ORIGINAL ARTICLE

Interleukin (IL)-17/IL-22-Producing T cells Enriched Within the Liver of Patients with Chronic Hepatitis C Viral (HCV) Infection

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Abstract

Background Effector CD4+ helper T cells have historically been classified into T helper 1 (Th1) and Th2 based on the production of signature cytokines. The recently identified interleukin (IL)-17 cytokine family plays important roles in host immunity against intracellular pathogens and in chronic inflammatory conditions; data have implicated IL-17 in autoimmune and viral liver disease.

Methods This study used three patient groups with HCV infection: acute HCV who either cleared spontaneously or became chronically infected ($n = 12$), endstage liver disease from whom both peripheral and intrahepatic lymphocytes were studied directly ex vivo ($n = 11$), and 134 patients with different stages of HCV-related fibrosis from whom serum was collected concurrently with liver biopsy. Normal healthy subjects ($n = 41$) served as controls.

Results Acute HCV was not associated with expansion of either CD4+ or CD8+ T cells producing IL-17 (Th17, Tc17) or IL-22, and frequencies did not differ in the blood of patients who cleared versus became persistently infected. The hepatic compartment of chronic HCV patients demonstrated statistically more CD4+ and CD8+ that produced IL-17, IL-22 or both as compared to peripheral blood. These T cells displayed a distinct phenotypic profile, high expression of the homing receptor CD161 and low levels of inhibitory receptors, mucin-domain-containing-molecule-3 (Tim-3) and programmed-death 1. Using a sensitive ELISA, we found no significant differences in serum levels of IL-17 according to HCV-related fibrosis.

Conclusions In chronic HCV, T cells producing IL-17/IL-22 may home to the liver; however, circulating levels of IL-17 do not correlate with fibrosis.

Keywords HCV · Liver · Th17 · IL22 · Fibrosis

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Introduction

Effector CD4+ T helper T cells have historically been classified into T helper 1 (Th1) and T helper 2 (Th2) cells based on the production of signature cytokines, e.g., IFN-γ for Th1 and interleukin-(IL-4) and IL-13 for Th2 effectors [1]. The recently identified interleukin-17 (IL-17) family of cytokines plays important roles in the host immunity against intracellular pathogens and in chronic inflammatory conditions, such as rheumatoid arthritis, psoriasis, and multiple sclerosis [2], and has been recently suggested as an important factor in the pathogenesis of primary biliary cirrhosis [3], autoimmune hepatitis [4], and hepatitis B [5]. Data from other studies indicate that plasma levels of IL-17 are elevated in patients with alcoholic hepatitis and cirrhosis [6], and that the serum levels of IL-17 and frequency of Th17 cells in peripheral blood increase as disease progresses in hepatitis B [5, 7], implicating these cells in the

pathogenesis of liver injury. The Th17 lineage of CD4+ T cells produces effector molecules that include IL-17A, IL-17F, IL-21, and IL-22. In addition, CD8+ T cells (Tc17), natural killer cells, monocytes, and $\gamma\delta$ T cells can produce these cytokines [8]. Furthermore, the IL-17 receptor is ubiquitously expressed, indicating that IL-17 can act on a broad range of cellular targets.

Hepatitis C virus (HCV) infection affects an estimated 180 million people globally, and is a leading cause of chronic hepatitis, cirrhosis, liver cancer, and primary indication for liver transplantation in the Western world [9, 10]. Very limited data exist on the potential role of IL-17-producing cells in HCV infection. Rowan et al. [11] identified HCV-specific Th17 cells that were suppressed by TGF-β and IL-10 derived from HCV NS4 protein-treated monocytes. More recently, Tc17 (IL-17 producing CD8+ T cells) were found to be enriched within the liver of HCV-positive patients (as well as the livers of patients with nonalcoholic steatohepatitis); these Tc17 cells expressed high levels of CD161, an important marker of tissue-homing populations at diverse sites [12].

Based on these collective and emerging data, the current study had several aims: (1) determine whether the frequency of Th17 and Tc17 cells in acute HCV infection predicts spontaneous recovery or persistence, (2) comprehensively characterize the phenotype of intrahepatic CD4+ and CD8+ that produce IL-17 directly ex vivo from the livers of HCV-infected patients and compare to the peripheral compartment, and (3) determine whether serum levels of IL-17 are associated with fibrosis stage in chronic HCV infection.

Methods

Study Population

The study protocol was approved by the University of Colorado Institutional Review Board, and each subject gave informed consent. The study population utilized was comprised of three groups of HCV-infected subjects. Group 1 consisted of 12 patients with acute HCV infection, 6 of whom never became jaundiced (serum total bilirubin <2.0 mg/dl) and remained persistently infected with a 12-month follow-up and 6 of whom became jaundiced (serum total bilirubin >4.0 mg/dl) and spontaneously eradicated HCV RNA without antiviral therapy. As described previously [13], these acutely infected patients were identified by the following 2 methods: (1) a positive HCV antibody or HCV RNA polymerase chain reaction (PCR) assay in a participant with a documented negative anti-HCV antibody test result within the past year, and (2) a positive anti-HCV antibody assay in a participant with clinical hepatitis, detectable serum HCV RNA level, serum alanine aminotransferase level >10 times

the upper limit of normal, and negative test results for hepatitis B surface antigen and hepatitis A immunoglobulin M antibody. Twelve healthy subjects negative for hepatitis C and human immunodeficiency-1 (HIV) antibody were used as controls. The acute patients who resolved infection (acute to resolve), the acute patients who went on to be chronically infected (acute to chronic), and the normal healthy subjects (normal) patients were matched by age, gender, and HCV genotype when available. Group 2 was comprised of 11 patients with HCV-related end-stage liver disease who underwent liver transplantation and from whom intrahepatic lymphocytes and peripheral blood mononuclear cells were studied directly ex vivo. Group 3 (used to estimate circulating serum IL-17 levels) were comprised of 163 patients with a wide range of HCV-related fibrosis stages. Serum samples were separated and stored at −80°C until analysis. The matching liver biopsies were graded and fibrosis was staged 0–4, according to the Batts-Ludwig system [14].

### Sample Preparation and Storage

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll (Amersham Biosciences, Piscataway, NJ, USA) density gradient centrifugation or cellular preparation tubes (Becton–Dickinson, Franklin Lakes, NJ, USA; anticoagulant sodium citrate). PBMCs were viably frozen in 80% FBS (BioWhittaker, Walkersville, MD, USA), 10% DMSO, and 10% RPMI 1640 Media (Life Technologies, Grand Island, NY, USA) in liquid nitrogen for subsequent analyses. Hepatic mononuclear cells were isolated from explanted liver tissue at time of liver transplantation for HCV-related liver disease.

Cell Stimulation

Cells were thawed at 37°C and placed into RPMI media plus 10% human serum andplated in 24-well ultra low adherence plates at a concentration of 5 million cells per ml. Each well was stimulated with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml Ionomycin for 5 h. Golgistop (BD Biosciences) was added to each well at the beginning of the stimulation based on the manufacturer's suggested concentration. After 5 h, cells were harvested from the plate and washed twice with 2 ml phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.01% sodium azide (Facs Wash).

Antibodies for Analysis of Cell Surface and Intracellular Antigen Expression/FACS Analysis

Multiparameter flow cytometry was performed using a BD FACSCanto II instrument (BD Biosciences) compensated with single fluorochromes and analyzed using Diva™
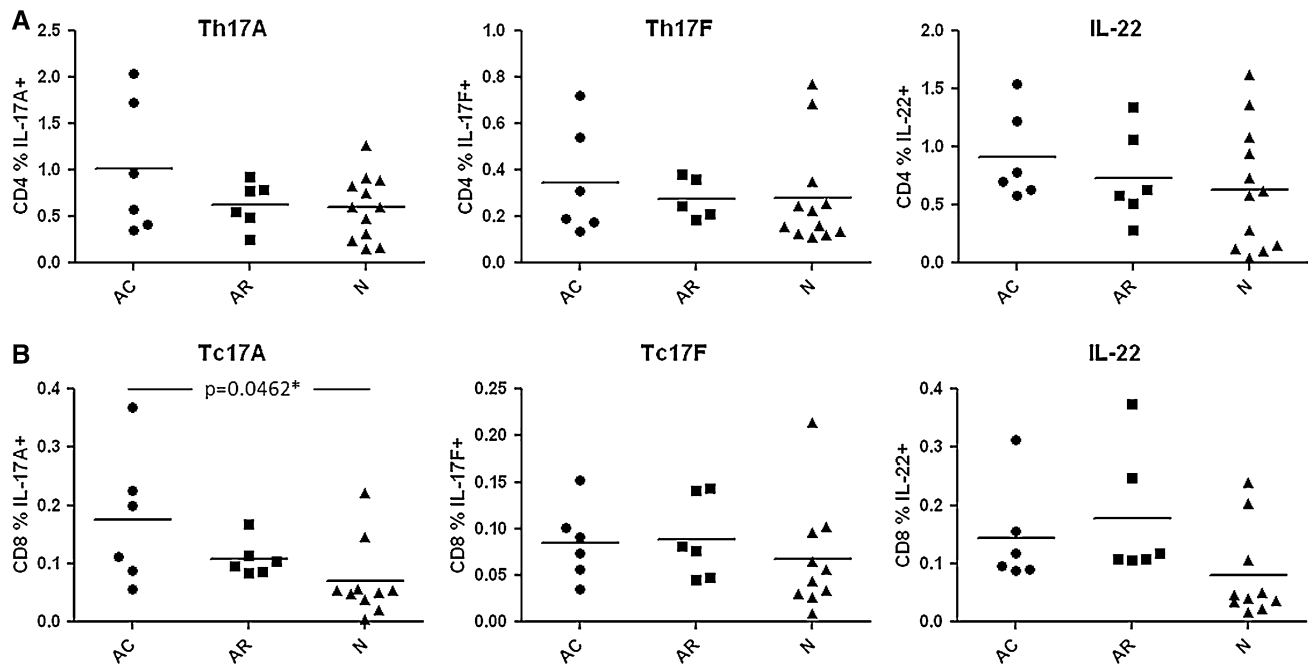
Fig. 1 The virologic outcome of acute HCV infection does not correspond to the frequency of circulating IL-17A-, IL-17F-, or IL-22-expressing T cells. **a** Peripherally circulating IL-17A-, IL-17F-, and IL-22-expressing CD4+ T cells (*Th17A, Th17F, IL-22*) as percentages of total circulating CD4+ cells, measured by flow cytometry. There were no statistically significant differences between acute-to-chronically infected (*AC*), acute-to-resolved infection (*AR*), and normal healthy (*N*) patients for the cytokines tested. **b** Peripherally circulating IL-17A-, IL-17F-, and IL-22-expressing CD8+ T cells as percentages of total circulating CD8+, measured by flow cytometry. IL-17A-expressing CD8+ T cells (*Tc17A*) were statistically more frequent in the acute-to-chronic infection (*AC*) patients compared to acute-to-resolved (*AR*) and normal healthy (*N*) patients, $P = 0.0462$, but there were no statistically significant differences between acute-to-chronic infection compared to acute-to-resolved patients alone. There were no statistically significant differences for IL-17F- and IL-22-expressing CD8+ T cells (*Tc17F, IL-22*)

software (BD Biosciences). Fluorochrome-labeled (FITC/PE/APC/eflour 710) monoclonal antibodies (MAb) specific for IL-17A, IL-17F, IL-22, and Programmed Death Receptor-1 (PD-1) were obtained from eBioscience (San Diego, CA, USA). Fluorochrome-labeled (APC/PerCP/V450/V500) MAb specific for CD3, CD4, CD8, and CD161 were obtained from BD Biosciences. Phycoerythrin-conjugated Anti-T cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) was supplied by R&D systems (Minneapolis, MN, USA). PBMCs or intrahepatic mononuclear cells (1×10^6) were stained for cell surface antigen expression at 4°C in the dark for 30 min. Cells were subsequently washed twice in 2 ml Facs Wash, and left overnight in 100 µl of Fix and Perm medium A (Caltag Laboratories, Carlsbad CA, USA). Cells were then washed and were stained for intracellular cytokine expression in Fix and Perm medium B (Caltag) at room temperature in the dark for 60 min. Cells were again washed twice and subsequently fixed in 200 µl of 1% paraformaldehyde (Sigma–Aldrich, St. Louis, MO, USA).

ELISA Assay for IL-17 Detection in Serum

The Human IL-17A (homodimer) Enzyme-linked immunosorbent assay (ELISA) Ready-Set-Go! kit was purchased

from eBioscience. The assay was performed according to the manufacturer's instructions. In brief, capture antibody was incubated overnight at 4°C. Wells were then washed and incubated for 1 h at room temperature in Assay Diluent. Serum samples were thawed overnight at 4°C then brought to room temperature. Next, 100 µl of standard or serum sample was added to each well, and incubated at 4°C overnight. Detection antibody was incubated for 90 min at room temperature. Wells were washed and Avidin-HRP was added and incubated at room temperature for 50 min. Wells were again washed, and 100 µl of substrate solution was added to each well and incubated at room temperature for 30 min. Then, 50 µl of stop solution (2 N sulfuric acid) was added to each well prior to analysis using Spectra-MAX 250 instrument (Molecular Devices, Sunnyvale, CA, USA) and SoftMax Pro software (Molecular Devices) at 450 nm with subtraction of 570 nm wavelength. IL-17A sensitivity is reported by the manufacturer to be 4 pg/ml.

Statistical Analyses

The JMP 6.0 (SAS Institute, Cary, NC, USA) statistical package and Prizm 4.03 (GraphPad Software, San Diego, CA, USA) was used. Comparisons of multiple groups were
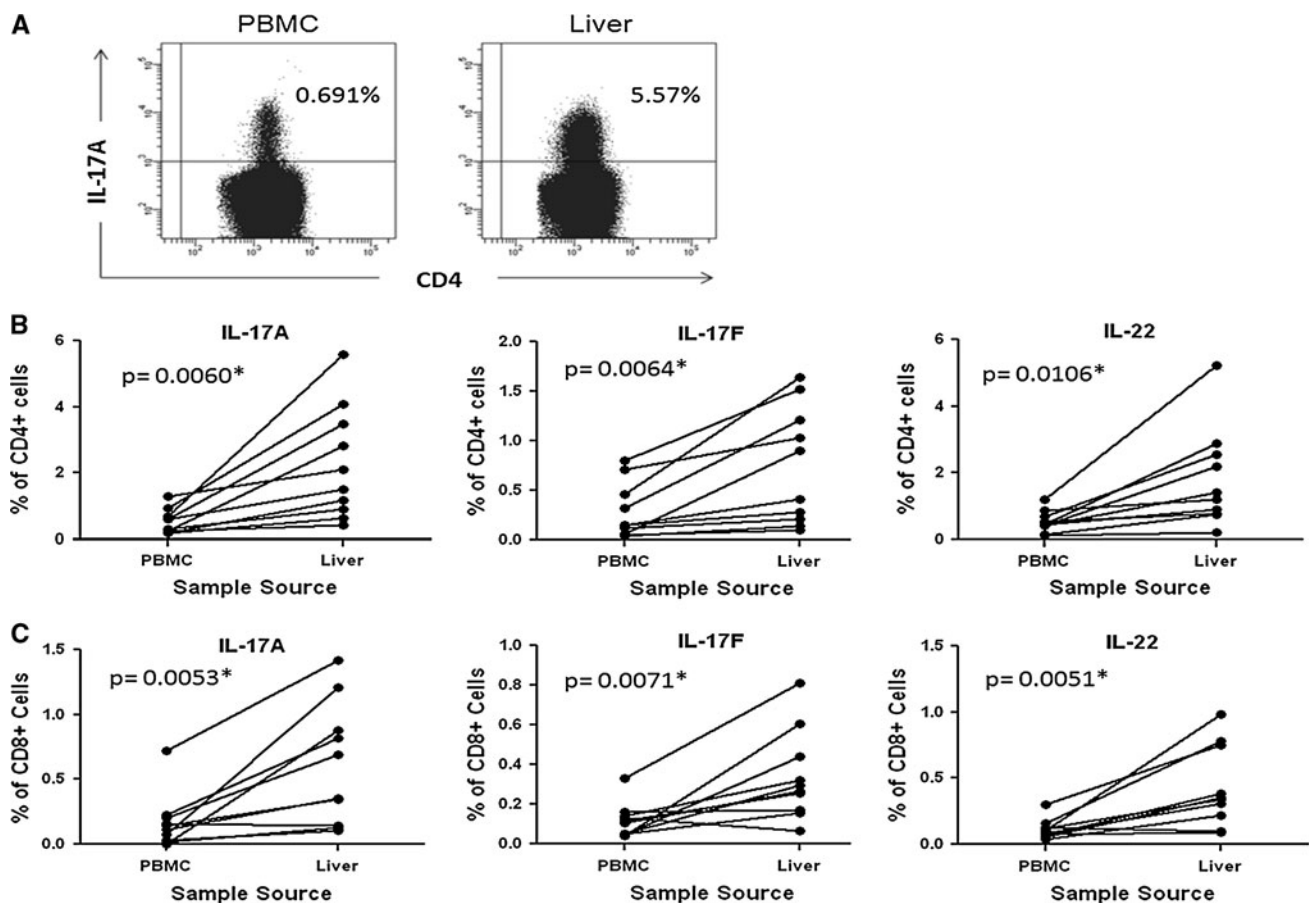
Fig. 2 IL-17- and IL-22-expressing T cells are enriched in the liver compared to peripheral blood. **a** Representative flow cytometry dot plots of IL-17A expression in CD4+ T cells as a percentage of total CD4+ T cells in peripheral blood (*PBMC*) and in intrahepatic lymphocytes (*Liver*). Frequencies of expression by CD4+ (**b**) and CD8+ (**c**) T cells of *IL-17A*, *IL-17F*, and *IL-22* as percentages of total CD4+ or CD8+ cells, respectively, in peripheral blood (*PBMC*) and intrahepatic (*Liver*) compartments. *Lines* connect measurements within the same patient. Statistically significant intrahepatic enrichment was noted for CD4+IL-17A+ ($P = 0.0060$), CD4+IL-17F+ ($P = 0.0064$), CD4+IL-22+ ($P = 0.0106$), CD8+IL-17A+ ($P = 0.0053$), CD8+IL-17F+ ($P = 0.0071$), and CD8+IL-22+ ($P = 0.0051$) cells

performed using one-way Analysis of Variance (ANOVA). A repeated measures ANOVA was substituted for analyses of groups of patients when multiple measurements were taken from each patient. For comparisons between only two groups of measurements obtained from the same patients, a two-sided paired t test was performed. For comparison of two groups of patients, a Mann–Whitney test was performed. For all analyses, a P value of <0.05 was considered significant.

Results

Th17-Positive Cells in Acute HCV Infection Do Not Predict Outcome

It has been known for some time that the development of jaundice is associated with a higher likelihood of spontaneous recovery in the early stages of acute HCV infection [13]. We hypothesized that the expansion of Th17-positive cells might enhance liver immunopathology and be associated with a higher rate of recovery from acute HCV infection. Using a well-characterized cohort of acute HCV patients, we compared the frequency of IL-17-producing CD4+ and CD8+ T cells in patients who were symptomatic (all had total serum bilirubin >4.0 mg/dl) and then spontaneously cleared HCV and asymptomatic patients who developed persistence. The patients were matched in terms of age and gender with each other and with a control group of HCV-uninfected normal controls (mean age 37.3 years; 83.3% of cohort female for entire cohort); the difference in total bilirubin at time of enrollment was statistically significant (mean 11.2 mg/dl vs. 1.05 mg/dl in resolved vs. persistence, respectively; $P = 0.0047$, Wilcoxon rank sum).

As shown in Fig. 1, there were no significant differences in the circulating frequency of CD4+ T cells that produced

Fig. 3 Co-expression of IL-17A and IL-22 is more frequent in intrahepatic CD4+ T cells compared to peripheral blood. **a** Representative flow cytometry *dot plot* of intrahepatic IL-17A and IL-22 co-expression in CD4+ T cells. Percentages are of total intrahepatic CD4+ T cells. **b** *Pie graphs* for intrahepatic (*Liver*) and peripheral blood (*PBMC*) cytokine co-expression in CD4+ T cells. Percentages are mean (*n* = 11) frequencies of single or dual cytokine-expressing CD4+ T cells as a component of all cytokine-positive cells (IL-17A+ or IL-22+) for that compartment. A statistically greater proportion of intrahepatic CD4+ T cells co-express IL-17A and IL-22 compared to peripheral blood, 12 versus 7% (*P* = 0.027)
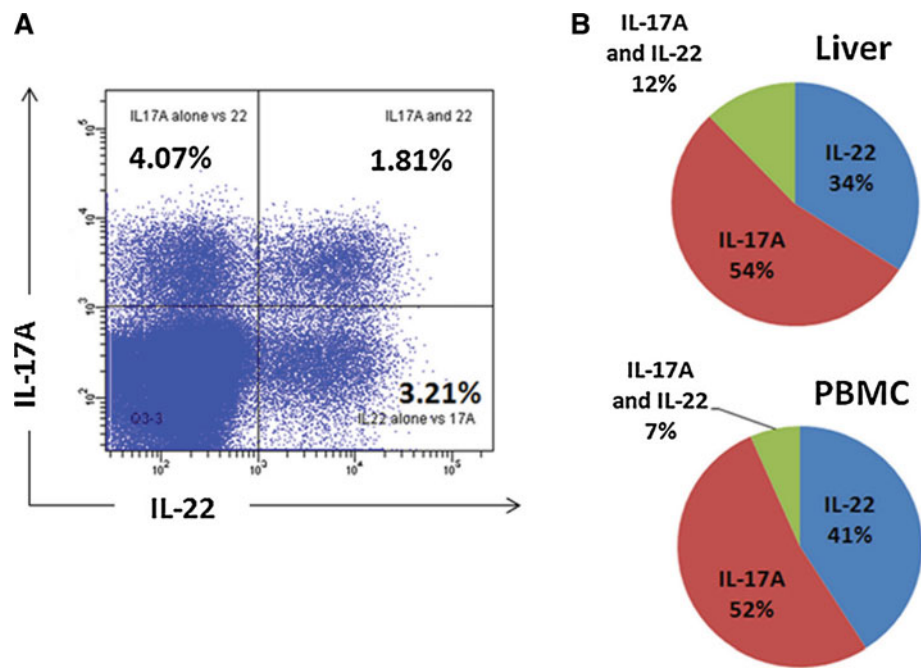

IL-17A, IL-17F, or IL-22 relative to outcome of infection or compared to normal controls. There was a slightly higher number of CD8+ T cells that produced IL-17A in patients with acute-to-chronic infection compared to the other two groups (Fig. 1b), but there was no statistically significant difference between acute-to-chronic versus acute-to-resolved infection. Moreover, there was no significant difference in the relative proportion that produced either cytokine alone or in combination according to virologic outcome (data not shown) when we examined the peripheral compartment.

Liver Demonstrates Marked Enrichment of CD4+ and CD8+ T cells that Produce IL-17 and IL-22: Association with Phenotypic Profiles

Because it is difficult to justify performing liver biopsies in the setting of acute HCV infection, we instead evaluated intrahepatic lymphocytes isolated directly ex vivo (without in vitro expansion) from chronically HCV-infected patients undergoing liver transplantation with paired peripheral cells. As shown in Fig. 2, IL-17A-, IL-17F-, and IL-22-producing cells were enriched within the liver of patients with chronic HCV infection. Moreover, this was consistent for CD4+ and CD8+ T cells. IL-22 is a cytokine previously implicated in tissue repair and epithelial defense [12, 15]; we found that the proportion of IL-17A-positive CD4+ T lymphocytes that co-secreted IL-22 was statistically higher within the liver than peripheral blood (Fig. 3).

Next, we comprehensively evaluated the phenotype of the cytokine-producing cells. Recent studies have shown that CD161 (NKRP1A) is highly expressed on Th17 cells and on Tc17 CD8+ T cells that potentially differentiate responses targeting specific organs [12, 16]. For both CD4+ and CD8+ T cells, we defined three groups according to its relative expression, i.e., CD161++, CD161+, and CD161−. As shown in Fig. 4b, a statistically greater proportion of the high CD161-expressing CD4+ T cells produced IL-17 as compared to the CD161 intermediate and negative fractions, and these patterns were consistent within the intrahepatic and peripheral compartments. The same pattern was also observed in CD8+ T cells (Fig. 4d–f).

In prior studies of HCV-infected patients, the liver has been shown to accumulate high levels of apoptotic, activated T cells relative to the peripheral blood [17]. Recently, we demonstrated that the co-expression of T cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3) and programmed death 1 (PD-1) demarcates particularly exhausted CD8+ T cells within the liver; moreover, these cells are concentrated within the CD45RA−CD27+CCR7+ central memory cell subset [18]. As shown in Fig. 5, intrahepatic IL-17-positive cells were less likely than IL-17-negative cells to also express either Tim-3 or PD-1. This pattern was not observed in the peripheral compartment where there was no difference in Tim-3 or PD-1 expression between IL-17+ and IL-17− cells (data not shown). Within the liver, the proportion of IL-17-producing cells was greater within the Tim-3−PD-1− (dual negative) fraction.
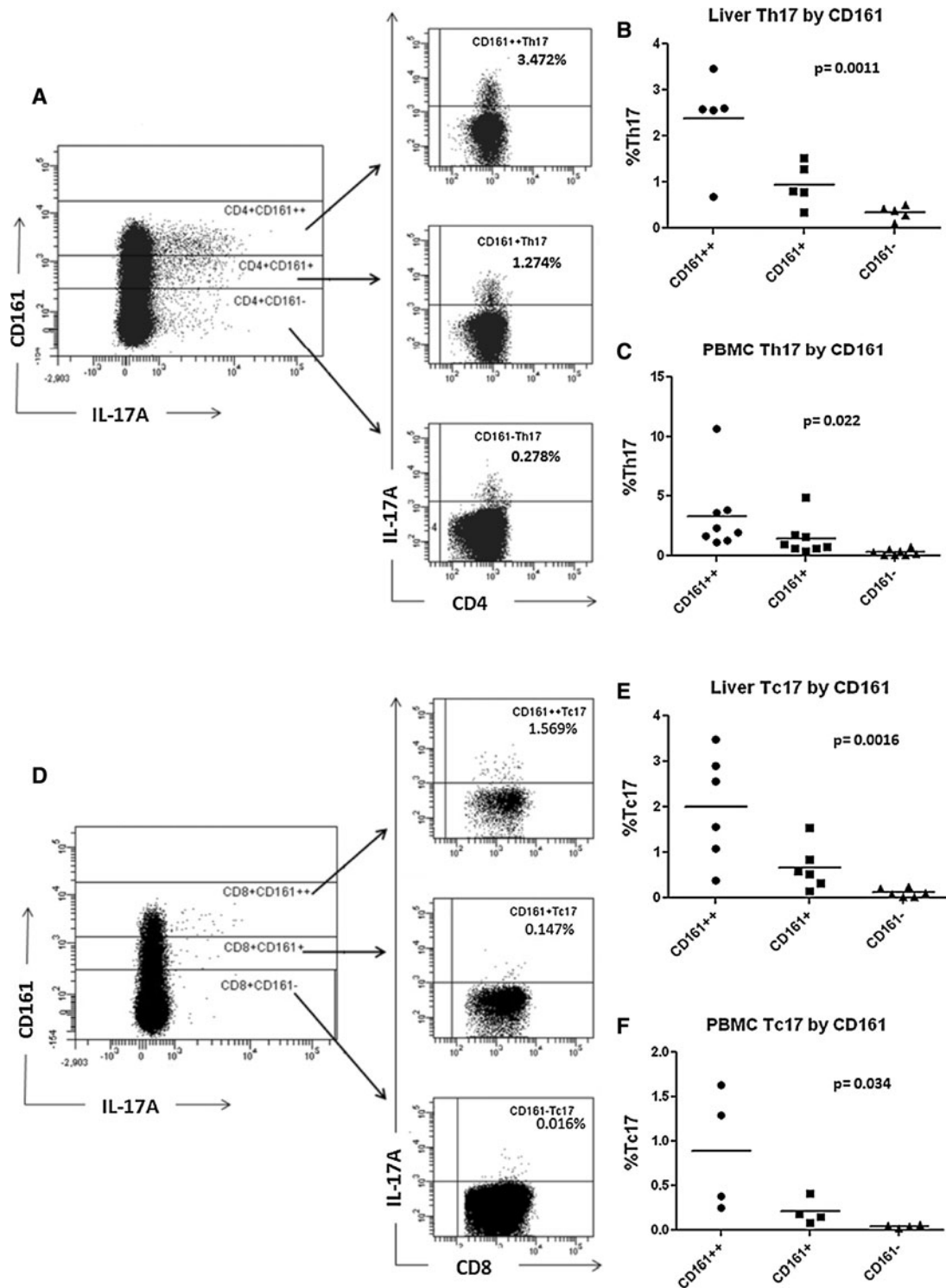
Fig. 4 CD161 is co-expressed as a marker of Th17 and Tc17 cells. **a** Representative flow cytometry *dot plots* of CD4+ T cell IL-17A and CD161 expression. Three levels (CD161++, CD161+, and CD161−) of CD161 expression in intrahepatic CD4+ T cells are defined (*left*), and corresponding dot plots with percentages of cells positive for IL-17A are shown (*right*). A statistically greater Th17 frequency was found in the CD161++ group compared to the CD161+ and CD161− groups in both the intrahepatic (**b**) and peripheral (**c**) compartments. **d** Representative flow cytometry *dot plots* of CD8+ T cell IL-17A and CD161 expression. Three levels (CD161++, CD161+, and CD161−) of CD161 expression in intrahepatic CD8+ T cells are defined (*left*), and corresponding *dot plots* with percentages of cells positive for IL-17A are shown (*right*). **e** A statistically greater Tc17 frequency was found in the CD161++ group compared to the CD161+ and CD161− groups in both the intrahepatic (**e**) and peripheral blood (**f**) compartments

Intrahepatic Th17 cells express lower levels of markers of cellular exhaustion than IL-17−C4+ cells. The frequencies of intrahepatic (*Liver*) Tim-3 expression (**a**) and PD-1 expression (**b**) are lower in Th17 cells compared to IL-17A−CD4+ cells. **c** Representative flow cytometry *dot plot* (*left*) of intrahepatic CD4+ T cells showing PD-1 and Tim-3 expression, defining quadrants with PD-1+Tim-3+ (dual positive) and PD-1−Tim-3− (dual negative) populations. *Dot plots* (*right*) show frequency of Th17 cells within the dual positive (*top*) and dual negative (*bottom*) populations. **d** Dual PD-1+Tim-3+ (exhausted phenotype) CD4+ T cells produce a lower amount of IL-17 when compared to dual negative cells
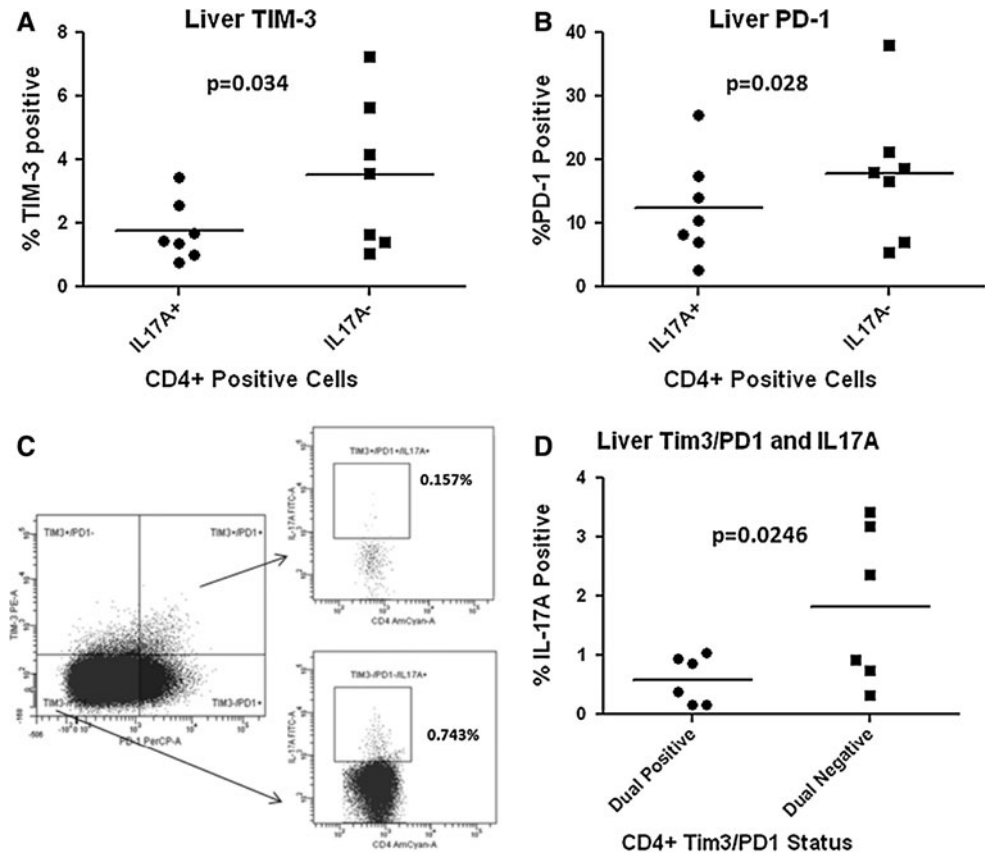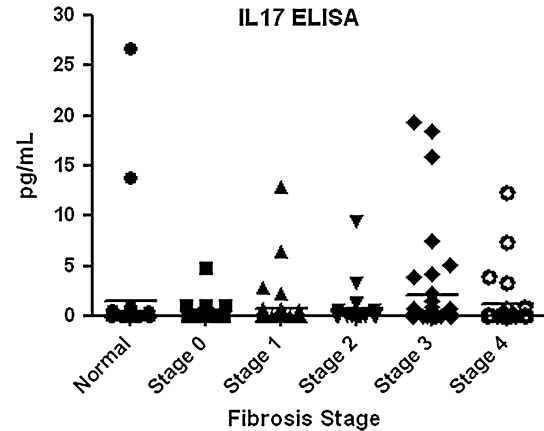

Circulating Levels of Serum IL-17 and Stage of Fibrosis

Using serum samples, we evaluated for total serum IL-17 concentrations ex vivo in 29 normal healthy control patients and compared those with 134 chronically HCV-infected patients with a range of liver fibrosis as determined by liver biopsy (stage 0, 15 patients; stage 1, 35 patients; stage 2, 21 patients; stage 3, 39 patients; stage 4, 24 patients). The majority of patient samples tested had serum IL-17 concentrations below the lower limit of detection for the kit used (4 pg/ml), and no significant differences were found between fibrosis stages or between HCV and normal patients (Fig. 6).

Fig. 6 Serum IL-17 levels do not correspond to hepatic fibrosis level in HCV patients. Serum IL-17 levels, measured by ELISA, did not statistically differ between HCV patients and normal controls or between HCV patients according to fibrosis level. The lower limit of this assay is 4 pg/ml

Discussion

T cells that produce IL-17 have recently been identified as a third distinct subset of effector T cells, and emerging data implicate Th17 cells as important in the pathogenesis of liver diseases by regulating innate and adaptive immunity, including autoimmunity [4]. In recent studies of patients with autoimmune hepatitis [4] and hepatitis B [5], the degree of hepatic inflammation was directly correlated with

Th17 cell infiltration. Our initial hypothesis was that, in patients with acute HCV infection who develop jaundice and spontaneously eradicate the virus, higher levels of circulating IL-17-producing cells would be found as compared to patients without jaundice who develop viral persistence. Our analysis failed to show any significant

difference in the frequency of IL-17- or IL-22-producing cells and the outcome of acute HCV infection, but this may have been due to the fact we initially examined the peripheral compartment. Comparison with the intrahepatic compartment revealed that IL-17A, IL-17F, and IL-22 were produced by CD4+ and CD8+ T cells at significantly higher levels within the liver as compared to peripheral blood. Recently, using immunohistochemical analysis of paraffin-embedded liver tissue, Chang et al. [19] suggested that IL-17-positivity correlated directly with severity of liver inflammation, whereas Billerbeck et al. [12], following nonspecific in vitro expansion and direct stimulation with PMA/ionomycin, found that the frequency of intrahepatic Tc17/IFN-γ cells was inversely correlated with stage of fibrosis, concluding that these cells play an important role in the control of HCV disease progression.

One of the limitations of our study was that, in order to isolate sufficient cells without in vitro expansion, we needed to use explanted livers (at the time of transplantation), and thus these patients all had advanced fibrosis. Nonetheless, our results provide novel insights into the nature of these cells in an HCV-infected liver. First, we found that a greater proportion of intrahepatic lymphocytes co-secreted IL-22 along with IL-17 than in the peripheral blood. Although the tissue-specific roles of IL-17 and IL-22 are not completely understood, the higher co-expression of IL-22 may mitigate some of the IL-17-mediated inflammation [15]. In accord with the Billerbeck [12] study, which found that the Tc17 subset was contained within the CD161++ population, we found that negligible levels of Tc17 cells were found in the CD161− population. Further, we found the same pattern within the liver and peripheral blood and for CD4 (Th17) cells, underscoring the utility of CD161 as a phenotypic marker for IL-17 cells, irrespective of CD4 or CD8 lineage. We have recently proposed a hierarchal model of T exhaustion [18] in the liver that follows a pattern of progressive loss of function (decreased IFN-γ and TNF-α production) and its association with dual expression of PD-1 and Tim-3. In the current study, we found that both Tim-3 and PD-1 were inversely correlated with IL-17 production, but this association was tissue-specific, i.e., demonstrable in the liver and not peripheral blood. In the present study, we did not perform experiments aimed at determining the antigenic specificity of IL17-producing cells. Future experiments uncovering the specific antigens eliciting IL-17 production in the liver would provide an understanding of the tissue-specific roles of these cells. Finally, we explored whether serum levels of IL-17 might be indicative of fibrosis in hepatitis C infection as has been shown in hepatitis B infection [5]. Despite utilizing a large cohort of patients with varying degrees of fibrosis, we were unable to find any significant difference in circulating levels of IL-17 in HCV-positive patients versus normal controls. Our results are similar to the study by Lemmers and colleagues [6] that focused on alcoholic liver disease but included HCV (cirrhotics and non-cirrhotics) as control groups. Whether IL-17 is upregulated nonspecifically in various chronic inflammatory diseases of the liver or whether differences in counter-regulatory responses account for the diverse findings across autoimmune and viral hepatic diseases warrants further investigation.

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