute the widely accepted "gut homing" hypothesis as the primary driver of PSC and indicate that aberrant hepatic recruitment of gut-derived T cells is not unique to PSC, but is a panetiological feature of CLD.

Abbreviations: AIH, autoimmune hepatitis; ALD, alcohol-associated liver disease; CLD, chronic liver disease; IBD, inflammatory bowel disease; LMNC, liver mononuclear cells; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; PSC-IBD, primary sclerosing cholangitis with associated inflammatory bowel disease.

INTRODUCTION

The recruitment of T lymphocytes from the peripheral circulation to secondary lymphoid organs and peripheral tissues is a highly coordinated process enacted through sequential interactions between homing receptors and their respective ligands expressed on endothelial cells. Tissue-specific combinations of chemokine ligands and adhesion molecules create a molecular "address" that is recognized by specific chemokine receptors and integrins expressed on lymphocytes. One of the most notable examples of tissue-specific recirculation of memory lymphocytes occurs within the gut. The gut molecular address consists of the chemokine ligand 25 (CCL25) and mucosal addressin cellular adhesion molecule 1 (MAdCAM-1), both of which regulate the recruitment of T lymphocytes to the gut through interaction with their cognate receptors: G protein coupled chemokine receptor 9 (CCR9) and alpha 4 beta 7 integrin ($\alpha4\beta7$).[1] Under physiological conditions the expression of CCL25 and MAdCAM-1 are largely confined to gut-associated lymphoid tissues. However, evidence of ectopic MAdCAM-1 and CCL25 expression was demonstrated in the liver of adults with chronic inflammatory liver disease, especially in primary sclerosing cholangitis (PSC), a condition frequently associated with inflammatory bowel disease (IBD), whereas their expression is absent in normal adult and fetal liver.[2] The liver inflammatory lymphocytic infiltrate of patients with PSC has been reported to consist mainly of nonactivated memory T lymphocytes, a substantial proportion of which expressed the co-receptors $\alpha4\beta7$ and/or CCR9.[3] Grant et al., extended this line of research showing hepatic MAdCAM-1 expression in a variety of chronic liver diseases (CLD), but particularly in patients with PSC and autoimmune hepatitis (AIH).[4] In addition, peripheral T lymphocytes from patients with PSC, ulcerative colitis (UC) and healthy controls adhered to histologically MAdCAM-1 positive PSC liver sections, indicating that the aberrant hepatic expression of MAdCAM-1 was functional. Further findings demonstrated that CCL25 could also be aberrantly expressed within the liver, particularly in patients with PSC (increased hepatic CCL25 mRNA levels and positive histological expression).[5] Moreover, up to 20% of PSC hepatic T cells expressed CCR9 compared with relatively low expression in primary biliary cholangitis (PBC) liver and nondiseased liver from organ donors (<2%).[5] It has been well documented that induction of gut tropism (expression of CCR9 and $\alpha4\beta7$) on T lymphocytes requires the presence of retinoic acid (RA).[1] Gut dendritic cells (DCs) are a unique subset of DCs defined by their expression of retinol hydrogenases which metabolize retinol to RA giving them the ability to induce gut tropism on T lymphocyte priming. Elevations in the expression of CCR9 and $\alpha4\beta7$ on hepatic T cells in patients with PSC raised the question whether the ability to induce the expression of gut tropic integrins on T cells could also be induced extraintestinally by liver DCs. Subsequent work reported that liver DCs isolated from normal and PSC livers were not able to induce or maintain expression of this gut tropic phenotype on T cells. Activation by gut DCs, however, did imprint high levels of both CCR9 and $\alpha4\beta7$ on T cells, leading the authors to conclude that hepatic CCR9 and $\alpha4\beta7$ expressing T cells are indeed of gut origin.[6]

Together these data prompted the proposal of a model of enterohepatic lymphocyte circulation in which long-lived gut activated memory T cells recirculate through the liver and are recruited by aberrantly expressed gut adhesion molecules during hepatic inflammation, thereby providing a theoretical mechanistic explanation for the striking association between PSC and IBD, including how these related conditions often display an asynchronous onset and the observation of the former arising even in patients in whom the colon had been removed.

Recent data have challenged this model, with a number of studies reporting hepatic MAdCAM-1 expression and elevations in $\alpha4$ and $\beta7$ gene expression in various nonautoimmune CLDs.[7–9] Furthermore, there is a lack of response of patients with PSC to the anti-$\alpha4\beta7$ treatment vedolizumab reported by several centes, including our own.[10–13]

Here, we appraise the gut lymphocyte homing hypothesis in PSC with associated IBD (PSC-IBD), by characterizing the hepatic expression of MAdCAM-1, CCL25, and E-Cadherin and the expression on both hepatic and peripheral T cells of their cognate receptors $\alpha4\beta7$, CCR9, $\alpha E\beta7$, and of the colonic homing receptor, GPR15, in paired blood and fresh liver tissue samples from patients with PSC-IBD or various other CLDs. Moreover, we define the inflammatory phenotype of gut-derived cells within the liver of patients with PSC-IBD, alcohol-associated liver disease (ALD) and in the normal liver.

PATIENTS AND METHODS

Patients

Explanted liver tissue was collected from 28 patients undergoing OLT for CLD. Nondiseased, age-matched control liver tissue was obtained from unused donor liver after organ reduction or split transplantation ($n = 4$) or from patients undergoing partial hepatectomy for removal of colorectal metastases ($n = 2$). Liver mononuclear cells (LMNC) were isolated from liver tissue from 17 of these patients; NASH ($n = 3$), ALD ($n = 7$), and PSC-IBD ($n = 7$) and from all nondiseased control samples ($n = 6$). Peripheral blood samples were taken at the time of transplantation from 10 patients with CLD (NASH [$n = 3$], ALD [$n = 3$], and PSC-IBD [$n = 4$]) and from 14 age-matched healthy donors. For demographic and clinical details, see Tables S1 and S2. All included

patients provided written informed consent, and this study was approved by the local research ethics committee.

Immunohistochemistry

CLD explanted tissue sections obtained from PBC (*n* = 2), ALD (*n* = 8), NASH (*n* = 6), chronic HCV (*n* = 2), AIH (*n* = 2), PSC-IBD (*n* = 8) and nondiseased liver tissue (*n* = 4) were collected for immunohistochemical analysis. In addition, diagnostic liver biopsy tissue sections obtained from PSC-IBD (*n* = 6), AIH (*n* = 5) and viral hepatitis (*n* = 5) were also collected for comparative MAdCAM-1 immunoreactivity analysis. Samples were formalin fixed, paraffin embedded. Sections were subsequently deparaffinised in xylene, dehydrated in ethanol and rehydrated in water. Following rehydration, sections were incubated overnight at 4°C with rabbit polyclonal anti-human MAdCAM-1 (cat.# ab178549, Abcam), mouse monoclonal anti-human E-Cadherin (cat.# NCL-L-E-Cad, clone # 36B5, Leica) and polyclonal rabbit anti-human CD25 (clone # EPR12388), used at a dilution of 1:500, 1:50, and 1:250, respectively. Following endogenous peroxidase blocking with 3% H_2O_2, sections were incubated with goat anti-rabbit or goat anti-mouse (both from Vector Laboratories) secondary antibodies, used at 1/1.000 and 1/400 respectively, for one hour at room temperature. After treatment with Vectastain Elite ABC kit (Vector Laboratories), ImmPACT DAB (Vector Laboratories) was applied, and sections were examined by light microscopy.

RT-PCR

Expression of *MAdCAM-1*, *CCL25*, and *E-Cadherin* was determined by RT-PCR, as previously described,[14] following total RNA extraction using TRIzol reagent (ThermoFisher Scientific) and mRNA reverse transcription using iScript cDNA synthesis kit (Bio-Rad Laboratories), according to the manufacturer's instructions. The primer sequences were as follows:

MAdCAM-1: Forward, 5′-GTATTGCGCCGCTAGAGGTG-3′; Reverse, 5′-CTGAACGCCACTTGT CCCTC-3′

CCL25: Forward, 5′-TTTGAAGACTGCTGCCTGG-3′; Reverse, 5′-GTCTTCTTCCTAACAAGCC-3′

E-Cadherin: Forward, 5′-CTGATGCTGATGCCCCCAATA-3′; Reverse, 5′-CAGTTTCTGCATCTTGCCAGG-3′

Cell isolation

LMNC were freshly isolated from explanted liver tissue or resected liver tissue according to established methodology.[15] Briefly, approximately, 50-100 g of liver tissue was placed in RPMI 1640 supplemented with 10% fetal bovine serum and placed on ice for up to 4 hours before homogenization. Liver was cut into 1 cm^3 pieces using a sterile No11 scalpel. Tissue cubes were then washed twice with cold RPMI 1640 before being placed into gentleMACS C Tubes (Miltenyi Biotec). C Tubes were then inserted into gentleMACS Tissue Disassociator (Miltenyi Biotec) and homogenized for 1 minute. Tissue homogenate was then diluted in cold RPMI 1640 and passed through a 63μm filter (Fisher Scientific). Homogenate was then further filtered through a 30μm filter (Miltenyi Biotec) before being centrifuged at 50 × *g* (acceleration 5 and deceleration 3) at 20°C in order to separate larger cellular debris. Supernatant was then extracted and layered onto 15 ml Ficoll-Paque density gradient solution (1.077 g/ml) in 50 ml sterile Falcon tube and centrifuged at 800 × g for 20 minutes at 20°C without centrifuge braking enabled. Lymphocyte interface was then aspirated and filtered through a 20 μm filter (pluriSelect) before being centrifuged once more. Cell pellet was then resuspended in 5 ml of RPMI 1640 before cell counting. The viability of freshly isolated LMNCs consistently exceeded 90%.

Flow cytometry

Flow cytometry was performed following incubation of PBMCs and LMNCs with anti-human antibodies against cell surface antigens CD3 (clone # OKT3), CD4 (clone # RPA-T4), CD45RO (clone # UCHL1), CCR9 (clone # L053E8), GPR15 (clone # SA302A10), αE (clone # Ber-ACT8) all from Biolegend and to CD8 (clone # 1G1), CD127 (clone # 11A9), CD25 (clone # M-A251), Integrin β7 (clone # FIB504) and α4 (clone # HIL-7R-M21) from BD Biosciences Systems. Expression of intracellular cytokines was assessed following cell stimulation with eBiosciences cell stimulation cocktail 500X plus protein transport inhibitors at 37°C, 5% CO_2 for 4 hours. Intracellular staining was conducted following cell fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences) and incubation with antibodies to human IFN-γ (clone # 4S.B3), TNF-α (clone # MAB11) and IL-17 (clone # BL168) all from eBioscience. Cell events were acquired on a BD LSRFortessa II and analyzed using FlowJo 3 software (version 10, TreeStar). Positively stained cell populations were gated based on fluorescence-minus-one method. Compensation was adjusted using Ultra Comp eBeads Plus Compensation Beads (Invitrogen).

Statistics

Results are expressed as mean + SEM. Normality of variable distribution was assessed by Kolmogorov-Smirnov goodness-of-fit test. Comparisons were

performed using parametric (paired or unpaired Student t test) or nonparametric (Wilcoxon signed-rank or Mann-Whitney test) tests according to data distribution. One-way ANOVA or Kruskal-Wallis test, followed by Tukey or Dunn multiple comparison tests, were used when comparing more than two sets of data. Statistical significance was defined as a p value <0.05 based on nominal p values, and different levels were noted on graphs: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Correlation between variables was determined by Pearson correlation coefficient, if the data conformed to a normal distribution, or by Spearman rank correlation coefficient, if the data were not normally distributed. p values < 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism, version 7.0a (GraphPad Software, San Diego, CA).

RESULTS

Expression of gut adhesion molecules are elevated in the chronically inflamed liver

MAdCAM-1

Immunohistochemical detection of MAdCAM-1 in human liver samples revealed a variable pattern of expression. Positive MAdCAM-1 immunostaining was found on 6/8 PSC-IBD explanted liver sections but in none of the normal livers (0/4). Marked MAdCAM-1 expression, however, was also detected in 16/20 patients with other CLDs (Table 1). For a significant proportion (19/28) of the MAdCAM-1 positive explanted livers, MAdCAM-1 was detected on the endothelial cell lining of central veins and small vessels, although not all vessels in a given tissue section stained positive, indicating a patchy distribution of the MAdCAM-1 protein (Figure 1). Occasionally, MAdCAM-1 expression was seen within the hepatocyte cytoplasm, as well as in structures that had the morphology of dendritic cells. RT-PCR confirmed the histological findings, as MAdCAM-1 gene expression was significantly up-regulated in CLD compared with normal liver tissue, the highest expression being found in PSC-IBD and ALD tissue (Figure 2A). Despite observing MAdCAM-1 positive immunoreactivity in multiple explanted CLD tissue sections, MAdCAM-1 staining was absent in all diagnostic liver biopsies (PSC-IBD 0/6, AIH 0/5 and chronic viral hepatitis (0/5) analyzed (Figure S1).

TABLE 1 MAdCAM-1 staining in CLD

Disease	Endothelial vessels	Lymphoid aggregates	No staining
PSC-IBD (n = 8)	6/8	0/8	2/8
PBC (n = 2)	1/2	2/2	0/2
ALD (n = 8)	6/8	0/8	2/8
NASH/NAFLD (n = 6)	4/6	0/6	2/6
AIH (n = 2)	1/2	0/2	1/2
HCV (n = 2)	2/2	2/2	0/2
NL (n = 4)	0/4	0/4	4/4

Abbreviations: NL, normal liver.

CCL25

On immunohistochemical staining, CCL25 immunoreactivity was negative in all PSC (n = 7) and all PBC (n = 2) liver tissue sections stained (Figure S2). Despite being unable to detect histological expression of CCL25, RT-PCR demonstrated significant up-regulation in gene expression in explanted CLD tissue compared with normal tissue (Figure 3D). Similarly, to MAdCAM-1, CCL25 gene expression was highest in patients with PSC-IBD and patients with ALD (Figure 3B).

E-Cadherin

Analysis of liver sections of explanted liver tissue stained for E-Cadherin revealed strong immunoreactivity on all cases, with expression confined to hepatocyte and biliary epithelial cell membranes (Figure 2). We did not observe any difference in the staining intensity or pattern between disease groups, nor was there any observed between explanted liver sections and normal liver. Consistent with our histological findings, RT-PCR showed that CDH1 is highly expressed within the liver in CLD (Figure 3D), particularly in patients with PSC-IBD (Figure 3C).

Gut integrin expression is increased on hepatic T cells

Significant increases in the frequency of $\alpha4\beta7$+ expressed on hepatic CD4+ T effector memory cells were observed in PSC-IBD, but also in patients with NASH and ALD compared with normal liver (p < 0.001, Figure 4B) with no differences among CLD groups. We also observed a significant reduction in peripheral $\alpha4\beta7$ expressing CD4+ and CD8+ memory T cells in patients undergoing OLT for CLD compared with healthy controls (Figure 4B, p < 0.01). Despite observing significant increases in hepatic CD4+ T effector memory cells expressing $\alpha4\beta7$ in patients with CLD compared with healthy controls, there were no differences in CD8+ memory T cells between normal liver and CLD (Figure 5A). In contrast to the findings on hepatic $\alpha4\beta7$ expression, we observed significant increases in $\alpha E\beta7$

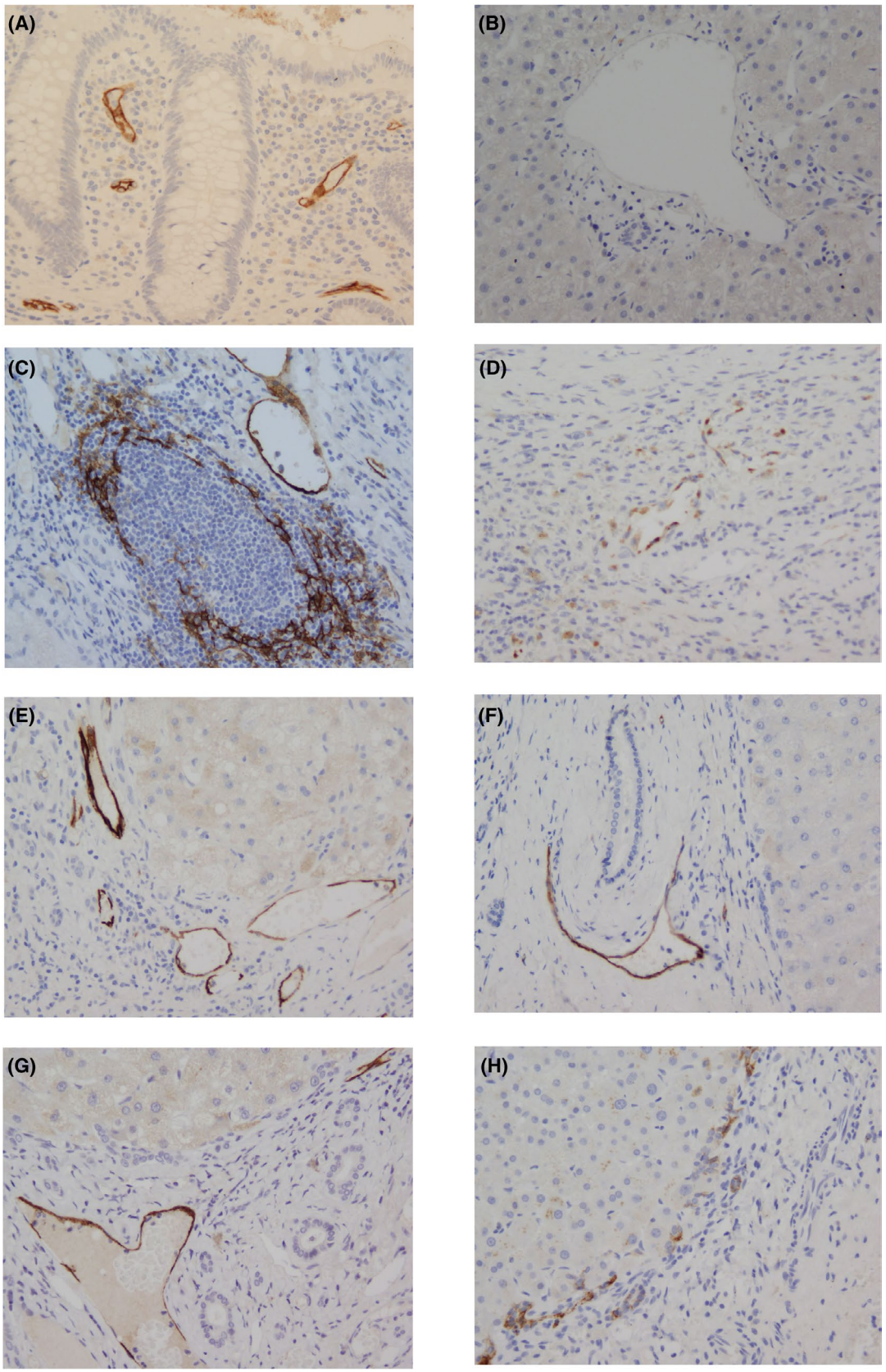

FIGURE 1 Representative MAdCAM-1 immunoreactivity patterns in patients with CLD explants. (A) Colon control tissue; (B) normal liver; (C) chronic hepatitis C; (D) PSC; (E) ALD; (F) PBC; (G) NASH; (H) AIH. Vessel MAdCAM-1 staining is present in panels C-G; lymphoid aggregate MAdCAM-1 staining in panel C (patient with HCV). Similar patterns were also observed in PBC sections). No staining is present in panel A

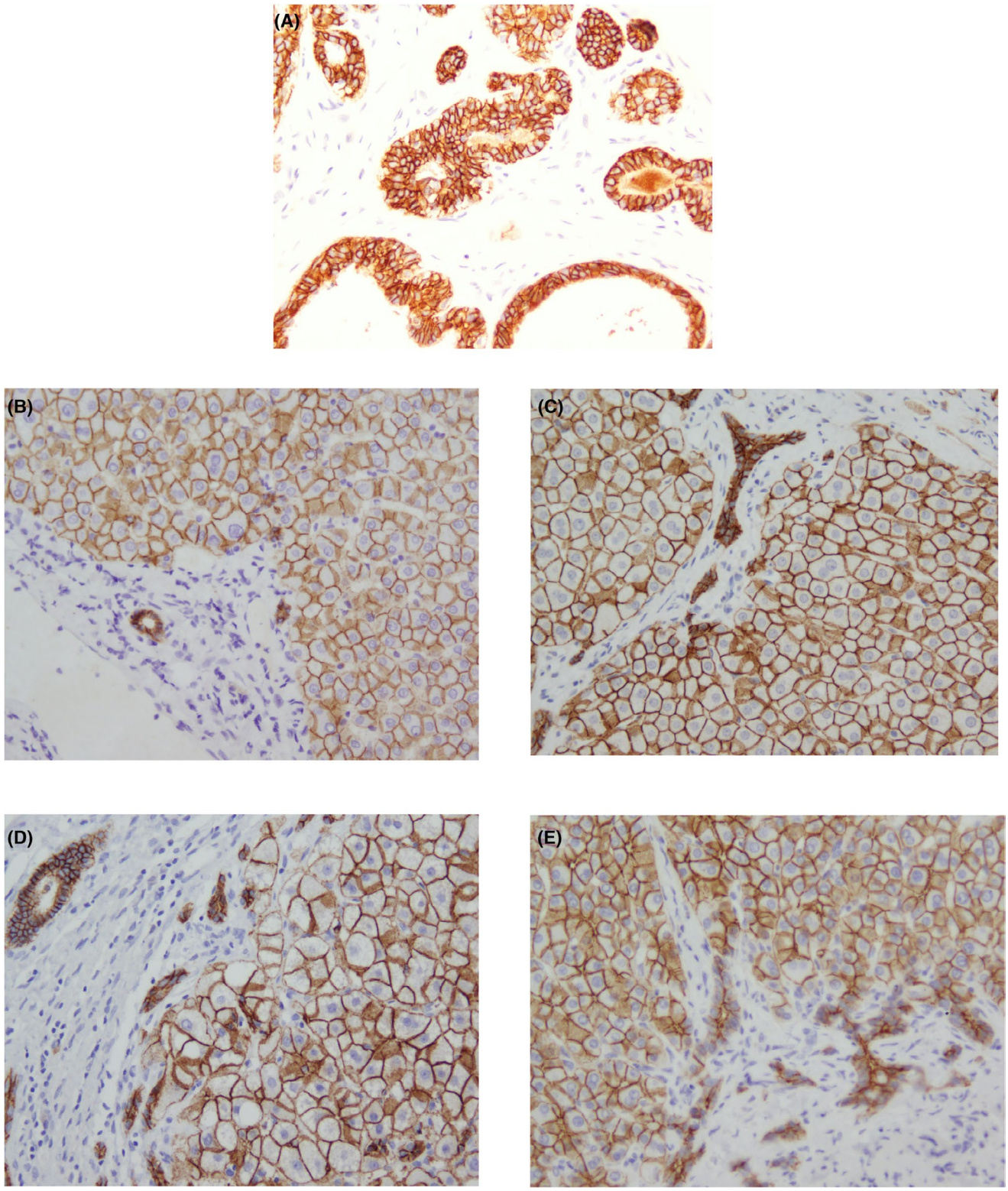

FIGURE 2 E-Cadherin immunoreactivity patterns in control colon tissue, donor liver and in CLD explants. (A) Colon, (B) normal liver, (C) PBC, (D) ALD; (E) PSC. Positive immunostaining with strong membranous expression is present on hepatocytes and cholangiocytes in all sections. The pattern and intensity of expression was consistent throughout all CLD and normal liver sections

expression on CD8+ memory T cells (Figure 5B, $p < 0.01$), but not CD4+ T effector memory cells, in patients with PSC-IBD and ALD when compared with normal liver. On both CD4+ and CD8+ T cells there was a significantly increased expression of CCR9 in patients with PSC-IBD when compared with normal liver (Figures 4D

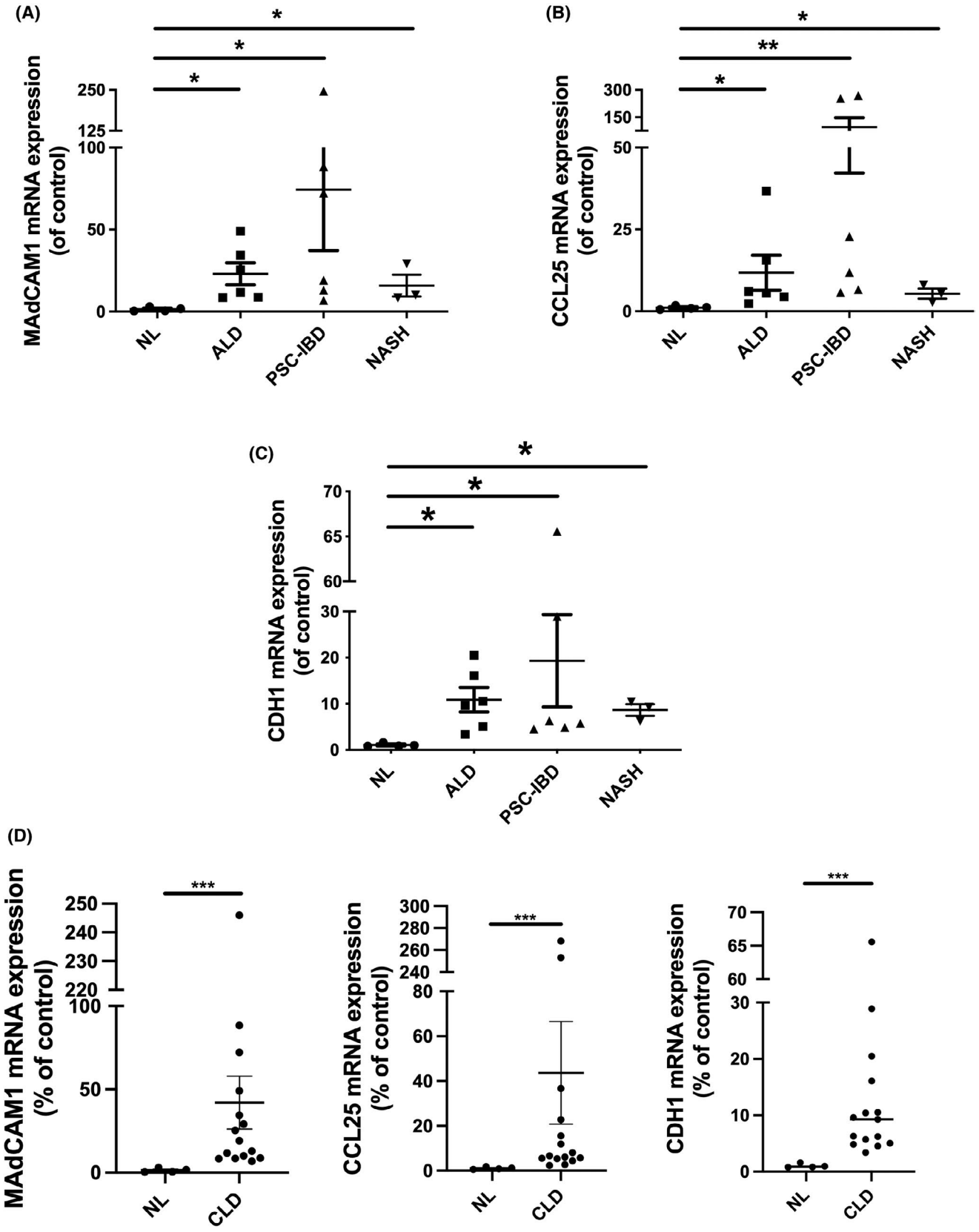

FIGURE 3 Expression of gut adhesion molecules is up-regulated in CLD. (A) MAdCAM-1, (B) CCL25 and (C) CDH1 (E-Cadherin) mRNA expression in normal liver, ALD, PSC-IBD, and NASH. (D) MAdCAM-1 (left), CCL25 (center) and CDH1 (right) mRNA expression in normal liver compared with combined CLD groups (ALD, PSD-IBD, and NASH). Results are expressed as mean ± SEM: Expression is presented as a percentage deviation from the normal liver. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001

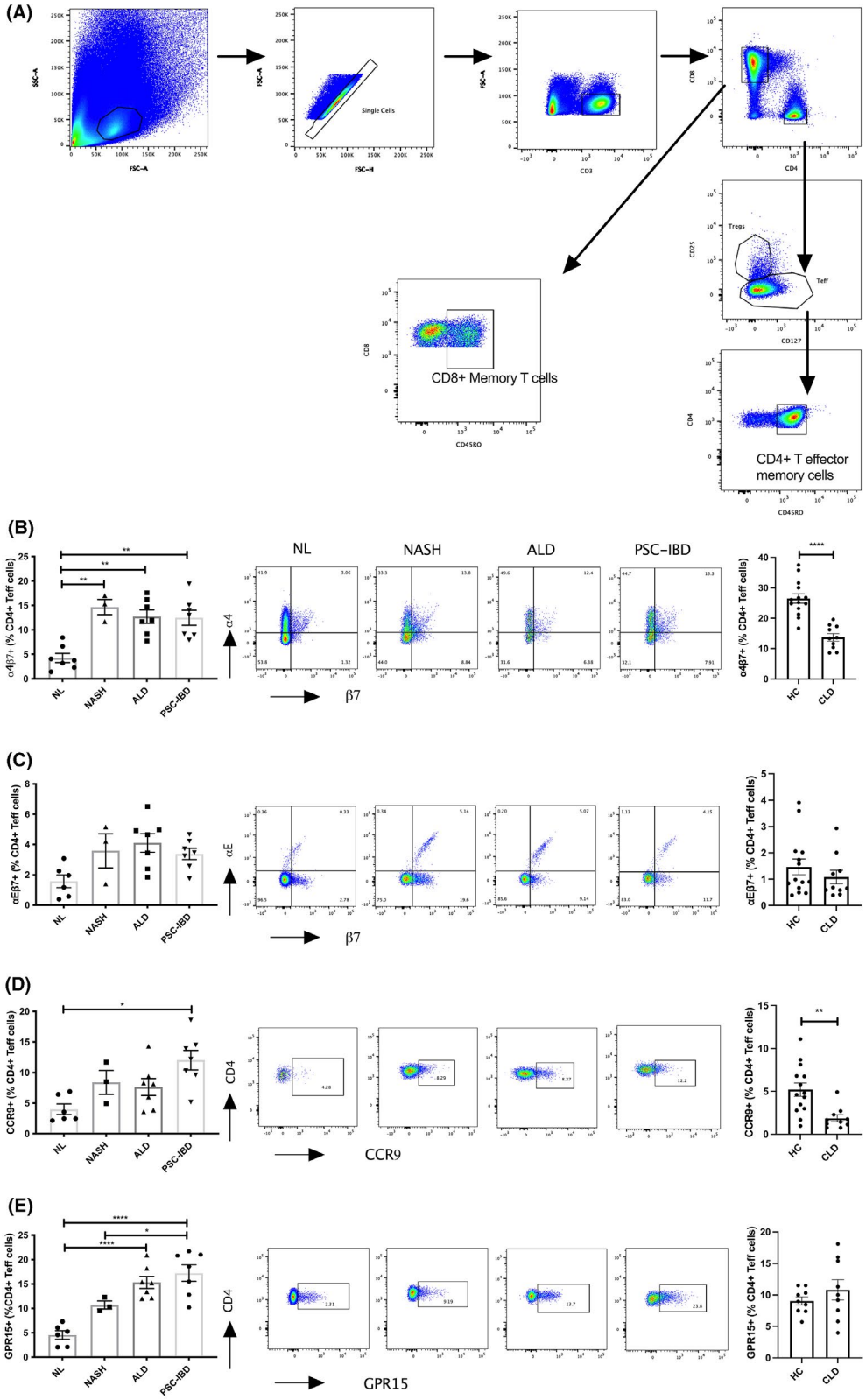

FIGURE 4 Differential expression of gut homing integrins on hepatic and peripheral CD4+ T cells in CLD. (A) Flow cytometry gating strategy for CD4+ T effector memory and CD8+ memory T cells. Left panels: expression of gut homing integrins: (B) α4β7, (C) αEβ7, (D) CCR9 and (E) GPR15 on hepatic CD4+ T cells (numbers expressed as a percentage of CD4+ T effector memory cells). Center panels: Representative flow cytometry plots of gut homing integrins on hepatic CD4+ T cells in normal liver (NL), NASH, ALD, and PSC-IBD (B-E). Right panels (B-E): Differential expression of gut homing integrins on peripheral CD4+ T effector memory cells in NL and patients with CLD. Integrin positive cells were gated on and expressed as a percentage of CD3+CD4+CD127+CD45RO+ cells (T effector memory cells). *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001

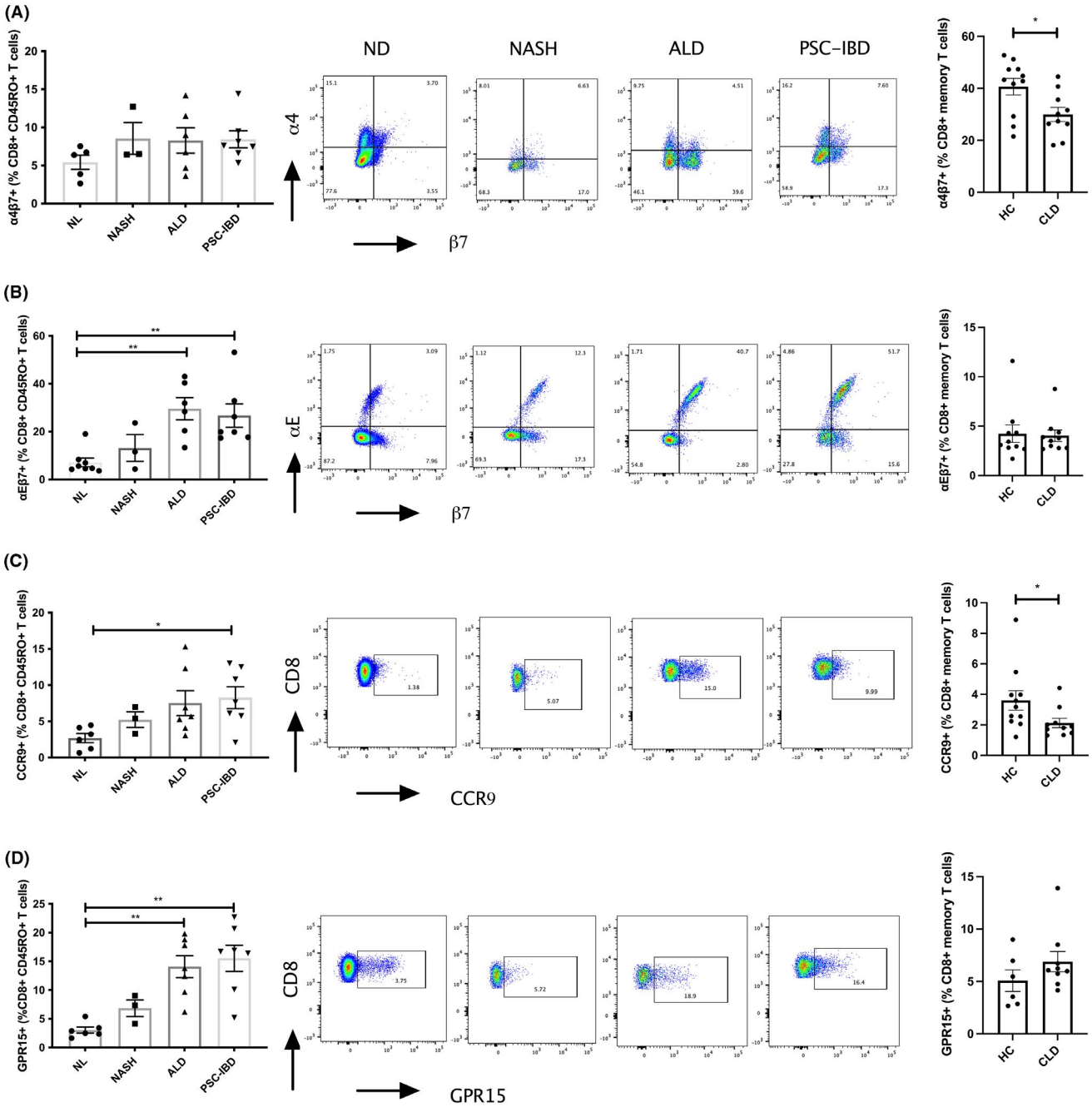

FIGURE 5 Differential expression of gut homing integrins on hepatic and peripheral CD8+ T cells in CLD. Left panels: expression of gut homing integrins: (A) α4β7, (B) αEβ7, (C) CCR9 and (D) GPR15 on hepatic CD8+ T cells (numbers expressed as a percentage of CD8+ memory T cells). Center panels: Representative flow cytometry plots of gut homing integrins and on hepatic CD8+ T cells in normal liver (NL), NASH, ALD, and PSC-IBD. Cells were gated on CD3+CD8+CD45RO+ cells (memory T cells). (B-E) Differential expression of gut homing integrins on peripheral CD8+ memory T cells in patients with HC and patients with CLD. *p ≤ 0.05

and 5C, $p < 0.01$). Although not statistically significant, CCR9 expression on CD4+ and CD8+ T cells was also elevated both in NASH and ALD with comparable levels to patients with PSC-IBD. Peripheral T cells showed significant reductions in CCR9 expression on CD4+ and CD8+ T cells in patients undergoing OLT for CLD compared with healthy controls (Figure 4C, $p < 0.01$).

Like for CCR9 and β7 integrin, the expression of GPR15 was increased on hepatic T cells in CLD. GPR15+ T cells were found within the hepatic infiltrate with level of expression comparable between CD4+ and CD8+ T cells for all disease groups and normal liver tissue (Figures 4E and 5D, $p < 0.01$, $p < 0.0001$). In patients with ALD and PSC-IBD there was significantly increased GPR15 expression on both CD4+ and CD8+ T cells compared with normal liver tissue. The level of expression was significantly greater in patients with PSC-IBD on CD4+ T cells when compared with NASH.

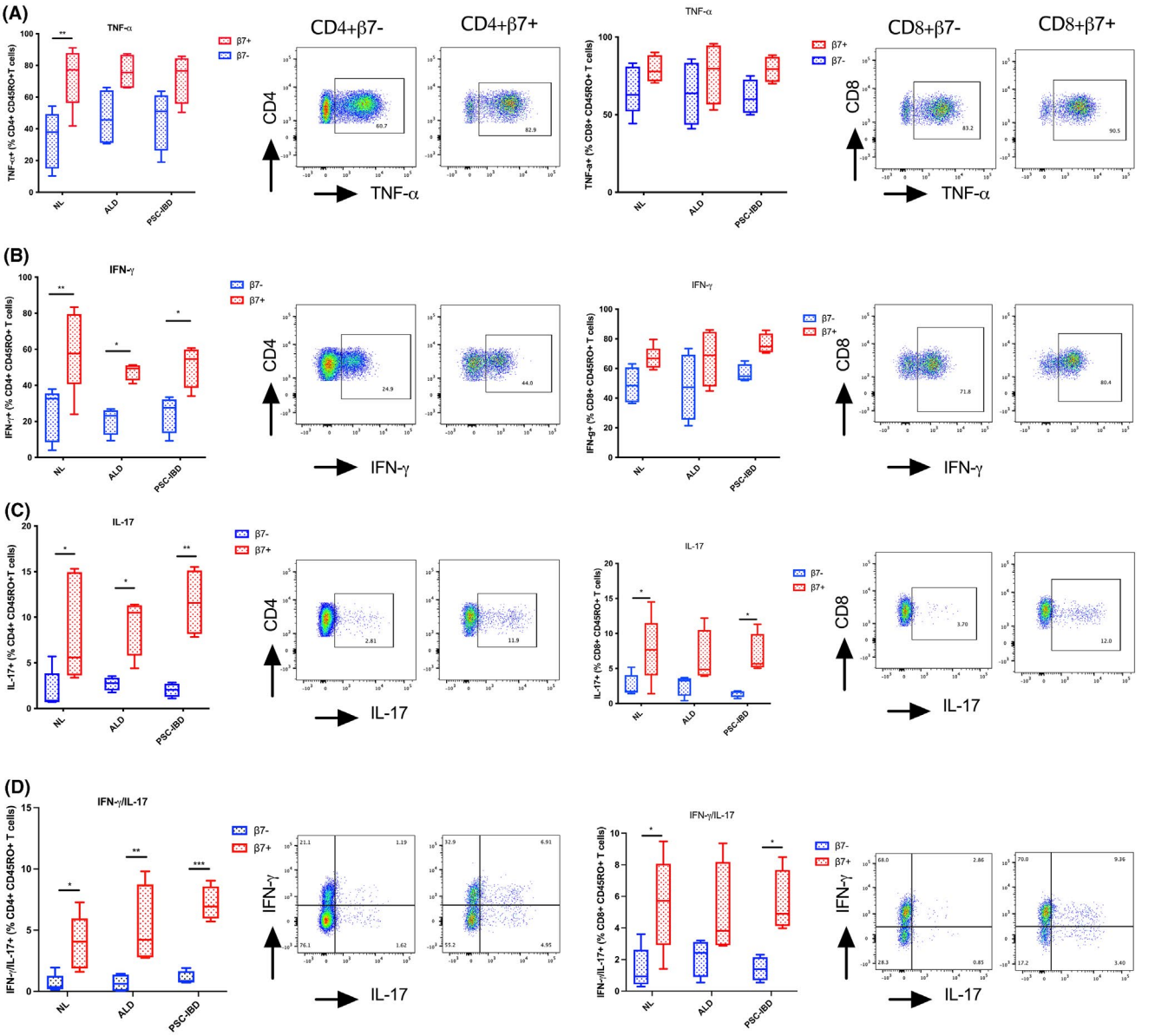

FIGURE 6 Gut-derived hepatic infiltrating memory T cells are associated with increased Th1 and Th17 cytokine production. Expression of proinflammatory cytokines TNF-α (A), IFN-γ (B), IL-17 (C) and dual IFN-γ/IL-17 (D) and representative flow cytometry analyses between gut-associated CD4+CD45RO+β7- and CD4+CD45RO+β7+ hepatic T cells (Left Panels) and CD8+CD45RO+β7- and CD8+CD45RO+β7+ hepatic T cells (Right Panels). Cytokines are expressed as a percentage of CD4+/CD8+CD45RO+β7-/β7+ hepatic T cells. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Although, not significant, we also observed a trend toward increased expression on CD4+ and CD8+ T cells in patients with NASH compared with normal liver.

Hepatic infiltrating β7 expressing T cells display an increased proinflammatory phenotype

We observed increased expression of TNF-α, IFN-γ, IL-17 and IFN-γ/IL-17 in hepatic memory CD4+ and CD8+ β7+ T cells when compared with hepatic β7- T cells (Figure 6A-D). This increased inflammatory phenotype was more marked within CD4+ T cells with significant increases observed in IFN-γ, IL-17, and IFN-γ/IL-17 expression in β7+ versus β7- T cells (Figure 6C-D). Despite the presence of IBD and cirrhosis, there were no differences in the inflammatory phenotype of CD4+ or CD8+ T cells between PSC-IBD and ALD when compared with normal liver.

DISCUSSION

Our data, demonstrating that expression of both MAdCAM-1 and CCL25 is up-regulated not only in the

liver of patients with PSC-IBD, but also in the liver of those with other CLDs, challenge the long-standing hypothesis formulated by Adams' group that the pathogenesis of PSC-IBD is mediated by enterohepatic T cell trafficking from the gut to the liver.[4–6,16] In the original report, it had been postulated that an aberrant upregulation in hepatic expression of the gut specific adhesion molecules MAdCAM-1 and CCL25 was a feature largely confined to patients with PSC-IBD, supporting the recruitment of pathogenic mucosal T cells.

Early studies did report a strong expression of MAdCAM-1 and CCL25 in the PSC liver with minimal expression in other CLDs, lending support to the gut homing theory in the pathogenesis of PSC,[4,5] but more recent reports challenged this notion, as hepatic MAdCAM-1 expression was observed in multiple CLDs.[2,7,9] Consistent with the latter data are our findings in the present study, which investigates hepatic expression of both MAdCAM-1 and CCL25 and quantifies expression of their cognate receptors CCR9 and α4β7, in addition to other colonic homing receptors αEβ7 and GPR15, on matched blood and tissue infiltrate in a sizeable group of patients with CLDs, including PSC-IBD. Not only do we describe positive hepatic MAdCAM-1 immunoreactivity in more than 75% of patients with CLD regardless of etiology, but we also show an increased expression of both CCL25 and MAdCAM-1 mRNA across all CLD groups compared with normal liver. In addition, by investigating paired tissue and blood samples, we show that expression of hepatic MAdCAM-1 and CCL25 induces tissue infiltration of α4β7 and CCR9 expressing CD4+ T cells, which is not confined to PSC-IBD, but is present also in other CLDs, with a concomitant reduction in peripheral frequencies.

The importance of investigating not only patients with PSC, but also with other liver diseases at comparable stages of disease is illustrated by the discrepant reports in the literature. Henriksen et al. reported memory T cells of common clonal origin in the gut and liver tissue of patients with PSC, seemingly supporting the gut homing theory,[16] but no other CLDs were investigated as controls, questioning the PSC specificity of their finding. More recently, a study did show elevations in hepatic MAdCAM-1 expression in patients with PSC-IBD referred for liver transplantation compared with controls (PBC and HCV).[17] However, control samples were biopsies not taken at a comparable stage of disease being either diagnostic or resected samples, whereas comparisons between "short-term" PSC, defined as disease duration of less than one year revealed no differences in MAdCAM-1 expression indicating the stage of disease is a strong determinate of expression. Supporting this notion, on analysis of diagnostic liver biopsies we failed to identify MAdCAM-1 immunoreactivity in PSC-IBD and other CLDs, indicating aberrant hepatic MAdCAM-1 expression likely occurs in the later stages of CLD thereby, does not mediate hepatic inflammation through gut lymphocyte recruitment in the early stages of disease. In further agreement with our data, expression of MAdCAM-1 and α4 and β7 integrins were shown to be up-regulated in the liver of patients with late-stage NASH[9]; moreover hyperexpression of α4, αE and β7 integrins was reported in the liver of patients with severe alcohol-associated hepatitis compared with normal tissue.[8] A recent report also showed no differences between PSC and other liver disease controls in CCR9 or β7 integrin gene expression in hepatic lymphocytes isolated by fine needle aspirate or in the expression of CCR9 on CD3+ cells in liver needle biopsies,[18] in line with our observation that MAdCAM-1/α4β7 and CCL25/CCR9 mediated hepatic recruitment of T cells is not unique to PSC-IBD.

To further understand whether gut-derived T cell homing to the liver is a common feature of CLD, we investigated the hepatic expression of the homing receptor GPR15 and of αEβ7 integrin, which control trafficking and retention of T effector cells to the colon.[19,20] As IBD in patients with PSC is characterized predominantly by colonic inflammation, it is plausible to speculate that the hepatic PSC infiltrate should be rich in GPR15 and αEβ7 expressing lymphocytes, should the gut homing theory prove true. Indeed, frequencies of both GPR15 and αEβ7 expressing T cells were increased in the PSC-IBD liver compared with normal liver, but as for CCR9 and α4β7 expressing T cells, elevations were also observed in other CLDs. In particular, the frequency of αEβ7 expressing CD8+ T cells was high across disease groups, possibly because of the increased CDH1 gene expression observed by RT-PCR. An alternative possibility that should be addressed in future studies, is the role of transforming growth factor-beta (TGF-β), which participates in all stages of liver disease progression, from initial injury through inflammation and fibrosis, to cirrhosis and cancer,[21] in down-regulating α4 integrin and up-regulating αE integrin, CD8+ T cells being particularly responsive to TGF-β-mediated αE induction.[22,23] To our knowledge, there are no published data on GPR15, and this study investigates its expression within the liver. However, a limitation of this work is the absence of data concerning the expression of its cognate receptor GPR15L, whose expression is reported to be absent or very weak in the human liver.[24,25] It could be suggested that GPR15+ cells infiltrating the liver may have been recruited through α4β7/MAdCAM-1 interactions, because GPR15 cells enriched in the colon may also express α4β7. However, we found that <20% of GPR15+ T cells expressed α4β7 (data not shown), suggesting that GPR15+ T cells are likely to be recruited to the liver by hepatic expressed GPR15L, which similarly to other adhesion molecules, is up-regulated in the liver in response to inflammation.[7,26–28] The dense infiltration of both GPR15+ CD4+ and CD8+ T cells we observed

in the cirrhotic tissue suggests that GPR15L is indeed expressed within the liver, but the distribution and functional role of hepatic GPR15/GPR15L interactions in CLD require further investigation.

Expression of αEβ7 has been shown to characterize a more proinflammatory subset of CD4+ T cells within the inflamed UC colon.[19] It is thereby possible that the hepatic β7 expressing T cells derived from an inflamed bowel, as is the case in patients with PSC-IBD, could display a similarly enhanced inflammatory phenotype. We, therefore, investigated the inflammatory profile of hepatic β7+ versus β7- T cells in CLD with IBD (PSC-IBD), without IBD (ALD) and normal liver. In all conditions, expression of β7 was associated with increased levels of Th1 (IFN-γ, TNF-α) and Th17 (IL-17) cytokines in both CD4+ and CD8+ memory T cells compared with β7- T cells, but the presence of IBD or CLD did not influence type and density of cytokine expression in hepatic β7+ T cells, with normal liver displaying a cytokine profile similar to patients with PSC-IBD and patients with ALD. This, however, does not exclude an increased tissue inflammatory environment in patients with or without concomitant IBD compared with health, as we have not quantified the relative abundance of β7 expressing T cells within the different CLD livers. It is possible that the increased expression of MAdCAM-1 in the diseased liver is accompanied by the recruitment of a high number of α4β7+ T cells and therefore enhanced inflammation. An increase in hepatic α4 and β7 integrin gene expression, has indeed been reported in both ALD and NASH,[8,9,18] and increases in intestinal MAdCAM-1 expression are associated with infiltration of β7+ T cells in the gut of patients with IBD.[19,23,29] Whether hepatic MAdCAM-1 mediated recruitment of inflammatory β7 expressing T cells contributes to fibrogenesis and disease progression in CLD remains to be defined. In this context it is of interest that in murine models of both NASH and concanavalin A induced hepatitis, MAdCAM-1 ablation resulted in reduced oxidative stress and inflammation,[30,31] and that in the NASH model, blockade of α4β7 attenuated hepatic inflammation and fibrosis,[9] implicating a pathogenic role for hepatic MAdCAM-1/α4β7 mediated cell recruitment in CLD. Further understanding of the mechanisms and in particular, identification of the time of hepatic MAdCAM-1 induction throughout the course of CLD could pave the way for the use of targeted anti-integrin therapies, as used in IBD, with the aim of blocking hepatic MAdCAM-1/α4β7 mediated cell recruitment and inhibiting the progression of CLD.

In summary, we have demonstrated that aberrant hepatic expression of MAdCAM-1 and CCL25 and subsequent infiltration of α4β7 and CCR9 expressing T cells into the liver is a common feature of CLD, questioning previous reports suggesting that this phenomenon is confined to PSC-IBD. The functional relevance of gut-derived hepatic infiltrating T cells displaying an inflammatory phenotype and their role in the progression of liver damage in CLD with or without IBD remains unknown and requires further investigation.

CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

Jonathon J. Graham: acquisition of data; analysis and interpretation of data; statistical analysis; drafting of the manuscript. Sujit Mukherjee, Rebeca Sanabria Mateos, Muhammad Yuksel, Zhenlin Huang, Tengfei Si, Xiaohong Huang: acquisition of data. Yoh Zen, Hadil Abu Arbouq: analysis and interpretation of data. Vishal Patel, Mark McPhail, Nigel Heaton, Michael Heneghan: Patient recruitment and consent. Rodrigo Liberal, Maria Serena Longhi, Diego Vergani, Giorgina Mieli-Vergani, Yun Ma, Bu'Hussain Hayee: critical revision of the manuscript. Diego Vergani, Giorgina Mieli-Vergani, Bu'Hussain Hayee: obtained funding.

ETHICS APPROVAL

This study was approved by the local research ethic committee (LREC (15/LO/0363)) and was performed in accordance with the Declaration of Helsinki.

ORCID

Jonathon J. Graham https://orcid.org/0000-0003-1637-7024
Vishal Patel https://orcid.org/0000-0001-6616-3628

REFERENCES

1. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. Immunity. 2004;21(4):527–38.
2. Hillan KJ, Hagler KE, MacSween RN, Ryan AM, Renz ME, Chiu HH, et al. Expression of the mucosal vascular addressin, MAdCAM-1, in inflammatory liver disease. Liver. 1999;19(6):509–18.
3. Ponsioen CY, Kuiper H, Ten Kate FJ, van Milligen de Wit M, van Deventer SJ, Tytgat GN. Immunohistochemical analysis of inflammation in primary sclerosing cholangitis. Eur J Gastroenterol Hepatol. 1999;11(7):769–74.
4. Grant A, Lalor PF, Hübscher SG, Briskin M, Adams DH. MAdCAM-1 expressed in chronic inflammatory liver disease supports mucosal lymphocyte adhesion to hepatic endothelium (MAdCAM-1 in chronic inflammatory liver disease). Hepatology. 2001;33(5):1065–72.
5. Eksteen B, Grant AJ, Miles A, Curbishley SM, Lalor PF, Hübscher SG, et al. Hepatic endothelial CCL25 mediates the recruitment of CCR9 $^{+}$ gut-homing lymphocytes to the liver in primary sclerosing cholangitis. J Exp Med. 2004;200(11):1511–7.
6. Eksteen B, Mora JR, Haughton EL, Henderson NC, Lee-Turner L, Villablanca EJ, et al. Gut homing receptors on CD8 T cells are retinoic acid dependent and not maintained by liver dendritic or stellate cells. Gastroenterology. 2009;137(1):320–9.
7. Ala A, Brown D, Khan K, Standish R, Odin JA, Fiel MI, et al. Mucosal addressin cell adhesion molecule (MAdCAM-1) expression is upregulated in the cirrhotic liver and immunolocalises to the peribiliary plexus and lymphoid aggregates. Dig Dis Sci. 2013;58(9):2528–41.
8. Riva A, Patel V, Kurioka A, Jeffery HC, Wright G, Tarff S, et al. Mucosa-associated invariant T cells link intestinal immunity

with antibacterial immune defects in alcoholic liver disease. Gut. 2018;67(5):918–30.
9. Rai RP, Liu Y, Iyer SS, Liu S, Gupta B, Desai C, et al. Blocking integrin α4β7-mediated CD4 T cell recruitment to the intestine and liver protects mice from western diet-induced non-alcoholic steatohepatitis. J Hepatol. 2020;73(5):1013–22.
10. Christensen B, Micic D, Gibson PR, Yarur A, Bellaguarda E, Corsello P, et al. Vedolizumab in patients with concurrent primary sclerosing cholangitis and inflammatory bowel disease does not improve liver biochemistry but is safe and effective for the bowel disease. Aliment Pharmacol Ther. 2018;47(6):753–62.
11. Tse CS, Loftus EV, Raffals LE, Gossard AA, Lightner AL. Effects of vedolizumab, adalimumab and infliximab on biliary inflammation in individuals with primary sclerosing cholangitis and inflammatory bowel disease. Aliment Pharmacol Ther. 2018;48(2):190–5.
12. Arijs I, De Hertogh G, Lemmens B, Van Lommel L, de Bruyn M, Vanhove W, et al. Effect of vedolizumab (anti-α4β7-integrin) therapy on histological healing and mucosal gene expression in patients with UC. Gut. 2018;67(1):43–52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27802155
13. Pavlidis P, Graham J, Gulati S, Dubois P, Heneghan M, Joshi D, et al. Letter: vedolizumab for autoimmune liver disease associated inflammatory bowel disease. Aliment Pharmacol Ther. 2018;47(10):1422–3.
14. Liberal R, Grant CR, Yuksel M, Graham J, Kalbasi A, Ma Y, et al. Regulatory T-cell conditioning endows activated effector T cells with suppressor function in autoimmune hepatitis/autoimmune sclerosing cholangitis. Hepatology. 2017;66(5):1570–84.
15. Liaskou E, Jeffery LE, Trivedi PJ, Reynolds GM, Suresh S, Bruns T, et al. Loss of CD28 expression by liver-infiltrating T cells contributes to pathogenesis of primary sclerosing cholangitis. Gastroenterology. 2014;147(1):221–32.e7.
16. Henriksen EKK, Jørgensen KK, Kaveh F, Holm K, Hamm D, Olweus J, et al. Gut and liver T-cells of common clonal origin in primary sclerosing cholangitis-inflammatory bowel disease. J Hepatol. 2017;66(1):116–22.
17. de Krijger M, Visseren T, Wildenberg ME, Hooijer GKJ, Verstegen MMA, van der Laan LJW, et al. Characterization of gut-homing molecules in non-endstage livers of patients with primary sclerosing cholangitis and inflammatory bowel disease. J Transl Autoimmun. 2020;3:100054.
18. Lynch K, Curion F, Yeung H-Y, Fryer E, Slater A, Ferry H, et al. PS-127-Analysis of liver infiltrating lymphocytes in primary sclerosing cholangitis by surface antigen and single cell RNAseq. J Hepatol. 2019;70(1):e78–9.
19. Lamb CA, Mansfield JC, Tew GW, Gibbons D, Long AK, Irving P, et al. αEβ7 integrin identifies subsets of pro-inflammatory colonic CD4+ T lymphocytes in ulcerative colitis. J Crohns Colitis. 2017;11(5):610–20.
20. Nguyen LP, Pan J, Dinh TT, Hadeiba H, O'Hara E, Ebtikar A, et al. Role and species-specific expression of colon T cell homing receptor GPR15 in colitis. Nat Immunol. 2015;16(2):207–13.
21. Fabregat I, Moreno-Càceres J, Sánchez A, Dooley S, Dewidar B, Giannelli G, et al. TGF-β signalling and liver disease. FEBS J. 2016;283(12):2219–32.
22. Casey KA, Fraser KA, Schenkel JM, Moran A, Abt MC, Beura LK, et al. Antigen-independent differentiation and maintenance of effector-like resident memory T cells in tissues. J Immunol. 2012;188(10):4866–75.
23. Zundler S, Schillinger D, Fischer A, Atreya R, López-Posadas R, Watson A, et al. Blockade of αeβ7 integrin suppresses accumulation of CD8 + and Th9 lymphocytes from patients with IBD in the inflamed gut in vivo. Gut. 2017;66(11):1936–48.
24. Suply T, Hannedouche S, Carte N, Li J, Grosshans B, Schaefer M, et al. A natural ligand for the orphan receptor GPR15 modulates lymphocyte recruitment to epithelia. Sci Signal. 2017;10(496):eaal0180.
25. Ocón B, Pan J, Dinh TT, Chen W, Ballet R, Bscheider M, et al. A mucosal and cutaneous chemokine ligand for the lymphocyte chemoattractant receptor GPR15. Front Immunol. 2017;8:1111.
26. Lalor PF, Edwards S, McNab G, Salmi M, Jalkanen S, Adams DH. Vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. J Immunol. 2002;162(2):983–92.
27. Salmi S, Jalkanen DH, Adams PF, Lalor S, Edwards G, Mcnab M, et al. Cells lymphocytes on human hepatic endothelial adhesion and transmigration of vascular adhesion protein-1 mediates vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. J Immunol. 2002;169(2):983–92.
28. Eksteen B, Miles A, Curbishley SM, Tselepis C, Grant AJ, Walker LSK, et al. Epithelial inflammation is associated with CCL28 production and the recruitment of regulatory T cells expressing CCR10. J Immunol. 2006;177(1):593–603.
29. Fischer A, Zundler S, Atreya R, Rath T, Voskens C, Hirschmann S, et al. Differential effects of α4β7 and GPR15 on homing of effector and regulatory T cells from patients with UC to the inflamed gut in vivo. Gut. 2016;65(10):1642–64.
30. Drescher HK, Schippers A, Clahsen T, Sahin H, Noels H, Hornef M, et al. β7-integrin and MAdCAM-1 play opposing roles during the development of non-alcoholic steatohepatitis. J Hepatol. 2017;66(6):1251–64.
31. Schippers A, Hübel J, Heymann F, Clahsen T, Eswaran S, Schlepütz S, et al. MAdCAM-1/α4β7 integrin-mediated lymphocyte/endothelium interactions exacerbate acute immune-mediated hepatitis in mice. Cell Mol Gastroenterol Hepatol. 2020;11(4):1227–50.e1.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Graham JJ, Mukherjee S, Yuksel M, Sanabria Mateos R, Si T, Huang Z, et al. Aberrant hepatic trafficking of gut-derived T cells is not specific to primary sclerosing cholangitis. Hepatology. 2022;75:518–530. https://doi.org/10.1002/hep.32193