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Hepatic IL-17 Responses in Human and Murine Primary Biliary Cirrhosis

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Abstract

The emergence of new regulatory and pro-inflammatory immune cell subsets and cytokines dictates the need to re-examine the role of these subsets in various diseases involving the immune system. IL-17 has been recently identified as a key cytokine involved in numerous autoimmune processes. However, its role in liver autoimmune diseases remains unclear. Primary biliary cirrhosis (PBC) is characterized histologically by autoreactive CD4 and CD8 T cells surrounding damaged bile ducts. CD4⁺ T cells are a major source of IL-17, which compose a distinct T helper subset (Th17). Thus we set out to determine the role of IL-17 in both human and a murine model of PBC in a liver-targeted manner. Our data demonstrate an increase in the frequency of IL-17⁺ lymphocytic infiltration in liver tissues from PBC patients and those with other liver dysfunctions as compared to healthy livers. IL-2 receptor α knockout mice, a recently identified murine model of human PBC, also demonstrate marked aggregations of IL-17 positive cells within portal tracts and increased frequencies of Th17 cells in the liver compared to the periphery. Interestingly, CD4⁺ T cells from livers of normal C57BL/6J mice also secreted higher levels of IL-17 relative to those from spleens, indicating a preferential induction of Th17 cells in liver tissues. Importantly, C57BL/6J cocultures of splenic CD4⁺ T cells and liver non-parenchymal cells increased IL-17 production approximately 10 fold compared to T cells alone, suggesting a role of the liver microenvironment in Th17 induction in cases of liver autoimmunity and other liver inflammatory diseases.

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Keywords

IL-17; primary biliary cirrhosis; IL-2 receptor alpha; liver; CD46; Tr1; microenvironment; CD4+ T cells

Introduction

Primary biliary cirrhosis (PBC) remains an enigmatic autoimmune liver disease much like several other autoimmune diseases characterized by the presence of automitochondrial antibodies and damage to small bile ducts particularly in women [1-5]. There is an important role for cytokines and tissue microenvironments in the regulation and propagation of inflammatory responses [6]. While increased serum levels of IL-2, IL-4, and IL-10 have been reported in PBC, the most significant increases were noted for IFN- γ and IL-5 when compared to normal controls [7]. Cytokine profiles from total RNA extractions have been performed on liver biopsies revealing an increase in the levels of IL-5 and IL-6 mRNA compared to both normal controls and patients with chronic hepatitis C (CHC). Furthermore, mRNA levels of IL-10 were lower in liver tissues of PBC than in CHC patients [8]. As IL-10-producing T cells have been theorized to play an attenuating role in autoimmunity, the low IL-10 levels could suggest a dysregulation of the control of autoreactive T cells in the liver.

Recently, IL-17, and IL-17 producing CD4⁺ T cells (Th17), have been identified as a key inflammatory cytokine involved in a number of autoimmune diseases including rheumatoid arthritis, experimental autoimmune encephalomyelitis (EAE), and colitis [9, 10]. Of the few studies that have examined the role of Th17 cells in liver diseases include studies that have reported its elevation in serum of patients with acute hepatic injury [11], in infection induced hepatic granulomas [12, 13], and in animal models of hepatic ischemia-reperfusion [14]. The role of Th17 cells in autoimmune liver diseases, however, remains unclear. Importantly, IL-2R α knockout mice (IL-2R α KO) mice exhibit an increase in Th17 cells due to the suppressive effect of IL-2 on Th17 induction [15, 16]. This observation is particularly interesting for us since IL-2R α KO mice have been identified as a small animal model of human PBC; these mice spontaneously produce anti-mitochondrial antibodies with specificity for the E2 subunit of pyruvate dehydrogenase and develop portal inflammation and biliary ductular damage both of which are characteristic of PBC [17]. The above knowledge prompted us to examine the role of Th17 cells in PBC and we thus began analyzing the potential role of IL-17 in PBC in two ways: we analyzed patient biopsies for the presence/influx of Th17 T cells and in parallel by an assessment of the degree of Th17 participation in diseased livers of IL-2R α KO mice.

IL-10 and IL-10 secreting regulatory T cells (Tr1) have been identified as playing a key role in maintaining tolerance and preventing autoimmunity [10, 18, 19]. Because a de-regulation in IL-10 has been observed in PBC patients and a recent report suggests that IL-10 affects IL-17 production [10], we reasoned that the PBC phenotype could possibly be due to a defect in the induction of IL-10-secreting Tr1 cells in PBC patients. Tr1 cells can be induced in vitro via drugs, specialized APCs and the concurrent cross-linking of the TCR along with the complement regulator CD46 in the presence of IL-2 [20, 21]. Thus, we assessed the capacity of patient CD4⁺ T cells to convert to IL-10 secreting Tr1 cells via CD3/CD46 co-activation.

We report herein that whereas liver tissues from PBC patients, as well as similar liver tissues from patients with other liver diseases, exhibit readily detectable frequency of IL-17 producing cells, no IL-17⁺ staining was detected in liver tissues from otherwise healthy

control donors. Unlike humans, both IL-2R α KO and normal B6 mice display IL-17⁺ cells not only in the liver but also spleen. However, there was a significant increase in the frequency of hepatic Th17 cells as compared to splenic Th17 cells in the IL2R α KO mice as compared with B6 mice indicating a potential role of the liver microenvironment on Th17 induction. This was supported by the induction of IL-17 synthesizing cells from splenic CD4⁺ T cells in the presence of liver nonparenchymal cells from normal mice.

Materials and methods

Human Studies

Patients—For immuno-histochemical analysis, a total of 67 well-preserved liver biopsies or formalin-fixed and paraffin-embedded blocks were obtained from patients with PBC (n = 17), and for purposes of control, liver tissues from patients diagnosed with chronic hepatitis C (CHC; n = 26), nonalcoholic steatohepatitis (NASH; n = 15), autoimmune hepatitis (AIH; n = 4), and normal controls subjects (NL; n = 5) registered in our universities and associated hospitals. All cases were considered early stage disease and had no evidence of cirrhotic changes. Peripheral blood samples were obtained for Tr1 studies from 20 patients with PBC and 15 healthy controls. All subjects provided written and informed consent.

Immunohistochemistry—Liver sections were immunostained using our standard microwave protocol as described previously [22]. All tissues were fixed in 10% neutral buffered formalin and embedded in paraffin, and 4 μ m-thick sections were cut from each paraffin block. In brief, after de-paraffinization and antigen retrieval using a pressure cooker for 15 min, 4 μ m thick formalin-fixed paraffin-embedded sections were incubated with 5% bovine serum albumin for 10 min. to inhibit non-specific reactions. Specimens were then incubated with primary antibodies in a wet chamber and irradiated intermittently for 15 min using a microwave. This was followed by further incubation for an additional 2-hours under room temperature. After rinsing with Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the sections were incubated with a secondary antibody and irradiated intermittently for 15 min and the incubated for 2 hrs at room temperature. Tris-buffered saline (TBS) was then used to rinse the specimens, after which 3,3'-diaminobenzidine (DAB) (Sigma, Steinheim, Germany) was applied as a substrate for the secondary antibody. Hematoxylin was applied as the counter stain, and the stained specimen cover slipped for observation under a light microscope.

The following antibodies were used for the detection of IL-17 in human liver specimens: rabbit polyclonal antibody against IL-17 (Santa Cruz Biotechnology, Inc, CA, USA; 1:100 dilution), anti-human IL-17 antibody (R & D Systems, Inc., MN, USA; 1:100 dilution) and Envision-peroxidase (Envision-PO) (DAKO, Glostrup, Denmark). In all cases, pre-determined optimal dilutions were used and positive and negative samples included with each assay. To evaluate and compare the distribution and frequency of cells positive for IL-17, three small to medium sized portal tracts from each sample were randomly selected for examination using an optical microscope. An unbiased observer counted the number of IL-17-positive cells contained within the portal tracts of blind-coded specimens at a magnification of 200 $\times$.

Tr1 induction—Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation with Histopaque-1077 (Sigma Chemical Co., St. Louis, MO). CD4⁺ T cells were enriched from unfractionated human PBMC's by negative selection using the RosetteSep CD4⁺ Enrichment Mixture (StemCell Technologies, Vancouver, British Columbia, Canada). The resulting CD4⁺ T cells were further enriched by the magnetic cell separation technique utilizing CD4 microbeads of the MiniMACS system (Miltenyi Biotech,

Auburn, CA). For the generation of Tr1 cells, 2×10^5 CD4⁺ T cells were incubated in 96-well flat bottom culture plates coated with monoclonal antibodies to CD3 (1 µg/ml, Biolegend), CD28 (1 µg/ml, Biolegend), and CD46 (1 µg/ml, provided by Dr. J.P. Atkinson, Washington University School of Medicine, St. Louis, MO) in the presence of rIL-2 (25U/ml). Controls consisted of aliquots of the same cells cultured in an identical manner except for the absence of anti-CD46 antibodies. Aliquots of these CD4⁺ T cells were either cultured for 2 days, after which the cells were incubated with Brefeldin A (10 µg/ml, Sigma, St. Louis, MO) for 5 hours in preparation for intracellular staining of IL-10 or for three days for the collection of supernatant fluid and cytokine measurement by ELISA.

Flow Cytometry—Flow cytometric assisted analysis was performed using 1×10^6 PBMCs suspended in 25 µl of staining buffer (0.5% bovine serum albumin, 0.04% EDTA, 0.05% sodium azide in PBS) which was pre-incubated with anti-human FcR blocking reagent (Biolegend) for 15 min. Surface stains include PE/Cy5-conjugated anti-human CD8α Ab (eBioscience, San Diego, CA), APC-conjugated anti-human CD3 Ab (Biolegend), and APC/Cy7-conjugated anti-human CD4 Ab (Biolegend). The cells were then fixed and permeabilized with BD Cytfix/Cytoperm solution (BD Biosciences, San Jose, CA) for 20 minutes at 4°C and washed once. Subsequently, intracellular staining was performed with combinations of PE-conjugated anti-human IL-10 antibody (BD Biosciences), FITC-conjugated anti-human IFN-γ antibody (Invitrogen, Carlsbad, CA), and PE-conjugated anti-human Granzyme-B antibody (eBioscience). Stained cells were analyzed on a FACScan flow cytometer (BD Immunocytometry Systems) that had been upgraded by Cytex Development (Fremont, CA) to allow for 5-color analysis. The acquired data were analyzed with Cellquest PRO software (BD Immunocytometry Systems). CD4⁺ T cell culture supernatants were analyzed for Th1 and Th2 cytokines (IFNγ, TNFα, IL-10, IL-5, IL-4, IL-2) using the BD Cytometric Bead Array kit (BD Biosciences) and examined through flow cytometric analysis.

IL-2RKO colangitis model

Mice—IL-2Rα heterozygous knockout male and female mice (B6.129S4-Il2ratm1Dw) on a C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME) as breeding pairs, maintained in individually ventilated cages under specific pathogen-free conditions. The homozygous offspring from the mating of the heterozygous mice IL-2Rα^{-/-} (IL-2Rα KO) were studied at 4-28 weeks of age. To prolong their lifespans, the IL-2Rα KO mice were fed with sterile Rodent helicobacter MDs (3 Drug Combo) diets (Bio-Serv, Frenchtown, NJ) and were provided sterile drinking water ad libitum, which included an antibiotic (sulfatrim). Wild-type B6 mice were also purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in individually ventilated cages under specific pathogen-free conditions. All studies were performed with approval from the University of California Animal Care and Use Committee.

Immunohistochemistry—Murine liver tissues were prepared as described in the immunohistochemistry section above. Tissue specimens were incubated with anti-mouse IL-17 antibodies (Biolegend, San Diego, CA, USA; 1:20 dilution), washed and then followed by incubation with Histofine simple stain MAX-PO (Rat)/HRP (Nichirei, Tokyo, Japan) as the secondary antibody.

CD4⁺ T cell enrichment—For CD4⁺ T cell isolation, livers and spleens were obtained from IL-2Rα KO and B6 mice. Livers were first perfused with PBS containing 0.2% BSA, passed through a nylon mesh, and re-suspended in PBS/0.2% BSA (EMD chemicals, Gibbstown, NJ) [17]. Hepatocytes were removed as pellets after centrifugation at 700 rpm for 1-minute. Spleens were also disrupted, passed through a nylon mesh, and suspended in

PBS/0.2% BSA. Lymphocytes from suspended liver and spleen cells were then isolated using Histopaque-1077 (Sigma Chemical Co. St. Louis, MO). After centrifugation, cells at the interface were washed with PBS/0.2% BSA, and the viability of cells confirmed by trypan blue dye (Sigma Chemical Co.) exclusion. Liver and splenic lymphocytes were first depleted of natural killer cells (NK), natural killer T cells (NKT), B cells and erythrocytes by incubating cell suspensions with biotin conjugated NK1.1, CD19, and TER119 antibodies, respectively, followed by negative selection with anti-biotin magnetic beads (Miltenyi Biotech Inc., Auburn, CA). Negative selection was performed utilizing a MiniMACS column (Miltenyi Biotech Inc.) with a 25-gauge needle mounted at the tip. After negative selection, liver and splenic lymphocytes were enriched for CD4⁺ T cells using positive selection with anti-CD4 magnetic beads (Miltenyi Biotech Inc.) utilizing a MiniMACS column.

Non-parenchymal cell isolation—Liver non-parenchymal cells (NPCs) were isolated similar to previously described methods [23]. Briefly, livers were first perfused with liver digest medium (Invitrogen, Carlsbad, CA), cut into small pieces, and incubated at 37°C for 20 min with constant shaking at 250 rotations per minute. The digested liver was then further disrupted with an 18 and 23-gauge needle and syringe. Next, the liver digest was centrifuged 3 times at 50g for 1 min intervals, the supernatants collected and the hepatocytes discarded. The NPC's in the supernatant were washed $\times 3$ by centrifugation at 300g for 5 min. NK, NKT, erythrocytes and T cells were removed by negative selection using biotin conjugated NK1.1, TER119, and CD3 antibodies, followed by negative selection with anti-biotin magnetic beads as mentioned above.

Intracellular cytokine analysis—Cell preparations to be analyzed by flow cytometry were first pre-incubated with anti-mouse FcR blocking reagent and then incubated at 4°C with a combination of fluorochrome-conjugated antibodies which included anti-CD4 PE-Cy5 (Biolegend), anti-CD3 APC-Cy7 (Biolegend), anti-NK1.1 APC (eBioscience), anti-CD44 FITC (BioLegend), anti-CD8 α PE-Cy5 (eBioscience), anti-IL-17 PE (BD Biosciences), and anti-IFN- γ FITC (eBioscience). Intracellular staining of IL-17 and IFN- γ was performed using the BD Perm/Wash system (BD Biosciences). The acquired data were analyzed with Cellquest PRO software and FlowJo Software (Tree star, Inc., Ashland, OR).

Supernatant cytokine quantification—TNF- α , and IFN- γ quantifications were performed using the mouse cytometric bead array (CBA) kits (BD Biosciences). Samples were analyzed using a FACScan flow cytometer. Quantitation of IL-17 was performed using the Mouse or Human IL-17 Quantikine ELISA Kit (R&D systems, Minneapolis, MN).

Statistical Analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM) and were evaluated using Mann-Whitney U tests for comparisons between samples from PBC patients and control subjects as well as IL-2R $^{-/-}$ mice and B6 controls. The paired, two-tailed, nonparametric Student's t-test was applied for comparing levels of cytokines in the culture supernatant with or without CD46 stimulation. Significant levels were defined as p values of <0.05 .

Results

PBC patients and IL-2R α KO mice exhibit liver IL-17

Because PBC by definition primarily targets the liver, we performed immunohistochemical analysis on liver tissues from control, PBC, CHC, NASH, and AIH subjects for the presence of IL-17-expressing cells. Liver tissue sections from 5 control and 17 PBC subjects were stained for the presence of IL-17 positive cells; approximately two portal areas per sample

were examined (Figure 1). Liver tissues from PBC patients as well as all liver disease controls demonstrated significantly higher numbers of IL-17 positive cells per portal tract compared to liver tissues from normal controls (Figure 1B; $p < 0.0001$). We next assessed the number of infiltrating Th17 cells in our mouse model. Similar to diseased PBC patient liver tissues, the IL-2R α KO liver tissues also exhibit marked aggregations of IL-17 positive cells near portal tracts compared to B6 control liver tissues (Figure 2). In contrast with serum IL-17 in PBC patients, serum IL-17 levels were elevated in IL-2R α KO mice (Figure 2A) while being undetectable in B6 mice (data not shown). This finding was consistent with a published study [15]. However, the increase in the serum IL-17 levels was not uniform over time, peaking between 8-13 weeks of age ($257.8 \text{ pg/ml} \pm 57.97$, $n=17$) and declining significantly over time (Figure 2A).

Increased Th17 population in livers of IL-2R α KO and wild-type mice

As previously reported, mesenteric lymph nodes from IL-2 $^{-/-}$ mice exhibit an increased Th17 frequency compared to wild-type mice [15]. In this study, liver-specific T cell infiltrates from IL-2R α KO mice were investigated for the presence of Th17 cells due to the recent identification of IL-2R α KO mice as a small animal model for primary biliary cirrhosis [17]. CD4 $^{+}$ T cells isolated from liver tissues of the IL-2R α KO mice comprised a significantly higher percentage of Th17 cells compared to those from the spleen, $20.3\% \pm 3.701$ vs $6.4\% \pm 2.180$, respectively ($P < 0.05$) (Figure 3). The increase in Th17 cells in IL-2R α KO livers was confirmed by analyzing the supernatants of liver and splenic CD4 $^{+}$ T cells cultured for 3 days in the presence of anti-CD3 and anti-CD28 antibodies. IL-2R α KO liver CD4 $^{+}$ T cells produced markedly higher amounts of IL-17 compared to those from IL-2R α KO spleens, 1204 ± 87.8 vs $329.4 \pm 59.2 \text{ pg/ml}$, respectively ($p < 0.01$) (Figure 3C). To determine if the liver microenvironment per se caused the relative increase in Th17 induction/migration in IL-2R α KO, CD4 $^{+}$ T cells were isolated from livers and spleens from normal B6 mice and cultured in the presence of anti-CD3 and anti-CD28 antibodies. Similar to IL-2R α KO mice, liver derived CD4 $^{+}$ T cells produced higher levels of IL-17 compared to their splenic counter part (35.8 ± 3.2 vs $8.6 \pm 2.1 \text{ pg/ml}$, $p < 0.001$) (Figure 3C).

Liver and splenic CD4 $^{+}$ T cell inflammatory cytokine production

Subsequently, we assessed the production of other pro-inflammatory cytokines from splenic and liver tissue sources of CD4 $^{+}$ T cells from the IL-2R α KO mice (Figure 4). The relative levels of IFN- γ and TNF- α synthesized by CD4 $^{+}$ T cells were significantly increased in both the spleens and liver tissues from the IL-2 α KO mice as compared to those from B6 mice. While the levels of IFN- γ production by CD4 $^{+}$ T cells from B6 liver tissues was higher than that from B6 spleens, the levels synthesized by splenic and liver tissue cells from the IL-2 R KO mice were essentially similar (Figure 4A). Ratios of IL-17 to IFN- γ production by CD4 $^{+}$ T cells in splenic versus livers tissues of IL-2R α KO mice were compared to determine the difference between the balance of Th1 and Th17 influences in the two organs. As seen in Figure 4C, the CD4 $^{+}$ T cells from liver tissues of IL-2R α KO mice skew considerably more towards a Th17 versus Th1 response than those from the spleen.

Liver APCs promote the induction of Th17 cells

To investigate whether the Th17 bias in the livers of IL-2R α KO and normal B6 mice is due to the preferential migration of Th17 cells to the organ or because of the unique cytokine and cellular milieu of the liver, we isolated liver non-parenchymal cells (NPCs) from B6 mice and co-cultured them with B6 splenic CD4 $^{+}$ T cells. Cultures containing NPCs and CD4 $^{+}$ T cells produced significantly higher levels of IL-17 in the presence of anti-CD3 and CD28 (Figure 5).

Generation of Tr1 cells in PBC and control subjects

The absence of IL-10 (an immuno-suppressive cytokine) in IL-10 KO mice has been shown to induce colitis mediated by IL-17 [10]. Thus, there is indication that IL-10 derived from T cells might regulate IL-17. Therefore, CD4⁺ T cells from PBC patients as compared with controls were assayed for their potential to synthesize IL-10. To accomplish this goal, we utilized the assay developed by Dr. Atkinson. Briefly, the engagement of the complement regulator CD46 on CD4⁺ T cells in addition to conventional T cell co-stimulation in the presence of IL-2 was shown to effectively induce IL-10 producing Tr1 cells [21]. CD4⁺ T cells were thus isolated from the PBMCs of control and PBC subjects and cultured *in vitro* in the presence or absence of anti-CD46 antibody, rIL-2 and antibodies to CD3 and CD28. Under these culture conditions CD46 stimulation resulted in a marked increase in the frequency of intracellular IL-10 and granzyme B synthesizing CD4⁺ T cells from both PBC and control subjects (Figure 6). However, there was no significant difference in the values obtained from control as compared with PBC patients (Figure 6A). When the supernatant fluids were analyzed, both PBC and control CD4⁺ T cells both responded to CD46 stimulation by increasing their production of cytokines such as IL-10 and IL-4 while decreasing levels of IL-2 production (Figure 6B). However, no significant differences were found in the levels of these cytokines synthesized by CD4⁺ T cells from patient and control groups (Figure 6B).

In summary, PBC patients and patients with other liver diseases such as NASH, AIH, and CHC exhibit readily detectable levels of IL-17⁺ hepatic infiltrates as compared with liver tissues from normal controls (Figure 1). Similarly, IL-2R α KO mice also demonstrate IL-17 staining in diseased portal areas (Figure 2). To determine if the liver environment promotes Th17 generation, we compared the frequency of Th17 cells in the livers of IL-2R α KO mice to that of a representative peripheral organ (spleen). Liver specific T cells contain an increased Th17⁺ population compared to that from the spleen (Figure 3). Liver specific CD4⁺ T cells from IL-2R α KO mice also produce a higher ratio of IL-17 compared to IFN- γ as compared to those from the spleen, suggesting a Th17 bias in the liver (Figure 4). This hypothesis was supported by the increase in IL-17 production by liver CD4⁺ T cells from wild type mice (Figure 3B) as well as the production of IL-17 by cocultures of wild-type splenic CD4⁺ T cells in the presence of liver NPCs (Figure 5). However, despite the association between IL-10 and Th17 induction in a colitis model, PBC patients do not demonstrate a deficiency in Tr1 induction via the cross-linking of CD3 and CD46 in the presence of IL-2.

Discussion

Due to the recent identification of the IL-17 secreting T helper cell subset, several disease paradigms had to be revisited in efforts to examine the role and contribution of such cells on the individual disease process. For example, it has been thought that Th1 responses are responsible for disease development in EAE and collagen-induced arthritis (CIA) [24]. However, neutralization or genetic knockout of IFN- γ in these models exacerbated these diseases [25]. Upon further investigation, it was revealed that IL-17 plays a critical role in the development of EAE and CIA [25, 26]. Augmented levels of IL-17 is also found in human diseases such as multiple sclerosis [27], rheumatoid arthritis [28], and systemic lupus erythematosus [29]. Traditionally, PBC has been associated with a Th1 prototype autoimmune response as represented by increased liver IFN- γ levels, along with “Th2” responses, which include IL-5 and IL-6 but not IL-10 or IL-4 [8, 30, 31]. Interestingly, IL-6 has recently been identified as a key inducer of Th17 cells [32]. Specifically, IL-1 β and IL-6 are able to induce IL-17A production from human central memory CD4⁺ T cells, whereas TGF- β and IL-21 are required for Th17 differentiation from naïve human CD4⁺ T cells. On

the other hand, TGF- β and IL-6 are required for the induction of murine Th17 cells from naïve CD4⁺ T cells. Therefore, we set out to determine the role of IL-17 in PBC.

In the present study, we detected an increased frequency of IL-17 producing cells in liver tissues of PBC patients as well as those from patients with CHC, NASH, and AIH compared with healthy control livers (Figure 1). These findings suggest that there is a potential preference for the induction of Th17 cells in an inflamed liver microenvironment, as its presence is a general phenomenon for a number of liver diseases.

Colitis exhibited by IL-10 KO mice was determined to be dependent on IL-17 [10]. Furthermore, retinoic acid receptor-related orphan receptor γ (ROR γ t), which is required for Th17 generation, is also expressed by IL-10 producing regulatory T cells. A homeostatic balance between these regulatory T and Th17 cells is required for proper inflammatory responses [33]. Since IL-10 is also key suppressive cytokine and Tr1 cells play critical roles in mucosal tolerance, we also investigated the role of this cytokine in PBC. However, CD4⁺ T cells from PBC patients were comparable to those from control subjects in their capacity to generate Tr1 cells (Figure 6).

It has recently been established that Th17 cells are preferentially induced in mice deficient in IL-2 due to the inhibitory effects of IL-2 on IL-17 production [15]. Thus, serum levels of IL-17 are significantly increased in IL-2R α KO mice (Figure 2). However, serum IL-17 levels appear to peak after 8 weeks of age and steadily decline after 13 weeks of age (Figure 2). This indicates that IL-17 maybe involved in earlier disease development, and that the participation of other immune mediators may also be critical. The lack of IL-2 signaling alone does not completely predispose naïve T cells to the Th17 lineage. In fact, naïve T cells from IL-2 deficient mice have not been demonstrated to have the capacity to develop into Th17 cells in the absence of IL-6 and TGF- β [15]. Thus, the cytokine milieu remains critical for the generation of Th17 cells. This is reflected by results of our study that show liver lymphocytes in IL-2R α KO mice which demonstrate a significantly higher frequency of Th17 cells compared to the periphery (spleen) (Figure 3). To address the possibility that the increase in liver-specific CD4⁺ T cell IL-17 production was not due to an overall increased inflammatory response in IL-2R α KO livers, the ratio of IL-17 to IFN- γ production was examined. In fact, Th17 responses were favored over Th1 (IFN- γ) responses considerably more in liver tissues than spleen cells of IL-2R α KO mice (Figure 4C). Subsequently, liver CD4⁺ T cells were isolated from non-diseased control B6 mice and investigated for their IL-17 producing capacity. Liver CD4⁺ T cells from normal mice produced higher levels of IL-17 compared to their splenic counterpart (Figure 3C). This led to the hypothesis that the liver environment may be conducive to Th17 induction.

Recently, various resident APC populations in the lamina propria have been identified to promote either the induction of Th17 or regulatory T cells, highlighting the importance of resident APCs in the shaping of local inflammatory responses [6]. To determine whether the increase in the liver Th17 population was due to the migration of previously primed Th17 cells or due to de novo Th17 induction due to the liver microenvironment, we cultured splenic CD4⁺ T cells in the presence of liver NPCs depleted of NK, NKT and T cells. The NPC population, all of which possess antigen presenting capabilities, consists of cell populations such as Kupffer cells, liver sinusoidal endothelial cells, stellate cells, and liver dendritic cells [23]. Results from this study suggest that NPCs may be quite efficient in the generation of splenic CD4⁺ T cells skewed to develop into a Th17 lineage (Figure 5). However, future will be necessary to investigate the mechanism of IL-17 induction, which may involve cell-to-cell interactions as well as cytokine milieus between liver NPCs and CD4⁺ T cells.

In the present study, the presence of IL-17 has been confirmed in both PBC patients as well as in a murine model of PBC. The data suggest that IL-17 responses is preferentially induced in the liver environment partly due to liver NPCs as reflected in the production of IL-17 in NPC/T cell co-cultures as well as the presence of IL-17⁺ cells in livers of IL2RαKO mice and diseased livers of patients suffering from PBC, CHC, AIH, and NASH. However, the importance of IL-17 in disease progression requires further investigation, such as studies involving the blocking of IL-17 signals. The exact mechanism of the exuberant Th17 induction in PBC is not clear. However, a dysregulation of Tr1 induction seems not to be the major underlying defect. It is possible that other inflammatory mediators may also be critical in liver disease induction due to the steady decrease in serum IL-17 levels over time in IL-2Rα KO mice. Further investigations into the role of IL-17 and autoimmune liver diseases may reveal potential therapeutic advances in this area.

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Abbreviations

AIH	autoimmune hepatitis
APC	antigen presenting cell
CHC	chronic hepatitis C
EAE	experimental autoimmune encephalomyelitis
IL-2Rα KO	interleukin 2 receptor alpha knockout
NASH	nonalcoholic steatohepatitis
NK	natural killer
NKT	natural killer T
NPC	non-parenchymal cell
PBC	primary biliary cirrhosis
PBMC	peripheral blood mononuclear cell
RORγt	retinoic acid receptor-related orphan receptor γ
TCR	T cell receptor
TGF	transforming growth factor
Th17	IL-17 producing CD4 ⁺ T cells
Tr1	IL-10 producing CD4 ⁺ regulatory T cells

Figure 1. Hepatic IL-17 expression of PBC and control subjects

A) Representative immunohistochemical stainings of IL-17 in the livers of PBC, CHC, NASH, AIH, and normal control subjects. B) Number of IL-17⁺ cells in portal areas of control and PBC subjects. Approximately 2 portal areas per subject were examined for the presence of IL-17 producing cells.

Figure 2. Serum and liver IL-17 in B6 and IL-2R α KO mice

A) Serum levels of IL-17 in IL-2R α KO mice. IL-2R α KO mice exhibit a peak in serum IL-17 around 8-13 weeks of age, which decreased over time. Statistically significant differences in serum IL-17 were observed between 8-13 wks old mice (257.8 pg/ml $\pm$ 57.97) and mice both at 1-7 wks (54.54 pg/ml $\pm$ 11.15) and over 19 wks of age (64.75 pg/ml $\pm$ 12.82), $p < 0.0001$ and $p < 0.001$, respectively. ***, $p < 0.001$. **, $p < 0.01$. B) In IL-2R KO Livers, marked aggregations of IL-17 positive cells were observed in portal tracts. In B6 Liver, no significant infiltration of IL-17 positive cells was observed.

Figure 3. IL-17 production by CD4⁺ T cells in livers and spleens of B6 and IL-2Rα KO mice
 A) Representative flow cytometric data of IL-17 producing CD4⁺ T cells from spleens and livers of B6 and IL-2Rα KO mice. B) The frequency of IL-17⁺ CD4⁺ T cells from IL-2Rα KO spleens is significantly lower than that from IL-2Rα KO livers, 6.36% ± 2.180 vs 20.39% ± 3.701, respectively. The data represent the mean + SEM (n=5). *, P<0.05. C) Levels of IL-17 production by CD4⁺ T cells from livers and spleens of B6 and IL-2Rα KO mice. IL-2Rα KO liver CD4⁺ T cells produced significantly higher levels of IL-17 compared to their splenic counter parts (1204 pg/ml ± 87.8 vs 329.4 pg/ml ± 59.2, p<0.01). Similarly, B6 liver CD4⁺ secreted higher levels of IL-17 compared to B6 splenic CD4⁺ T cells (35.8

pg/ml $\pm$ 3.2 vs 8.6 $\pm$ 2.1, $p < 0.01$). The IL-2R α KO and B6 data represent the mean + SEM (n=5), and mean+ SEM (n=4), respectively. *, $p < 0.05$, **, $p < 0.01$.

Figure 4. Levels of proinflammatory cytokines produced by CD4⁺ T cells from spleens or livers of B6 or IL-2Rα KO mice

CD4⁺ T cells from livers and spleens of B6 or IL-2Rα KO were cultured for 3 days in the presence of plate-bound anti-CD3 and anti-CD28. Supernatants were collected and analyzed for levels of inflammatory cytokines. A) Levels of IFN-γ production by CD4⁺ T cells. The data represent the mean + SEM (n=4). **, P<0.01. B) Levels of TNF-α production by CD4⁺ T cells from B6 and IL-2Rα KO mice. No significant differences were found between livers and spleens of B6 or IL-2Rα KO mice. C) Ratio of IL-17 to IFN-γ production in IL-2Rα KO mice. CD4⁺ T cells from livers of IL-2Rα KO mice demonstrate a greater proportion of

IL-17 relative to IFN- γ production compared to those from IL-2R α KO spleens. The data represent the mean + SEM (n=5). **, p<0.01.

Figure 5. Induction of IL-17 production in liver microenvironment

Splenic CD4⁺ T cells from normal B6 mice were cultured in the presence or absence of liver NPCs for 4 days and supernatants were analyzed for IL-17 levels. IL-17 levels were significantly increased in CD4⁺ T cell and NPC co-cultures compared to cultures with CD4⁺ T cells or NPCs alone. **, p<0.01.

Figure 6. Tr1 induction upon CD46 engagement in control and PBC peripheral CD4⁺ T cells
Peripheral CD4⁺ T cells from PBC or control subjects were cultured in the presence of recombinant IL-2, and anti-CD3 and anti-CD28 antibodies in the presence or absence of anti-CD46 antibodies. A) Intracellular staining for the presence of IL-10 or granzyme B was performed after a 2-day culture period. Mean frequency of IL-10⁺ ($N_{\text{Control}}=15$, $N_{\text{PBC}}=20$), granzyme B⁺ ($N_{\text{Control}}=12$, $N_{\text{PBC}}=12$), and IFN- γ ⁺ cells ($N_{\text{Control}}=12$, $N_{\text{PBC}}=12$) in patient and control CD4⁺ T cell samples. B) Supernatant cytokine profile of PBC and control subjects after a 3 day culture. Significant differences in cytokine production were noted between the different stimulation methods (with or without CD46) in both PBC and control groups. However, no significant differences were identified between normal and PBC subjects. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.