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Novel Model of Antigen-Specific Induction of Bile Duct Injury

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Abstract

Background & Aims—Biliary-directed inflammation is an important cause of acute and chronic liver disease. We developed and characterized a transgenic mouse model of immunemediated hepatobiliary injury.

Methods—Ovalbumin (OVA)-BIL mice were developed using 3.0 kilobase of the rat apical sodium-dependent bile acid transporter promoter to drive aberrant expression of a membrane form of ovalbumin (OVA) on biliary epithelium. Liver inflammation resulted from adoptive transfer of OVA-specific T cells. Liver immune cells were characterized to determine the mechanism of the response by assessing activation, proliferation, and intracellular cytokine expression.

Results—OVA-BIL transgenic mice were tolerant to OVA, without evidence of liver disease. Adoptive transfer of OVA-specific CD4⁺ and CD8⁺ T cells into naïve OVA-BIL mice led to biliary-centered necroinflammatory damage in a dose-dependent manner. This inflammation absolutely required CD8⁺ T cells and was augmented by CD4⁺ T cells. Adoptively transferred OVA CD8⁺ cells homed to and proliferated in the liver but not the spleen. These activated, adoptively transferred cytotoxic T lymphocytes produced elevated levels of tumor necrosis factor α and interferon γ .

Conclusions—T-cell recognition of antigen aberrantly expressed on bile duct epithelium induced an acute necroinflammatory response specific to the liver, with activation, proliferation, and cytokine production predominantly by the OVA-specific cytotoxic T cells. Thus, OVA BIL represents an antigen-specific animal model of inflammatory bile duct injury.

The liver is the first line of defense against gut-derived antigens and pathogens entering via the portal vein and must maintain a balance between tolerance to incoming antigens and generation of an immune response. It is rich in immune cells whose composition and state of activation differ from those of other lymphoid organs. Intrahepatic lymphocytes compose

25% of nonparenchymal cells in the normal liver and include natural killer T (NKT) cells, (NK) cells, and $\gamma\delta$ T cells.¹⁻³ In contrast to peripheral blood, CD8 T cells are more abundant than CD4 T cells in the liver.

Biliary damage can result from obstruction, infection, allograft rejection, and autoimmune diseases with varying outcomes from a fibrotic response to bystander hepatocellular damage. Although the tissue damage in many of these diseases appears because of an immune reaction, little is known about the way the disease is initiated or regulated because studies of pathogenesis are hampered because the patients typically present for care only after significant liver damage has occurred, many years after induction of disease. Similar pathologic and radiologic findings may be caused by diverse etiologies; for example, abnormalities remarkably similar to primary sclerosing cholangitis can be seen secondary to cryptosporidium infection in the immunocompromised host or to ischemic problems following liver transplantation.^{4,5} In addition, the phenotype of a specific disease may change, as in some children who transit from autoimmune hepatitis into sclerosing cholangitis over time.⁶ To begin to gain insight into these processes, we sought to develop a mouse model of immune-mediated hepatobiliary injury and to evaluate early immune responses after recognition of antigen on biliary epithelium.

Materials and Methods

DNA Construct

Apical sodium-dependent bile acid transporter (ASBT)-membrane-bound ovalbumin (mOVA) complementary DNA (cDNA) was constructed using the rat ASBT promoter,⁷ a membrane-bound ovalbumin (OVA) (amino acids 139–385)⁸ and a polyA tail (pDo15-polyA). The membrane-bound form of OVA consists of a fusion protein made up of the first 118 residues of the human transferrin receptor (including cytoplasmic tail and signal/anchor domain) linked to residues 139–385 of mature OVA, transferrin receptor-ova (TFR-OVA), targeting membrane expression of OVA on biliary epithelium. The 5' flanking region of the ASBT gene was cut using BamHI and BstXI sites (all restriction enzymes from New England Biolabs, Ipswich, MA), resulting in a 3.0-kb fragment that was used in the ASBT-mOVA construct. The TFR-OVA fragment was cut from the rat insulin promoter-mOVA plasmid⁸ by digestion with HindIII and XbaI, and then the HindIII site was blunted. The ASBT promoter and TFR-OVA fragments were then ligated into the pBSKS vector (Stratagene, La Jolla, CA). The polyA region was a rabbit β -globin gene isolated from the pDOI-5 plasmid from BamHI to XhoI and was blunted at 2 ends⁸ and was then inserted into the NotI site of the pBSKS-ASBT-mOVA. Its orientation was checked after insertion. After isolation of the plasmid, restriction enzyme mapping was performed, and TFR-OVA was sequenced to verify that the construct was correct. Vector sequence was excised by digesting with BamHI and SacII. The purified DNA was microinjected into the pronuclei of the C57BL/6 oocytes (Jackson Laboratory, Bar Harbor, ME).

Development of OVA-BIL Transgenic Mice

C57BL/6 oocytes were injected with the purified DNA constructs. Weanling mouse tail DNA was extracted and tested by polymerase chain reaction (PCR) for mOVA. Primers

specific for ASBT-OVA genotyping were 5'GGCGTGTGAAAGTAAGC and 3'CCAGACAGATTGGCTGAAGAGCTA. After 30 cycles, denaturation (95°C for 45 seconds), primer annealing (55°C for 45 seconds), and primer extension (72°C for 1 minute) produced a single band of 994 base pair (bp) on ethidium bromide-stained 1% agarose gels. Tail DNA from OVA-BIL mice was positive for the transgene by PCR, which was confirmed by Southern blot analyses: 6 founders lines of OVA-BIL were chosen. Mice were housed in sterile isolator cages maintained under clean, specific pathogen-free conditions, according to NIH guidelines (Publication 86-23). The animal studies committees of the University of California-San Francisco and the Washington University School of Medicine approved the protocol.

Development of Biliary Inflammation in OVA-BIL Transgenic Mice

OT-I T-cell receptor transgenic mice (Jackson Laboratory) produce OVA-specific, major histocompatibility complex (MHC) class I H-2^b restricted CD8⁺ cytolytic T cells (OT-I T cells) that recognize the SIINFEKL peptide sequence at residues 257–264 of OVA. OT-II T-cell receptor transgenic mice produce MHC class II I-A^b restricted CD4⁺ helper T cells (William Heath and J.F.A.P. Miller, the Walter and Eliza Hall Institute, Melbourne, Australia) that recognize a different T-cell epitope at residues 323–339 of OVA.⁸ OT-I and OT-II transgenic cells utilize V α 2 V β 5 as their T-cell receptor (TCR) compared with <2% of nontransgenic C57BL/6 mice. Because there is no clonotypic antibody, monoclonal antibody (mAb) staining for V α 2 was used to identify adoptively transferred OVA-specific T cells. Varying numbers of T cells from OT-I and OT-II mice or normal controls were injected intraperitoneally (IP) into OVA-BIL transgenic mice or control C57BL/6 mice. Serial serum alanine aminotransferase (ALT) was performed using the GP Transaminase kits (Sigma Chemical Co, St. Louis, MO) to evaluate liver inflammation, with a normal range of 10–23 U/L. Serum alkaline phosphatase was assayed in the clinical laboratory, with a normal range of 29–111 U/L. Serum ALT was found to be the more sensitive marker of inflammation and was used for routine studies. At various time points, mice were killed, and histologic sections of the liver were studied and scored without knowledge of the nature of the animal, for evidence of portal and lobular inflammation.⁹

Immunohistochemical Analyses

Mice were killed at varying days after T-cell transfer, and tissues were placed in neutralized 10% formalin solution (J. T. Baker, Phillipsburg, NJ) or frozen in Optimal Cutting Temperature compound (OCT: Miles Inc, Elkhart, IN). Briefly, cryostat sections of frozen tissues were fixed in ethanol at 4°C for 10 minutes, air-dried, and stored at –20°C. Rabbit anti-OVA antibody was obtained by immunizing rabbits with whole OVA (OVA grade V; Sigma Chemical Co) and was used to confirm OVA expression on biliary epithelium. Sections were preincubated in blocking serum for 15 minutes and then incubated with rabbit anti-OVA antibody (40 μ g/mL) at 4°C overnight followed by incubating with FITC or PE conjugated anti-rabbit IgG (Imgenex, San Diego, CA).

Hepatic Mononuclear Cells Isolation and FACS Analyses

OVA-BIL mice were killed at varying intervals after adoptive transfer of OT-I and OT-II splenocytes. Livers were perfused free of blood and then perfused with collagenase (0.1

mg/mL in phosphate-buffered saline [PBS; Sigma Chemical Co) via the portal vein. The livers were excised, strained through a 70-micron filter, and centrifuged on a discontinuous Percoll gradient. The interface contained mononuclear cells (MNC) >98% CD45 positive by flow cytometry. Spleens were isolated and homogenized through a 70-micron filter. Liver and spleen MNC cells were counted, centrifuged, raised in FACS buffer (15.4 $\mu\text{mol/L}$ NaN_3 in 2% fetal bovine serum [FBS]/PBS) to a concentration of $5\text{--}15 \times 10^6$ cells/mL and treated with antimouse CD16/CD32 (1 μg /million cells) to inhibit crystallizable fragment of immunoglobulin G-mediated binding. All mAb were obtained from BD Pharmingen, San Diego, CA. Cells were labeled for 45 minutes with fluorescein (FITC), phycoerythrin (PE), PE-Cy5.5, and APC conjugated mAb to TCR $\text{V}\alpha 2$ (B20.1) (CD45), B lymphocytes (CD19), T lymphocytes (CD3, CD4, and CD8), NK cells (NK1.1), Kupffer cells (CD16), macrophages (CD11b), activation antigens CD69 and CD25, and Fas ligand (CD178). They were subsequently washed with 3 mL of FACS buffer and fixed with 1% paraformaldehyde in FACS buffer and analyzed by flow cytometry. Peripheral blood B cells were evaluated by FACS after separation of whole blood with Lympholyte-M (Cedarlane Labs, Hornby, Ontario, Canada) and staining with B-cell markers as noted above.

Cell division in vivo was measured by labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes). OT-I spleens were excised, strained through a 70-micron filter, and raised in PBS to a concentration of 2×10^7 cells/mL. CFSE in DMSO (1 mg/mL) was added to a final concentration of 5 $\mu\text{mol/L}$ CFSE, and the cells were stained for 5 minutes at 37°C. The reaction was quenched with 10% FBS/RPMI, washed, confirmed to be >90% CFSE labeled by flow cytometry, and injected into OVA-BIL mice. At varying times, the mice were killed, and the liver and spleen MNC were analyzed by flow cytometry using phycoerythrin conjugated mAb to CD45, CD4, CD8, CD19, and NK1.1.

Intracellular cytokine staining was performed on liver and splenic MNC after treatment with Brefeldin A (1 μL /million cells; eBioscience, San Diego, CA) for 2 hours at 37°C. Next, MNC were washed with 3% FBS/PBS and stained with mAb to various cell surface antigens. MNC were then fixed with 4% paraformaldehyde/PBS, washed, permeabilized with 0.1% saponin/PBS, and stained with mAb to the intracellular cytokines interferon (IFN)- γ and tumor necrosis factor (TNF)- α (BD Pharmingen, San Diego, CA). After 15 minutes incubation, MNC were washed with 3% FBS/PBS and analyzed by multicolor flow cytometry. In a subset of experiments, the liver and splenic MNC were prestimulated with 80 ng phorbol myristate acetate (PMA) and 2 μg ionomycin (Sigma Chemical Co) per 1×10^6 cells, and then they were stained with mAb to intracellular cytokines interleukin (IL)2, IL-4, IL-10, IL-12, IFN- γ , and TNF- α and analyzed by multicolor flow cytometry. Autoimmune markers: serum was assayed for immunoglobulins, antinuclear antibodies, and antimitochondrial antibodies as described.^{10,11}

Results

Ovalbumin Expression in OVA-BIL Mice

The liver, heart, kidney, small intestine, colon, lung, and brain of OVA-BIL transgenic mice were studied for expression of OVA by immunofluorescence. OVA mAb stained the biliary

epithelium but not the hepatocyte membrane of all 4 transgenic murine livers studied (Figure 1A). OVA mAb also stained the apical border of ileal epithelial cells but not other organs studied (data not shown). Although the thymus did not stain for OVA by immunohistochemistry (IHC), reverse-transcription PCR analysis of the thymus revealed low-level OVA expression (data not shown). OVA-BIL mice developed normally. There was no liver inflammation, as assessed by serum ALT, serum alkaline phosphatase, or histology in OVA-BIL transgenic mice housed up to 2 years of age (Figure 1B).

Adoptive Transfer of OVA-Specific T Cells to OVA-BIL Mice Results in Bile Duct Inflammation

Transfer of naïve OT-I splenocytes and naïve OT-II splenocytes IP into OVA-BIL transgenic mice led to inflammation in the liver (Figure 1C–E) but not other organs as assessed by histology. At time of peak inflammation, 7–10 days after adoptive transfer, there was extensive inflammation in every portal triad in all 15 mice studied. This was centered on the bile ducts, with infiltrating lymphocytes in the bile duct epithelia, apoptotic hepatocytes, and loss of biliary epithelial cells as well as interface hepatitis (Figures 1C–E). There was no inflammation of the gallbladder (Figure 1F) or the ileum (Figure 1G) of OVA-BIL mice after adoptive transfer of OVA-specific CD4 and CD8 T cells (Figure 1F and G).

The inflammatory response was time dependent. Following adoptive transfer of 10×10^6 OT-I and 4×10^6 OT-II splenocytes, there was a dramatic expansion of liver MNC (Figure 2A) without change in the splenic MNC population. Prior to transfer, there were $6.1 \pm 0.5 \times 10^6$ MNC (mean $\pm$ SEM) in the liver. After adoptive transfer, the liver MNC population increased more than 8-fold to $55.0 \pm 10.9 \times 10^6$ MNC (mean $\pm$ SEM) by day 10. Serum ALT increased from baseline to peak at 10 days (Figure 2B) after adoptive transfer and returned to normal by 28 days. Histologic sections obtained daily, from 1-day post-adoptive transfer of OVA-specific T cells, all show bile duct damage with interface hepatitis around the portal triads increasing with time to a maximum by days 7–10. In addition, the liver inflammation in histologic sections also resolved by day 28 (data not shown). There was no change in mortality of mice following adoptive transfer of OT-I and OT-II splenocytes from untreated OVA-BIL or normal C57BL/6 mice.

The extent of inflammation and injury was dependent on the number of T cells transferred, as shown in Figure 3 by peak serum ALT. There was increasing serum ALT with adoptive transfer of increasing OT-I splenocytes. Serum ALT was less than 100 IU/L after adoptive transfer of 2×10^6 OT-I and 1×10^6 OT-II splenocytes (data not shown). Adoptive transfer of 5×10^6 OT-I and 2×10^6 OT-II splenocytes led to a peak serum ALT of 228 ± 52 IU/L (mean $\pm$ SEM; Figure 3B), approximately 8 times baseline. Following adoptive transfer of 10×10^6 OT-I and 4×10^6 OT-II splenocytes, the serum ALT peaked at 10 days at 846 ± 192 IU/L (mean $\pm$ SEM; Figure 3A), over 30 times the baseline values. In contrast, serum alkaline phosphatase only doubled at time of maximal ALT. Because serum ALT was found to be the more sensitive marker of necroinflammation, it was used for further studies. Only when OT-I cells were transferred into OVA-BIL recipient mice did inflammation result. Adoptive transfer of 2, 5, or 10×10^6 OT-II T splenocytes alone did not lead to liver inflammation as assessed by ALT or histology. Adoptive transfer of 5 million OT-I cells

alone induced less inflammation with peak serum ALT of 44 ± 13 IU/L (mean $\pm$ SEM), indicating that OT-II CD4⁺ T cells are required for maximal hepatobiliary inflammation in OVA-BIL (Figure 3C). Transfer of OT-I T splenocytes (primed or unprimed with OVA) into normal littermate C57BL/6 mice failed to generate inflammation (Figure 3D). In addition, in vitro activated nonantigen-specific C57BL/6 CD8 T cells adoptively transferred into OVA-BIL or normal C57BL/6 littermates failed to generate inflammation (data not shown). Thus, acute inflammation in this model required CD8⁺ OVA-specific cells adoptively transferred into OVA-BIL recipient mice and was maximal when antigen-specific CD4⁺ cells were transferred as well.

Characteristics of Liver MNC During Development of Hepatobiliary Inflammation

The MNC present in the liver during inflammation were then characterized. The largest increase was in the CD8⁺ population (Figure 4A), followed by a smaller increase in CD4. The liver B-cell population did not change (data not shown). The CD8⁺ cells were predominantly V α 2⁺ (Figure 4B), corresponding to the adoptively transferred OT-I T cells.

Innate immune cells, NK, and NKT cells also increased after initiation of hepatobiliary damage (Figure 4B). However, NKT cells in the liver are known to be heterogeneous and CD8⁺ T cells can express NK1.1 after activation.^{12,13} For this reason, we evaluated NK1.1 expression in the OVA-specific T-cell population. Total NKT cell population increased 6.6-fold after adoptive transfer of OT-I and OT-II splenocytes by day 10, but the NK1.1⁺, CD8⁺, TCR⁺ cells increased 21-fold. V α 2⁺ expression in this population was negligible (<0.1%) prior to adoptive transfer, but, 10 days after adoptive transfer of OT-I and OT-II splenocytes, 55% of the NK1.1⁺, CD8⁺, TCR⁺ cells were V α 2⁺ positive, indicating they were activated OVA-specific CD8 T cells.

To track homing and proliferation of OT-I cells, OT-I splenocytes were labeled with CFSE prior to adoptive transfer with unlabeled OT-II splenocytes into OVA-BIL mice. After 3 days, the CFSE-labeled OT-I splenocytes were found in the liver and were vigorously dividing (Figure 4C). In contrast, no proliferation of CD8⁺ /OT-I cells was seen in the spleen of OVA-BIL mice. The same results were obtained when purified CD8 lymphocytes from OT-I spleen were adoptively transferred after CFSE labeling (data not shown). Thus, the majority of CD8⁺ T cells in the liver after initiation of inflammation arose because of homing and proliferation of antigen-specific cells.

With development of liver inflammation, expression of activation markers CD25 and CD69 increased. After adoptive transfer of OT-I and OT-II splenocytes in OVA-BIL mice, the number of liver MNC expressing CD25 increased markedly from $1.0 \pm 0.3 \times 10^5$ to $21.1 \pm 7.6 \times 10^5$ (mean $\pm$ SEM). CD8 cells accounted for the majority of this increase; at baseline there were 0.1×10^5 CD8⁺ cells expressing CD25 and at peak inflammation $13.1 \pm 5.1 \times 10^5$ CD8⁺ cells expressed CD25 (mean $\pm$ SEM). During liver inflammation, expression of CD69 also increased from $4.5 \pm 1.6 \times 10^5$ to $26.7 \pm 6.9 \times 10^5$ (mean $\pm$ SEM). The cells most responsible for this increase were CD8 (baseline $0.5 \pm 0.2 \times 10^5$ to peak $11.4 \pm 3.3 \times 10^5$; mean $\pm$ SEM) and CD4 (baselines $0.7 \pm 0.3 \times 10^5$ to peak $8.3 \pm 2.9 \times 10^5$; mean $\pm$ SEM). These increases in CD69 and CD25 are consistent with the marked proliferative response of antigen-specific T cells.

Fas Ligand Expression During the Immune Response

To determine the role of Fas-mediated apoptosis, Fas ligand expression on T cells was measured at varying times after adoptive transfer of OVA-specific splenocytes. Fas ligand expression by CD8 T cells increased markedly from $0.014 \pm 0.007 \times 10^6$ at day zero to $1.2 \pm 0.2 \times 10^6$ (mean $\pm$ SEM) at peak inflammation on day 10. The increase was predominantly due to adoptively transferred V α 2 CD8⁺ cells whose Fas ligand expression increased from a baseline of $0.003 \pm 0.002 \times 10^6$ to $1.1 \pm 0.2 \times 10^6$ (mean $\pm$ SEM) during the peak immune response. There was a more modest increase in CD4 T-cell Fas ligand expression from $0.023 \pm 0.013 \times 10^6$ to $0.38 \pm 0.02 \times 10^6$ (mean $\pm$ SEM) at peak inflammation.

Serum Markers

Serum B-cell levels were evaluated and, like the intrahepatic B cell level, did not change following adoptive transfer. At day zero, B cells composed $50.8\% \pm 1.9\%$ of peripheral blood mononuclear cells, and, at peak inflammation on day 10, they composed $48.4\% \pm 0.6\%$ (mean $\pm$ SEM of 3 mice). Serum immunoglobulin G levels were 1.78 ± 0.39 mg/cc at baseline and 1.72 ± 0.25 mg/cc at peak inflammation. These levels are lower than levels seen in NZB/W lupus-prone mice.¹⁰ Serum anti-mitochondrial antibody and anti-nuclear antibody levels were measured at baseline and peak inflammation in 6 mice. Autoantibodies were not detected prior to adoptive transfer or during peak inflammation at day 10.

Cytokine Production During Hepatobiliary Inflammation

Intracellular cytokine expression by liver MNC and subpopulations was evaluated at varying time points after adoptive transfer of OT-I and OT-II splenocytes into OVA-BIL mice. Liver MNC were isolated, stimulated with PMA and ionomycin, and then analyzed. Following adoptive transfer, liver MNC had easily discernable expression of TNF- α and IFN- γ and few but discernable cells expressing IL-2, IL-4, and IL-10 (data not shown). As TNF- α and IFN- γ predominated after in vitro stimulation, we studied the number of TNF- α - and IFN- γ -producing cells in vivo (without in vitro PMA/ionomycin stimulation) during hepatobiliary inflammation. IFN- γ (Figure 5A) and TNF- α (Figure 5B) production by liver MNC was low at baseline and peaked at day 10. This increase was predominantly due to the dramatic increase in cytokine production by adoptively transferred V α 2⁺ CD8⁺ CTL (Figure 5A and B). Total IFN- γ production increased from $2.2 \pm 0.5 \times 10^5$ to $32.0 \pm 12.0 \times 10^5$ (mean $\pm$ SEM); the predominant cells expressing IFN- γ were V α 2⁺ CD8⁺. Total TNF- α production increased from $1.8 \pm 0.5 \times 10^5$ to $30.9 \pm 9.7 \times 10^5$ (mean $\pm$ SEM) and was mainly due to V α 2⁺ CD8⁺ CTL. Although the majority of both TNF- α and IFN- γ was produced by V α 2⁺ CD8⁺ T cells, there was also an increase in intracellular TNF- α and IFN- γ staining by NKT and NK cells and CD4⁺ lymphocytes (data not shown).

Discussion

OVA-BIL is a novel antigen- and organ-specific model of T-cell-mediated destruction of bile ducts with accompanying bystander hepatocellular necroinflammation. Our model is similar to other transgenic models of organ-specific disease in that there is immune tolerance in transgene-expressing mice and thus no organ damage without adoptive transfer of T cells. Although there were significant numbers of liver MNC (5 million per liver) in OVA-BIL

mice prior to adoptive transfer of antigen-specific T cells, these mice had no evidence of inflammation by histology or serum ALT elevation, and we presume that this level of MNC infiltration represents homeostasis. However, the introduction of naïve antigen-specific T cells was sufficient to initiate marked inflammation in the liver. Thus, there is no requirement for memory T cells in this response. Inflammation was triggered by naïve OVA-specific T-cell recognition of antigen on the biliary epithelium and was absolutely CD8⁺ dependent and markedly augmented by antigen-specific CD4⁺ lymphocytes.

Autoreactive and alloreactive CD4 and CD8 T cells have been shown in human autoimmune hepatitis and murine models of diabetes^{14,15} and in acute cellular rejection of liver allografts.¹⁶ After T-cell activation, TNF- α and IFN- γ were major cytokines produced, and there was spillover of necroinflammation to hepatocytes. This likely is because of cytokine-mediated bystander hepatitis that has been described in other transgenic models of hepatocellular damage¹⁷ but could result from epitope spreading, a process by which epitopes, distinct from and noncross-reactive with an inducing epitope, become major targets of an ongoing immune response.¹⁸ Because the majority of proliferating cells was OVA-specific, we have very little evidence for epitope spreading in the acute inflammation that results from adoptive transfer of OVA-specific T cells. The mechanism by which inflammation is terminated is likely complex and related to T-cell exhaustion, to T-regulatory-cell expansion, or to subsequent down-regulation of antigen-specific responses, as well as cytokine-mediated inhibition. We favor a role for active immune regulation, and this hypothesis is supported by the fact that, after adoptive transfer of OT-I and OT-II cells into OVA-BIL/RAG^{-/-} mice, down-regulation of ensuing inflammation does not occur (our unpublished data). Evaluating the mechanism(s) of down-regulation of inflammation is of great interest and part of proposed future avenues of research using this model. The histopathology of the T-cell-mediated inflammation in OVA-BIL mice is similar to both acute transplant rejection and autoimmune cholangiopathy, and, therefore, the model may be of value in furthering understanding of the immune responses that lead to these diseases. Because inflammation in OVA-BIL occurs in mice with intact immune responses, including normal innate immune systems, this model likely will be of value in dissecting the complex interplay between innate and adaptive immune responses in the liver.

This initial work reveals that hepatobiliary inflammation coincides with the expansion of OVA-specific CTL, which express high levels of the cytokines IFN- γ and TNF- α . Although the inflammation is strictly CD8 dependent, CD4 cells are required for maximal pathogenesis but alone are unable to generate a response. The mechanism of CD4 augmentation in this system will be the basis of future studies: how naïve cells are activated; which antigen-presenting cells in the liver are critical; and whether enhancement of MHC class II up-regulation on biliary epithelium is required for cooperation with CD8. We speculate that the CD4⁺ cells may have several roles, including augmentation of TNF- α production, leading to direct toxicity to hepatocytes and biliary epithelium; production of IL-2 and other cytokines to enhance activation, proliferation, and effector function of the CD8⁺ T cells; and perhaps even production of chemokines that enhance homing of antigen-specific CD8 cells.

Liver immune cells differ in their cell constitution and in their activation states compared with the lymph node and spleen, even in the absence of disease. Activated but not resting CD8⁺ T cells can be retained by the hepatic endothelium through CD54: CD11a/CD18 (ICAM-1: LFA-1) interaction. In addition, hepatic endothelium can be induced to express VCAM. This has led some investigators to suggest that the liver is a sink for circulating activated T cells, which undergo apoptosis in the liver and cause hepatocyte damage.^{19,20} However, we were unable to demonstrate that activated nonantigen-specific C57BL/6 CD8 T cells adoptively transferred into OVA-BIL or normal C57BL/6 littermates caused inflammation, and bile duct expression of antigen in the recipient mice was absolutely required for liver damage by antigen-specific cells. This suggests that hepatic inflammation, at least in these mice, is not a result of nonspecific trapping of activated T cells. This requirement for nominal antigen in necroinflammatory disease is seen in patients with primary biliary cirrhosis and autoimmune hepatitis, in which clonal populations of CD4 and CD8 cells can be found in the peripheral blood and liver, which recognize bile duct or liver antigens present at sites of destruction.^{21,22}

NKT cells in the liver are heterogeneous and include both CD1d-restricted populations, nonclassical populations, and CD8⁺ T cells, which express NK1.1 on activation.^{12,13,23} The rise in the total NKT cell population was driven by an increase in the OVA-specific CD8⁺ V α 2⁺ population during the immune response, suggesting that up-regulation of NK1.1 on the activated adoptively transferred CTL accounted for much of the observed increase in the NKT population. Which functional populations of NKT cells exist in this model is an area for future research.

All current animal models for immunologic diseases of the liver are imperfect.²⁴ Spontaneous multiorgan disease, including biliary and portal tract destruction, occur in MRL/lpr mice and aly/aly mice,^{25,26} but it is unclear that inflammation in these mice is initiated by T-cell recognition of biliary antigens. Nonsuppurative inflammatory cholangitis and portal inflammation is seen in mdr2 knockout mice, apparently because the biliary tree is exposed to bile salts uncomplexed with phospholipid.²⁷ A murine model of graft vs host disease, using minor histocompatibility mismatches, also produces a CD4-mediated nonsuppurative cholangitis.^{28,29} In the B10.D2 into BALB/c model, progressive peribiliary fibrosis occurs, but this is not seen in human primary biliary cirrhosis or acute cellular rejection, in which biliary injury is followed by loss of bile ducts.³⁰ Other attempts to emulate human disease have utilized injection of peripheral blood lymphocytes or bile duct epithelial cells from patients with primary biliary cirrhosis into immunodeficient mice, leading to inflammation and destruction of intra-hepatic bile ducts.^{31,32} The disadvantage of these models is that both innate and adaptive immunity in the host mouse are dramatically compromised, limiting the ability to generalize the mechanisms involved in this disease to circumstances in which innate immune responses are intact, as in most patients.

One goal of the OVA-BIL model was to induce disease in which inflammation was initiated in the biliary tree. There was no evidence of generalized autoimmunity in this model. However, the model addresses acute liver responses after antigen recognition of aberrant expression of a foreign antigen on biliary epithelium and may provide information about what is occurring during autoimmune or infectious damage to biliary epithelium in human

disease states. In the OVA-BIL mice, ovalbumin is found on the biliary and ileal epithelium, but pathologic and immunohistochemical studies confirm that inflammation only occurs at the intrahepatic biliary epithelium after adoptive transfer of OVA-specific T cells. While the inflammation was biliary centric, there was significant hepatocyte cell death. Our model shows bile duct damage with increasing interface hepatitis around the portal triads, and the interface hepatitis increases with time to a maximum at days 7–10. Although this suggests that the damage is initiated at the biliary epithelium, it was not possible to find lymphocytes within the biliary epithelium without some interface hepatitis. However, in another transgenic mouse with OVA expressed on hepatocytes alone, there is no biliary damage but only hepatocyte damage (unpublished observations, M. Peters, 2006). Clinically, biliary and hepatocellular injury is also noted in acute cellular rejection, autoimmune cholangiopathy, and in some children with sclerosing cholangitis, in whom, initially, the disease appears more like autoimmune hepatitis and with time transitions into a more fibrosing disease. Lack of inflammation in the intestine may be due to the cellular localization of antigen, as ASBT targets apical expression of OVA in enterocytes, or to different requirements for T-cell activation and subsequent injury in the intestine compared with the liver. Others have found it difficult to induce inflammation in the intestine using OVA as antigen, perhaps because it was not apically targeted and had a CD4-dependent response.³³ A transgenic mouse strain that expresses chicken ovalbumin (Act-mOVA) on the surface of all cells in the body has been produced.³⁴ Skin grafts from Act-mOVA mice were rapidly and completely rejected by wild-type recipients but only when both CD4 and CD8 T cells were present. Although these Act-mOVA mice might be useful as tissue donors, because all cells express OVA, OVA-BIL mice need to be used for studies of regional and tissue-specific responses. A benefit of OVA-BIL is that we are able to study the role of antigen-specific T cells in organ- and region-specific immune responses while exploiting all of the existing cells, mice, and reagents available for the OVA system, which has been widely used in other contexts, to address questions of liver-specific immunity. Given these features, we predict that OVA-BIL mice will provide a powerful tool to study the mechanism and regulation of immune bile duct destruction that occurs in several significant human diseases.

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Abbreviations used in this paper

ASBT	apical sodium-dependent bile acid transporter
CFSE	carboxyfluorescein diacetate succinimidyl ester
CTL	cytotoxic T lymphocytes
MNC	mononuclear cells
mOVA	membrane-bound ovalbumin

OVA	ovalbumin
PCR	polymerase chain reaction
PMA	phorbol myristate acetate
TFR	transferrin receptor

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Figure 1.

(A) OVA BIL expression on bile ductules but not hepatocyte epithelium shown by staining with fluorescent-tagged ovalbumin antibody (original magnification, 40×). (B) OVA-BIL mice were normal and showed no liver inflammation prior to adoptive transfer (original magnification, 10×). (C–E) Liver immunohistochemistry in OVA-BIL mice following adoptive transfer of OT-I and OT-II cells. At peak inflammation, bile ducts are disrupted with infiltrating lymphocytes (C, original magnification, 10×; and D, original magnification, 40×). This was accompanied by the loss of biliary epithelial cells and marked interface

hepatitis with apoptotic hepatocytes (*E*, original magnification, 40×). There was no inflammation in gallbladder (*F*, original magnification, 10×) and ileum (*G*, original magnification, 40×).

Figure 2.

(A) Increase in total liver MNC population following adoptive transfer of OT-I and OT-II splenocytes into OVA-BIL mice. (B) Time course in days of serum ALT (IU/L) after adoptive transfer of 10×10^6 OVA-specific OT-I and 4×10^6 OVA-specific OT-II T splenocytes into OVA-BIL mice. Mean $\pm$ SEM of 5 mice per time point.

Figure 3.

Peak serum ALT levels are shown for the following adoptively transferred T cells into recipient mice. (A) Adoptive transfer of 10×10^6 OT-I and 4×10^6 OT-II splenocytes into OVA-BIL mice resulted in a peak ALT level of 846 ± 192 IU/L. (B) Adoptive transfer of 5×10^6 OT-I and 2×10^6 OT-II splenocytes into OVA-BIL mice resulted in a peak ALT level of 228 ± 52 IU/L. (C) Adoptive transfer of 5×10^6 OT-I splenocytes into OVA-BIL mice resulted in a mean peak ALT level of 44 ± 13 IU/L. (D) Adoptive transfer of 5×10^6 OT-I splenocytes into normal C57BL/6 mice did not result in inflammation. ALT 11 ± 1 IU/L. Mean $\pm$ SEM of at least 4 mice per experiment.

Figure 4.

Liver MNC subpopulations and proliferation at varying days after adoptive transfer of OT-I and OT-II splenocytes. (A) CD8 (■), NK (□), NKT (○), and CD4 (●) populations in the liver are shown. (B) The majority of CD8+ (■) were Vα2+ CD8+ (◇) CTL and accounted for the majority of liver MNC and increased over time. Mean of 5 mice per time point for (A) and (B). (C) Proliferation of OT-I splenocytes (labeled with CFSE) 3 days after adoptive transfer with unlabeled OT-II splenocytes into OVA-BIL mice. Actively proliferating CFSE-labeled OT-I splenocytes were found in the liver (*solid upper line*) and not in the spleen (*lower dotted line*). Representative FACS analysis of 3 experiments.

Figure 5.

IFN- γ and TNF- α production by liver MNC with time (days) following adoptive transfer of OT-I and OT-II splenocytes into OVA-BIL mice. (A) IFN- γ expressing total liver cells (■), CD8+ lymphocytes (○), and V α 2+ CD8+ (●). (B) TNF α expressing total liver cells (■), CD8+ lymphocytes (○), and V α 2+ CD8+ (●). Mean $\pm$ SD 3 mice per time point.