Association between chronic liver and colon inflammation during the development of murine syngeneic graft-versus-host disease

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Brandon JA, Perez J, Jennings CD, Cohen DA, Sindhava VJ, Bondada S, Kaplan AM, Bryson JS. Association between chronic liver and colon inflammation during the development of murine syngeneic graft-versus-host disease. Am J Physiol Gastrointest Liver Physiol 299: G602–G613, 2010. First published July 15, 2010; doi:10.1152/ajpgi.00511.2009.—The murine model of cyclosporine A (CsA)-induced syngeneic graft-versus-host disease (SGVHD) is a bone marrow (BM) transplantation model that develops chronic colon inflammation identical to other murine models of CD4⁺ T cell-mediated colitis. Interestingly, SGVHD animals develop chronic liver lesions that are similar to the early peribiliary inflammatory stages of clinical chronic liver disease, which is frequently associated with inflammatory bowel disease (IBD). Therefore, studies were initiated to investigate the chronic liver inflammation that develops in the SGVHD model. To induce SGVHD, mice were lethally irradiated, reconstituted with syngeneic BM, and treated with CsA. All of the SGVHD animals that developed colitis also develop chronic liver inflammation. Liver samples from control and SGVHD animals were monitored for tissue pathology, RNA for inflammatory mediators, and phenotypic analysis and in vitro reactivity of the inflammatory infiltrate. Diseased animals developed lesions of intrahepatic and extrahepatic bile ducts. Elevated levels of mRNA for molecules associated with chronic liver inflammation, including mucosal cellular adhesion molecule –1, the chemokines CCL25, CCL28, CCR9, and TPT1- and TPT17-associated cytokines were observed in livers of SGVHD mice. CD4⁺ T cells were localized to the peribiliary region of the livers of diseased animals, and an enhanced proliferative response of liver-associated mononuclear cells against colonic bacterial antigens was observed. The murine model of SGVHD colitis may be a valuable tool to study the entero-hepatic linkage between chronic colon inflammation and inflammatory liver disease.

chronic liver inflammation; inflammatory bowel disease; T₄₁ immune; T₄₁7 immunity

Clinically, a high percentage of inflammatory bowel disease (IBD) patients also present with some form of portal/perportal liver inflammation, including autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC), and occasionally primary biliary cirrhosis (PBC) (30). In a recent study, elevation of serum aminotransferase was found in one-third of 500 IBD patients (39). Primary sclerosing cholangitis and AIH are common extraintestinal manifestations of IBD, with the majority of PSC patients having IBD (>75%); ulcerative colitis is present in most (~90%), with Crohn’s disease being present in the rest (5, 17). While PSC is typically diagnosed after the diagnosis of IBD, the development of PSC can precede IBD by several years (30). Autoimmune hepatitis is a more classic autoimmune disease, but some patients can be diagnosed with features of both PSC and AIH as an overlap disorder. These forms of chronic liver disease are characterized by portal/perportal inflammation that can lead to fibrosis.

IBD-associated chronic liver inflammation such as PSC appears to be mediated by IFN-γ-producing, microbial-antigen-specific CD4⁺ T cells activated in the gut and recruited to the liver by aberrant expression of mucosal adressin cellular adhesion molecule 1 (MadCAM-1) and chemokines (CCL25) that are typically expressed in the gut (for a review, see Ref. 1). Uregulation of these molecules on the portal endothelium of the liver enables the recruitment of α4β7⁺, CCR9⁺, and CD4⁺ memory T cells to the liver from the gut (1). α4β7 and CCR9 are the ligands for MadCAM and CCL25, respectively. It has been postulated that Toll-like receptors (TLR) on immune [macrophages, Kupffer cells, dendritic cells (DC)] and non-immune cells (cholangiocytes) in the liver are exposed to bacteria/bacterial products via enterohepatic circulation and bind pathogen-associated molecular patterns (PAMPs), become activated, and secrete proinflammatory cytokines and chemokines (TNF-α, IL-1β, IL-6, CCL25) (42) that can participate in the recruitment of adaptive immune cells to the liver (28).

Animal models have been developed to understand the pathophysiology associated with chronic liver inflammation. One class uses bacterial wall components and develop biliary sclerosis after bacterial overgrowth of the small bowel (35) or following injection of bacterial products and induction of experimental colitis in rats and rabbits (36). Non supplicative destructive cholangitis is the main lesion during murine allogeneic GVHD (58). The injection of 2,4,6-trinitrobenzene sulfonic acid into the extrahepatic bile duct induces an antigen-specific immune response that is similar to PSC clinically, including a CD4⁺ T₄₁ immune response with structuring of hepatic bile ducts and induction of portal fibrosis (43). In addition to models of PSC, other murine models have been developed to simulate AIH. The intravenous injection of concanavalin A (32) or the injection of antigen-specific T cells into transgenic mice that express novel antigen on the surface of hepatocytes results in the development of T-cell mediated AIH (11). More recently, PBC was shown to develop in IL-2Rα⁻/⁻ animals. In this model, the CD8⁺ effector cells differentiated in the liver and appeared not to be recruited from other sites (59).

Inflammatory bowel disease represents a chronic inflammation of the intestinal tract of unknown origin. It has been recognized that IBD results from a dysregulated immunological response to commensal bacteria in genetically susceptible patients. The pathophysiology associated with chronic colon inflammation and the development of chronic liver inflammation in the SGVHD model is described.
individuals (48). Experimentally, colitis develops in chemically treated and gene knockout animals and after adoptive transfer of naïve CD4+ T cells into immune-deficient recipients (for a review, see Ref. 49). The majority of these models has been thought to develop as a result of a Th1 cytokine-based immune response that is characterized by excessive production of IL-12/IFN-γ/TNF-α. Recently, however, it has been shown that in addition to Th1 cells, a distinct population of IL-17-producing CD4+ T cells (Th17 cells) can drive intestinal inflammation (for a review, see Ref. 23).

In murine syngeneic graft-versus-host disease (SGVHD), a colitis-like disease developed following lethal irradiation, syngeneic bone marrow transplantation (BMT), and a short course of cyclosporine A (CsA) therapy (7, 18, 19). Clinical symptoms (weight loss, diarrhea) typically occurred in 80–100% of the CsA-treated animals, with lymphocytic infiltration of the colon and liver being observed. In vivo depletion and adoptive transfer studies demonstrated that CD4+ but not CD8+ T cells, played a prominent role in the development of murine SGVHD-associated colitis (6, 10). Similar to murine colitis models, recent studies have demonstrated enhanced responsiveness of SGVHD CD4+ T cells against antigens isolated from cecal bacterial preparations (6), leading to the speculation that CD4+ T cells reactive against bacterial antigens mediate the chronic intestinal and liver inflammation observed during murine SGVHD.

There currently are no established animal models of IBD-associated chronic liver inflammation. In this article, data are presented that demonstrates an absolute linkage between liver inflammation and colitis in the SGVHD model and test the hypothesis that microbe-specific T cells migrate from the colon to the liver to induce chronic liver inflammation. Chronic liver inflammation of intrahepatic/extrahepatic bile ducts was associated with the aberrant expression of colon-associated CAM/chemokines and a Th1/Th17 inflammatory immune response. Given the complete enterohepatic linkage, the role of CD4+ T cells in the SGVHD disease model and the similarity in inflammatory response/lesions with the early lesions associated with clinical PSC, murine SGVHD is a useful model in which to study the immune mechanisms that are essential for the initiation and maintenance of chronic liver inflammation during IBD.

MATERIALS AND METHODS

Animals. Female C3H/HeN mice were purchased from Harlan (Indianapolis, IN) at 19–21 days of age and were used within 1 wk of arrival. Animals were housed in sterile microisolator cages (Lab Products, Maywood, NJ) and were fed autoclaved food and acidified water ad libitum. All animal protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Induction of SGVHD. Bone marrow (BM) was isolated from the femurs and tibias of syngeneic age-matched mice. Donor BM suspensions were prepared in RPMI 1640 (Cellgro, Herndon, VA) containing 100 U/ml penicillin and 100 μg/ml streptomycin and 2 mM glutamine (Gibco, Grand Island, NY) and depleted of Thy-1+ BM cells, as previously described (8). To induce SGVHD, recipient mice were lethally irradiated (900 cGy) in a Mark I137Cs irradiator (J. L. Aldrich, St. Louis, MO). Upon cessation of CsA therapy, the BMT control and CsA-treated animals were weighed 3 times/wk and monitored for the development of clinical symptoms of SGVHD (weight loss, diarrhea). Animals that developed clinical symptoms for three consecutive weighings were considered positive for the induction of SGVHD.

Histological analysis of SGVHD inflammation. Tissue were removed from euthanized animals at the indicated times after BMT and cessation of CsA therapy and placed into 10% buffered formalin. The fixed tissues were embedded in paraffin, cut into 4- to 6-μm sections, mounted onto glass slides, and stained with a standard hematoxylin-and-eosin (H&E) protocol. All slides were analyzed blindly and were graded for inflammation, according to a previously published grading scale (9).

Immunohistochemical staining. Liver and intestinal samples were taken and immediately embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance CA) and frozen in liquid nitrogen. Samples were then cut into 10-μm sections, mounted on glass slides, and stored in −20°C freezer. Tissues were fixed with 3% formalin for 15 min followed by 3 × 5 min washes in PBS. Slides were blocked to prevent nonspecific binding with 2 mg/ml normal donkey serum and 0.3% Triton X-100 in PBS for 30 min at 4°C. Slides were then incubated with FITC-conjugated mAb against CD4 (GK1.5; BD PharMingen, San Diego, CA) (1:1,000) in PBS supplemented with 2 mg/ml normal donkey serum and 0.3% Triton X-100 at 4°C overnight in a humidified chamber. Control staining was performed by using FITC IgG Rat antibody (1:1,000). After 2 washes in PBS, the samples were visualized on a Carl Zeiss microscope ×100 magnification and digitized with the camera AxioVision HR. Because the automatic exposure setting on the microscope camera overcompensated for tissues without T-cell infiltration, postcapture image processing was used to adjust tissue section images to the equivalent levels of background fluorescence.

Quantification of serum markers. Serum/plasma alanine aminotransferase (ALT) concentrations were performed as per manufacturer’s instructions. Briefly 100 μl of serum was mixed with 1 ml of 37°C prewarmed ALT reagent (Pointe Scientific, Canton, MI, USA) and further incubated at 37°C for 1 min before the absorbance at 340 nm was read. An additional two absorbance readings (340 nm) were taken 1 min apart with the sample being incubated at 37°C between readings. The ALT concentration (IU/l) was calculated by multiplying the average absorbance difference per minute (Δabs/min.) by the factor 1.768.

Levels of anti-ssDNA in the serum/plasma of SGVHD mice were determined by ELISA. Briefly, wells of a 96-well plate were coated with 100 μl of ssDNA (heat-denatured salmon sperm DNA at 10 μg/ml in citrate phosphate buffer, pH 5.0). The plates were incubated at 4°C overnight. After washing, the plates were washed twice with TBS containing 0.3% Tween-20 [TBS-T (pH 7.6)]. Plates were blocked with 1% BSA in TBS for 1 h at room temperature. Plates were washed with TBS-T, and experimental samples were diluted 1/20 in TBS-T containing 0.25% BSA, and 100 μl was added per well for 2 h at room temperature. The standard positive control was pooled serum from (3H9 × CD5−−) F1 mice at different dilutions (4). Plates were washed with TBS-T and alkaline phosphatase-conjugated goat anti-mouse Ig (heavy and light chain), diluted 1/1,000 in TBS-T containing 0.25% BSA, and 100 μl was added per well for 2 h at room temperature. Plates were washed with TBS-T and twice with TBS, p-nitrophenyl phosphohosphate was diluted to 1 mg/ml in substrate buffer (phenylamine buffer, pH 9.8) and added to each well for 30 min at room temperature, and the absorbance was read at 405 nm.

Detection of perinuclear antineutrophil cytoplasmic antibodies (p-ANCA) was performed on ethanol-fixed human neutrophil substrate slides (INOVA Diagnostics, San Diego, CA). Substrate slides were placed in a humidified chamber and 20 μl of either sample serum/plasma, positive or negative controls (INOVA Diagnostics) was added.
to the corresponding wells and incubated for 30 min at room temperature. Slides were then washed twice in PBS. Mouse IgG antibodies were detected by FITC-conjugated goat anti-mouse IgG antibodies (Sigma-Aldrich) or goat anti-human IgG antibodies for positive controls (Sigma-Aldrich). After 30-min incubation, the slides were washed twice in PBS and analyzed using a Carl Zeiss microscope at ×200 magnification and digitized with the camera AxioVision HR.

The grading of the p-ANCA results was determined by the level of fluorescence as described by the manufacturer. Depending on the intensity of the staining, the sample was given a fluorescent grade. Intensity grading criteria for fluorescence are as follows: brilliant fluorescent staining of nucleus (grade 4), bright fluorescent staining of nucleus (grade 3), nuclear staining clearly distinguishable, but not bright green (grade 2), lowest specific fluorescence that allows the differentiation of the nucleus from the background staining (grade 1), and no distinction between the nucleus and background staining (grade 0). For this study, a sample that had been graded 2 to 4 was considered positive, and those samples graded either 0 or 1 were considered negative for p-ANCA.

Analysis of cytokine gene expression by real-time PCR. Total mRNA was isolated from the livers using TriZOL reagent (Invitrogen, Grand Island, NY). mRNA (1 µg) from each group was reverse-transcribed into cDNA using the Promega (Madison, WI) reverse transcription system. cDNA was suspended in 1× master mix [0.5 U Platinum Taq (Invitrogen), 0.2 nM of each dNTP, 0.2 mM PCR buffer (Idaho Technology, Salt Lake City, UT, USA), 1 × SYBR Green (Molecular Probes, Eugene, OR)]. The reaction volume was made to 10 µl with ddH2O. Primers for IL-12, IFN-γ, TNF-α (21), IL-17, IL-23p19, CCR9, CCL25, CCL28, MadCAM-1, and GADPH (44) were purchased from Integrated DNA Technologies (Coralville, IA) and were used at 1 µM concentration. Real-time PCR was performed on a Roche LightCycler (Roche Diagnostics, Indianapolis, IN). Reaction conditions were as follows: 1 min at 95°C followed by 50 cycles of 6 s at 95°C, 10 s at 60°C, and 15 s at 72°C. All the primers listed were normalized to GADPH, and their expression was calculated by the comparative cycle threshold (Ct) method.

Isolation of liver-associated mononuclear cells. Livers were isolated from control BMT or SGVHD mice at 2–4 wk after cessation of CsA therapy and perfused with 5 ml of digestion buffer (RPMI 1640, 5% FCS, 0.05% collagenase IV (Sigma-Aldrich), 0.002% DNAse I (Sigma-Aldrich)); then single-cell suspensions were prepared in digestion buffer. The liver homogenate was placed into 50-ml centrifuge tubes and placed in a 37°C water bath for 40 min with intermittent shaking. The cells were washed twice, and the pellet was resuspended in 50-ml staining buffer (PBS containing 1% FBS, 0.1% NaN3). To minimize nonspecific binding, the samples were washed twice in PBS and analyzed using a Carl Zeiss microscope at 200 magnification and digitized with the camera AxioVision HR.

A cecal bacterial antigen (CeAg) preparation was prepared according to the procedure described by Cong et al. (13). Bone marrow-derived dendritic cells (DC) were generated by culturing C3H/HeN BM cells in RPMI 1640 containing 5% FCS, penicillin/streptomycin/glutamine, and 5 mM 2-ME containing 20 ng/ml of recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF). The nonadherent cells were removed on days 3 and 5, and GM-CSF-containing media were added. At 8–10 days after initiation of culture, cecal antigen-pulsed DC were prepared by incubation of DC overnight with 200 mg/ml of CeAg (6). This dose of CeAg was the minimal dose required to induce maximal proliferative response of CD4+ T cells from SGVHD animals. Furthermore, incubation of the DC with CeAg resulted in the maturation of DC resulting in increased expression of major histocompatibility complex (MHC) class II and B7 costimulatory molecules (J. S. Bryson, unpublished observation). To determine the proliferative capacity of liver-associated mononuclear cells (LAM) against bacterial antigens, 2 × 105 LAM from control or SGVHD mice were cultured with 1 × 104 irradiated (2,000 cGy) DC- or CeAg-pulsed DC in a 96-well flat-bottomed plate. Proliferation was measured by the addition of [3H]-thymidine during the last 18 h of a 96-h culture. Flow cytometry. LAM cells were harvested and placed into staining buffer (PBS containing 1% FBS, 0.1% Na3). To minimize nonspecific binding, cells were incubated with Ab against CD16/CD32 (2.4G2, Fc Block, BD Pharmingen). The cells were stained with antibodies against CD4 (RM-4 –5) (Caltag, Burlingame, CA), CD8 (CT-CD8, Caltag), CD11b (M1/70), CD11c, β7 receptor (BD Pharmingen), TLR-2 (eBioscience, San Diego, CA), or TLR-5 (Imgenex, San Diego, CA), and then they were analyzed by flow cytometry. To detect invariant natural killer T cells (iNKT), LAM were stained with αβ T cell receptor (TCR) (H57–597; BD Pharmingen) and α-galactosylceramide (α-GalCer)-loaded CD1 tetramers or unlabelled CD1 tetramers (kindly provided by Dr. Mitch Kronenberg, La Jolla Institute for Allergy and Immunology, San Diego, CA) (38). To determine intracellular cytokine levels, isolated LAM were placed in complete RPMI growth media and stimulated with anti-mouse CD3 ascites for 8 h at 37°C. Two micromoles monensin (eBioscience) was added during the last 4 h of culture, and the cells were harvested, counted, and placed in staining buffer (PBS containing 1% FCS, 0.1% Na3). To reduce nonspecific staining, cells were incubated with Ab against CD16/CD32 (Fc Block). 1 × 106 cells were then stained with fluorochrome-conjugated mAb against CD4 (Caltag). Intracellular staining for IL-17, IFN-γ, and TNF-α was performed using an Intracellular Cytokine Staining Kit (eBioscience, San Diego, CA), according to manufacturer’s directions. Stained cells were analyzed using a BD Biosciences FACSCalibur flow cytometer (San Jose, CA).

Statistical analysis. Statistical differences between groups were determined using Student’s t-test. Differences ≤ 0.05 were considered statistically different.

RESULTS

Chronic liver pathology associated with SGVHD. In addition to CD4+ T-cell-mediated inflammation in the epithelium and lamina propria of the colon (Fig. 1B vs. 1A) (8, 10) with similarity to that observed in other models of murine colitis, significant and reproducible chronic inflammatory lesions are observed in the livers of SGVHD mice. As opposed to other murine models of colitis, where chronic liver inflammation was observed in ~30% of the animals (40), histological analysis of both the colons and livers of SGVHD mice showed an absolute correlation between colitis and liver inflammation (9/9 in the current study). As shown in Fig. 1, the inflammation described for samples taken in the first 2–4 wk (Fig. 1D) after induction of SGVHD resembles the initial portal stage of clinical PSC, with inflammation limited to the portal area with no fibrosis being present (50). The lesions demonstrated significant portal lymphoid infiltrates in and around the intrahepatic bile ducts with cholangioyte necrosis (Fig. 1D). Liver samples taken at 8 wk after cessation of CsA therapy [Fig. 1E (H&E) and 1F (Trichrome)] show extension beyond the limiting plate, resembling the periporal stage (II) of PSC (50). In contrast, an occasional lymphocyte can be observed outside the bile ducts of transplant control animals (Fig. 1C) with no significant inflammatory response being observed. Examination of the extrahepatic bile ducts taken from the SGVHD animals showed apoptotic changes along with the bile duct invasion by inflammatory cells (Fig. 1H). Inflammatory debris was also present in the lumen, as well as inflammatory cells being located in the
surrounding adventitia. Minimal inflammation was detected in the extrahepatic bile ducts isolated from the control BMT animals (Fig. 1G). As shown previously (9), a significantly higher pathology grade was observed in the colon and livers obtained from the SGVHD vs. BMT control animals (Fig. 2). Although slightly reduced in severity, similar changes and pathology grades were observed 1 wk after cessation of CsA therapy, prior to clinical symptoms of SGVHD (J. S. Bryson, unpublished observations).

Similar to what is observed clinically in patients with chronic liver inflammation, elevated levels of ALT were observed in the serum/plasma from SGVHD vs. control BMT animals (Fig. 3A, left). These increases were associated with the liver pathology observed in these animals (Figs. 1 and 2) and were significantly elevated in SGVHD mice. Similar to the increased presence of liver enzymes, significantly increased levels of the autoantibody, anti-ssDNA (Fig. 3B, left), and importantly, perinuclear ANCA (p-ANCA), was observed during active disease as well (92% vs. 10%) (Fig. 3C, left). No differences in the levels of ALT, anti-ssDNA, or p-ANCA autoantibodies were observed between control and CsA-treated animals 1 wk after cessation of CsA therapy. However, the levels of these mediators increased with time after cessation until they were significantly increased by 5 or 6 wk after induction therapy (Fig. 3, A–C, right). Changes in ALT and p-ANCA have been associated with the development of chronic liver and colon inflammation, including AIH and PSC (52, 54, 61).

Phenotypic analysis of LAM isolated from SGVHD animals. In addition to studies monitoring pathology and liver-associated inflammatory markers, preliminary studies were initiated to monitor phenotypic changes that occur in the cellular immune response in the SGVHD liver. Liver-associated mononuclear cells were isolated from transplant control and SGVHD mice and monitored for the presence of CD4+H11001, CD8+H11001, iNKT cells, macrophages, and neutrophils. No significant change was observed in CD4+H11001, CD8+H11001 (Fig. 4A), and neutrophils (J. S. Bryson, unpublished observations) between BMT controls and SGVHD animals. However, although the number of CD4+ T cells did not change, immunohistochemistry analysis showed that CD4+ T cells were primarily localized around the hepatic bile duct in the SGVHD liver (Fig. 4D; white arrows) compared with being more diffusely dispersed throughout the BMT control liver (Fig. 4C). In addition, while no apparent changes in T-cell numbers were observed in the SGVHD liver, a significantly decreased proportion of iNKT cells staining with α-GalCer-CD1d tetramers, in LAM from BMT control vs.
SGVHD mice (Fig. 4E). These cells have been shown to be involved in development of oral tolerance (29, 57) and increase in SGVHD mice that recover from colitis (~30% of diseased animals) (J. Bryson, unpublished observations). Finally, significantly increased percentages of CD11b+ (Fig. 4F) or CD11b+GR-1− myeloid/macrophage cells were observed in LAM from SGVHD vs. control animals. As activation of innate effector cells via signaling through TLR may play a significant role in the generation of adaptive immunity and more pointedly in the initial stages of PSC pathology (53), increases in macrophages may be significant in the development in SGVHD-associated liver inflammation.

**TLR expression in SGVHD.** Gram-positive and Gram-negative bacteria were found in a significantly higher percentage of the livers from CsA-treated vs. control animals (18). As bacteria are a significant source of TLR ligands (PAMPs), it is likely that liver macrophages (Kupffer cells) and other tissue cells come into contact with PAMPs, resulting in activation and secretion of inflammatory mediators. Real-time PCR studies demonstrated that mRNA for all TLR (1–9) was increased in the liver and colon of SGVHD mice. Importantly, mRNA for four of the five TLR that are associated with binding bacterial PAMPs, TLR-1 (J. Bryson, unpublished observations), -2, -5, and -9, were significantly increased (see Supplemental Fig. 1 in the online version of this article). Finally, while antibodies are not available for all TLR, flow cytometry studies were performed using antibodies against TLR-2 and TLR-5 to determine the nature of the LAM cells expressing these TLR. A significant increase in TLR-2 and TLR-5 staining was found in CD11b+ cells isolated from the livers of SGVHD vs. control BMT animals (Supplemental Fig. 2).

**Increased inflammatory mediators in livers of SGVHD mice.** It has been widely suggested that IBD-associated chronic liver inflammation is mediated by Th1 CD4+ T cells (42). Initial studies demonstrated that SGVHD-mediated colon inflammation could be mediated by Th17 or Th1 immune responses.
To determine the nature of the T-helper immune response in the SGVHD liver, LAM were isolated from control and diseased animals, stimulated, and analyzed for the production of IL-17 (TH17) or IFN-γ (TH1) by intracellular cytokine-staining techniques. As shown in Fig. 5A, significantly increased percentages of both IL-17- and IFN-γ-producing CD4+ T cells were observed in LAM isolated from SGVHD vs. control animals. To support and expand these findings, mRNA was isolated from control and SGVHD livers and analyzed by real-time PCR for TH17 and TH1 cytokines and inflammatory mediators that are associated with mucosal/liver inflammation. In the SGVHD liver, there was a significant increase in mRNA for IFN-γ (P = 0.0009) as previously published in this model (19) and as demonstrated clinically (42). Since TH17 cells have been shown to be elevated in IBD (20, 27) and in the SGVHD liver (Fig. 5A), we sought to monitor the levels of TH17-associated cytokines. Real-time PCR analysis of liver RNA demonstrated increased expression of mRNA for IL-17 (P = 0.0075), IL-23p19, and TNF-α (Fig. 5B). In addition, increased mRNA for IL-12p40 (Fig. 5B), a subunit for both IL-23 and the TH1 cytokine IL-12 was found to be significantly increased in diseased livers as well. Finally, inflammatory chemokines and chemokine receptors involved in mucosal T-cell homing (CCL25, CCL28, and CCR9) (Fig. 6, A, B, and D) were also increased in the livers of SGVHD mice (D) were examined by immunohistochemistry for the presence of CD4+ T cells. Significance was determined using unpaired Student’s t-test.
in the percentage of CD4+ expressing the ligand for MAdCAM, β7 integrin, was observed in the LAM from diseased vs. control animals (Fig. 4B). Thus, there was an increase in CD4+ T cells in the SGVHD liver that have a phenotype typical of effector cells that are derived in the intestinal tract.

We have observed that at the end of the CsA therapy (day 21 post-BMT), prior to the development of clinical symptoms, there are increases in the expression levels of mRNA for proinflammatory cytokines, chemokines, and adhesion molecules in the colons of SGVHD animals (J. Perez, J. A. Brandon, D. A. Cohen, C. D. Jennings, A. M. Kaplan, J. S. Bryson, unpublished data). It is proposed that during PSC, CD4+ T cells migrate to the liver following activation in the colon via aberrant hepatic expression of MAdCAM and CCL25 (1) (Fig. 6). To investigate when T cells migrate into the liver of CsA-treated animals, liver tissue isolated from CsA-treated/SGVHD and control animals at 14, 21, and 37 days after BMT was stained for CD4+ T cells. CD4+ T cells were rarely observed in livers from control or CsA-treated animals at 14 and 21 days after BMT (Fig. 7A). Conversely, CD4+ T cells were easily detected around the bile ducts (arrow) of diseased animals (37 days or 2–3 wk post-CsA), as was shown in Fig. 7A.

Furthermore, on the basis of PCR analysis, MAdCAM mRNA was not elevated at day 14 post-BMT (J. Bryson, unpublished observations), and both MAdCAM and CCL25 trended toward increased levels in the liver of CsA-treated mice by day 21 post-BMT (Fig. 7B). Together, these findings suggest that an ordered expression of chemokines and CAM results in the timed migration of CD4+ T cells into the liver.

LAM from SGVHD mice display increased microbial reactivity. Previous studies have demonstrated increased proliferation of peripheral SGVHD CD4+ T cells against bacterial
antigens (6). Since it is known that increased bacteria were present in the livers of CsA-treated animals (18), studies were performed to analyze the antibacterial antigen-specific proliferation of LAM isolated from SGVHD mice. LAM isolated from the livers of SGVHD mice demonstrated a significantly enhanced proliferative response against cecal bacterial antigens compared with control liver cells \( (P = 0.0002) \) (Fig. 8).

Fig. 6. Increased levels of liver inflammatory chemokines and adhesion molecules in SGVHD livers. Hepatic mRNA was extracted from SGVHD mice and cDNA was prepared. Inflammatory chemokine and adhesion molecule mRNA was analyzed using real-time RT-PCR for the primers CCL25, CCL28, CCR9, and MAdCAM-1. Expression of these genes was normalized to GAPDH using the \( \Delta\Delta C_{\text{T}} \) method. Represents pooled data from 3 experiments; \( n = 12 \) per group with significance determined using unpaired Student’s \( t \)-test.

Fig. 7. \( CD^{4^+} \) T cells migrate to the liver after cessation of CsA therapy. Livers were isolated at 14, 21, and 37 days from SGVHD and control animals and analyzed by immunohistochemistry for the presence of \( CD^{4^+} \) T cells (A) or for MAdCAM mRNA (B) 21 days after BMT by real-time RT-PCR. Data presented are representative of 4 tissues from two experiments (A), or pooled samples (B; means \( \pm \) SE) from two experiments, \( n = 8 \) and were analyzed using unpaired Student’s \( t \)-test.
expression is elevated during CsA-therapy as early as post-BMT and CD4
molecules associated with IBD (30). The current study describes the pathogenesis of chronic liver lesions that develop during the induction of murine SGVHD. Murine SGVHD is a unique model of colon inflammation with 100% penetrance of chronic liver inflammation that involves intrahepatic and extrahepatic bile ducts. SGVHD-mediated liver disease was associated with increased ALT and p-ANCA levels similar to clinical markers of chronic liver inflammation. Liver mRNA levels of molecules associated with chronic inflammation and lymphocyte migration were increased along with TH1- and TH17-associated immune responses. Finally, increased reactivity of hepatic LAM to colonic bacterial antigen-pulsed DCs was observed, suggesting that in SGVHD, microbial antigen-specific T cells may be responsible for the chronic inflammation that is observed in the liver. The utilization of murine SGVHD provides an opportunity to study the immune responses involved in both the initiation and progression of IBD-associated chronic liver inflammation.

It has been suggested that the lymphocytic infiltration that is seen in IBD-associated liver inflammation comprises cells that were activated within the gut and have migrated to the liver, aided by the aberrant expression of gut homing molecules (1). Studies in the SGVHD model have shown that colon CAM expression is elevated during CsA-therapy as early as day 14 post-BMT and CD4+ T cells begin to accumulate in the colon at this time as well. (J. Perez, J. A. Brandon, D. A. Cohen, C. D. Jennings, A. M. Kaplan, J. S. Bryson, unpublished data) (10). Although the basis for this early induction of CAMs and T-cell accumulation in the colon is not known at this time, we hypothesize that pretransplant radiation and CsA treatment in the early post-BMT period result in the production of inflammatory mediators, including TNF-α. At least two possibilities exist to explain the early proinflammatory response that occurs during the induction of SGVHD. First, both radiation and CsA have been shown to induce oxidative stress and transcription factors (14, 51, 60, 64) that could mediate the upregulation of inflammatory cytokines (12, 25, 34, 63). In addition to direct involvement in the production of inflammatory mediators, irradiation and CsA have been shown to damage the gut, leading to increased leakage of bacteria (18). The interaction of TLR on innate effector cells with microbial products (PAMPs) is an alternative pathway that could focus the enhanced production of proinflammatory cytokines such as TNF-α within the colon, leading to increased expression of CAM and in accumulation of CD4+ T cells in the colons of CsA-BMT animals. Why the expression of these molecules is delayed in the liver, is not clear at this time but may relate to differences in the effects of the inductive therapy on the target organs. On the basis of real-time PCR data, MadCAM-1 mRNA was not elevated at day 14 post-BMT, but along with the chemokine, CCL25, trended toward increased levels in the livers of CsA-treated mice by day 21 post-BMT (Fig. 7). Few, if any, CD4+ T cells were observed in the livers in the early post-transplant period (days 14 or 21) (Fig. 7) but were observed during active disease (Figs. 4 and 7). Phenotypically, increased numbers of CD4+ T cells expressing markers of intestinal-derived T cells, β7 integrin, and CCR9, were found in the livers of SGVHD animals compared with controls. Together, these findings suggest CD4+ effector cells are activated in the intestinal tract and that an ordered migration of CD4+ T cells from the colon into the liver of SGVHD animals exists.

Hepatic T lymphocytes found in IBD-associated liver inflammation express the CCR9+ α4β7+ phenotype classically seen in lymphocytes that have been activated by gut DCs (2). Abnormal expression of the mucosal addressin, MadCAM-1, along with the gut-associated chemokine CCL25, has been observed at elevated levels in the liver endothelium in PSC (1, 26) and provided a mechanism by which CCR9+ T cells activated in the intestinal tract can migrate to the liver. The mechanisms by which these inflammatory molecules are upregulated in the liver are unknown but may involve the activation of TLR ligands with microbial PAMPs that arrive in the liver via entero-hepatic circulation resulting in the production of inflammatory mediators. Levels of TLR mRNA that bind bacterial PAMPs, as well as TLR-expressing myeloid LAM were elevated in livers from SGVHD animals (Supplemental Figs. 1 and 2). This is supported further by previous data demonstrating that increased numbers of bacteria can be detected in the livers of CsA-treated mice during the induction of SGVHD (18). Furthermore, similar to peripheral effector cells from diseased animals (6), the LAM isolated from SGVHD mice demonstrated an increased proliferative response to bacterial antigen-pulsed antigen-presenting cells. We have previously shown that isolated CD4+ T cells from the peripheral lymphoid tissues of SGVHD mice could adoptively transfer colitis and liver inflammation into secondary recipient animals (6). The ability of LAM isolated from the livers of SGVHD mice to transfer disease to secondary recipient animals has yet to be determined.

Damage to liver bile ducts during IBD-associated liver inflammation is thought to be mediated by TH1, CD4+, IFN-γ-producing T cells. Hepatocellular damage through IFN-γ is thought to occur by a variety of mechanisms, including direct cellular injury, modulation of Ag presentation, and both the recruitment and activation of other immune cells (15, 55).

**Fig. 8.** LAM from SGVHD livers showed an increased proliferative response to cecal bacterial antigen (CeAg)-pulsed dendritic cells (DC). LAM were isolated from BMT control and SGVHD mice 4 wk after cessation of CsA therapy. Proliferation against CeAg-pulsed DC in the presence or absence of anti-CD4 mAb was measured by thymidine incorporation during the last 18 to 96 h of the assay. Data comparing CeAg-induced proliferation are representative of 3 experiments and represent means ± SE of triplicate samples, and significance was determined using unpaired Student’s t-test. CD4 blocking represents results from a single experiment. *Significantly different from control LAM stimulated with DC-CeAg: *P = 0.0029. **Significantly different from SGVHD DC-Ag: *P = 0.0231.

**DISCUSSION**

Chronic inflammatory processes of the liver are frequently associated with IBD (30). The current study describes the pathogenesis of chronic liver lesions that develop during the induction of murine SGVHD. Murine SGVHD is a unique model of colon inflammation with 100% penetrance of chronic liver inflammation that involves intrahepatic and extrahepatic bile ducts. SGVHD-mediated liver disease was associated with increased ALT and p-ANCA levels similar to clinical markers of chronic liver inflammation. Liver mRNA levels of molecules associated with chronic inflammation and lymphocyte migration were increased along with TH1- and TH17-associated immune responses. Finally, increased reactivity of hepatic LAM to colonic bacterial antigen-pulsed DCs was observed, suggesting that in SGVHD, microbial antigen-specific T cells may be responsible for the chronic inflammation that is observed in the liver. The utilization of murine SGVHD provides an opportunity to study the immune responses involved in both the initiation and progression of IBD-associated chronic liver inflammation.

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However, the immune phenotype of the cells that induce autoimmune chronic liver inflammation has come into question, with Th17 immunity being observed as well (22, 45). Data presented here indicate that Th17 cells, determined by increased intracellular cytokine production of IL-17 by CD4\(^+\) T cells and increased mRNA levels of Th17-associated cytokines (IL-17, IL-23p19, TNF-\(\alpha\)), were increased in the livers of SGVHD mice. We have also found a significant increase in Th17-associated cytokines in the colons of SGVHD mice (J. A. Brandon, C. D. Jennings, A. M. Kaplan, J. S. Bryson, unpublished data). This increase was observed even at day 21 post-BMT in CsA-treated mice, suggesting that Th17 T cells may have an important role in SGVHD-associated colitis. As increased percentages of CD4\(^+\) Th17 were also found in diseased livers, the role that Th17 and Th1 CD4\(^+\) T cells play during SGVHD-induced chronic liver inflammation remains to be determined. It should be noted that while Th17 cells are present in the PBC livers of IL-2R\(\alpha^{-/-}\) animals, a model of chronic liver inflammation, the data suggest that the IL-17-secreting cells differentiate in the liver and appear not to be recruited from other sites (33), with no enterohepatic linkage being observed in the development of the autoimmune PBC. Finally, both Th17 and Th1 cells have both been detected in an autoimmune model of skin inflammation. These cells developed with different kinetics with Th17 cells emerging early, being replaced by a Th1 CD4\(^+\) immune response later in the disease process (37).

It is likely that altered immune regulation in the periphery and in the liver following induction of SGVHD contributes to the development of the chronic inflammatory responses described in the current manuscript. CD4\(^+\) regulatory T cells have been shown to regulate the development of spontaneous and inducible murine colitis (3). Hess et al. (24) have demonstrated that altered regulatory T-cell activity contributed to the development of rat SGVHD with reduced numbers of regulatory lymphocytes being observed in diseased animals (24). Similarly, in a model of CsA-induced autoimmunity, it was shown that reconstitution of CD4\(^+\)CD25\(^+\) regulatory T cells was delayed until after cessation of CsA treatment (62). With these findings in mind, we have demonstrated that at the time of cessation of CsA therapy, significantly reduced numbers of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T cells are present in the spleen and mesenteric lymph node of CsA-treated animals (J. S. Bryson, unpublished observations). Similar to the reduced expression of regulatory T cells in the SGVHD animal, results presented in the current article demonstrated that another T-cell population with regulatory potential, inNKT cells, was significantly reduced in the livers of SGVHD vs. control BMT animals (Fig. 4E). Invariant NKT cells in the mouse express a single invariant V\(\alpha\) TCR chain (V\(\alpha14\alpha18\)) (for a review, see Ref. 31), are positively selected to the nonclassical MHC molecule CD1d, have strong reactivity to the glycosphingolipid \(\alpha\)-GalCer, and are present in high numbers in the liver. Liver iNKT cells have been shown to participate in the induction of oral tolerance (29, 57), tolerance to antigens in privileged sites (47), and inhibit the development of experimental colitis in mice (41, 46, 56). Significant reductions in CD4\(^+\) Treg and inNKT are observed in the periphery and livers of SGVHD animals, respectively. Thus, as a result of CsA therapy, reconstitution of these important regulatory immune populations is delayed, and uncontrolled expansion of T-effector cells occurs, resulting in the development of chronic colitis and ultimately, liver inflammation. In line with this hypothesis, we have demonstrated that recovery from SGVHD (~30% of diseased animals) is associated with increases in regulatory T cells and iNKT cells and that CD4\(^+\)CD25\(^+\) regulatory T cells from normal mice inhibited the adoptive transfer of SGVHD (6) (J. S. Bryson, unpublished observations), demonstrating the potential role of these cells in controlling chronic inflammation in this model system.

In other murine models of colitis, the development of chronic liver inflammation was only observed in ~30% of mice (40). Significant and reproducible inflammatory liver lesions of bile ducts were observed in 100% of SGVHD mice in association with increased inflammatory mediators within the liver that are normally associated with colitis. Furthermore, significant changes in inflammatory markers associated with chronic liver inflammation were observed, suggesting a mechanistic relationship between the development of SGVHD-induced liver inflammation and that observed in the early stages of clinical disease. Similarities include the induction of inflammatory molecules that result in the influx of CCR9\(^+\) \(\beta\) integrin\(^+\) CD4\(^+\) T cells and inflammation of the intrahepatic and extrahepatic bile ducts. And while the model mimics many aspects of the disease process associated with chronic liver inflammation, at the time points analyzed thus far, little to no sclerosis has been observed. Whether this is a limitation of the model or merely a timing issue in relation to disease induction remains to be elucidated. With the enterohepatic linkage and the previously published role of CD4\(^+\) T cells in the SGVHD model (6, 10), the inflammatory response seen in these animals may provide an opportunity to study the pathobiology involved in both the initiation and progression of IBD-associated, chronic liver inflammation.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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