Analysis of mononuclear cells in the adult mouse liver revealed that B cells represent as much as half of the intrahepatic lymphocyte population. Intrahepatic B cells (IHB cells) are phenotypically similar to splenic B2 cells but express lower levels of CD23 and CD21 and higher levels of CD5. IHB cells proliferate as well as splenic B cells in response to anti-IgM and LPS stimulation in vitro. VDJ gene rearrangements in IHB cells contain insertions of N,P region nucleotides characteristic of B cells maturing in the adult bone marrow rather than in the fetal liver. To evaluate whether B cells can have an impact on liver pathology, we compared CCl$_4$-induced fibrosis development in B cell–deficient and wild-type mice. CCl$_4$ caused similar acute liver injury in mutant and wild-type mice. However, following 6 weeks of CCl$_4$ treatment, histochemical analyses showed markedly reduced collagen deposition in B cell–deficient as compared with wild-type mice. By analyzing mice that have normal numbers of B cells but lack either T cells or immunoglobulin in the serum, we established that B cells have an impact on fibrosis in an antibody- and T cell–independent manner.
Attenuated liver fibrosis in the absence of B cells

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Introduction

The functions of the liver, such as removal of pathogens and antigens from the blood, protein synthesis, and metabolism, require an immune response that is adapted to these tasks and is locally regulated. The liver is rich in immune cells. In addition to the presence of large numbers of resident macrophages (Kupffer cells), the liver contains T cells, NK cells, and NKT cells (1, 2). Interestingly, no mention of B cells in the adult mouse liver is found in most reviews, even though embryonic liver is a well-studied site of origin for B cells and B lymphopoiesis persists in liver for 2 weeks after birth (3). B cells originating from the embryonic liver have the phenotype of B1 cells (CD5+, CD43+, Mac-1+), encode a particular set of B cell receptor specificities, reside mostly in the peritoneal cavity (PC) and the pleural cavity, and carry little, if any, N nucleotide insertions in their VDJ joints (4). All these features are distinctly different in B2 cells, the predominant population of B cells in the adult mouse (4).

Since little is known about B cells in the adult liver, we decided to look for adult mouse hepatic B cells and, if they were found, to characterize them phenotypically and with respect to their possible involvement in the response to liver injury. We chose the carbon tetrachloride–induced (CCl4-induced) liver degeneration model of liver disease to avoid targeted activation of a specific subset of lymphocytes a priori, as LPS or concanavalin A–induced liver damage stimulates B cells/macrophages or T cells, respectively (5, 6). The hallmarks of chronic liver diseases, such as alcohol-induced liver degeneration, hepatitis C infection, and nonalcohol-induced steatohepatitis, are chronic inflammation, cellular damage, regeneration, and fibrosis. All of these features can be evoked by repeated CCl4-induced liver injury.

The hepatotoxicity of CCl4 is thought to involve 2 phases. First, CCl4 is metabolized by cytochrome P450 (expressed at high levels in centrilobular hepatocytes; ref. 7) to produce trichloromethyl radicals, which cause lipid peroxidation and membrane damage. The second phase is an inflammatory response launched by resident hepatic macrophages, the Kupffer cells, which upon activation, secrete cytokines, chemokines, and other proinflammatory factors (IL-18, TNF-α, IL-1, IL-6, IL-8, eicosanoids, and NO). In addition to having direct cytotoxic effects, these factors attract and activate other monocytes as well as neutrophils and lymphocytes, which all contribute to tissue damage. Initial damage is followed by a phase of repair that includes a TGF-β–induced increase in collagen I (coll1) production (8). Repeated cycles of injury, inflammation, and repair result in fibrosis. Accumulation of coll occurs in the space between hepatocytes and endothelial cells, where it replaces a low-density basement membrane-like matrix containing collIV. This conversion of the subendothelial matrix to a matrix rich in fibrillar coll is a pivotal event mediating the loss of differentiated functions characteristic of progressive liver disease.

In an alternative model, liver injury is induced by biliary toxin α-naphthylisothiocyanate (ANIT), mimicking biliary cirrhosis and sclerosing cholangitis (9). ANIT, similarly to CCl4, induces nonimmune cell–targeted hepatotoxicity followed by inflammatory and fibrotic responses, although at a different hepatic anatomic location compared with CCl4.

Here we characterize intrahepatic B (IHB) cells with respect to cell phenotype, N nucleotide insertions at the VDJ junction, and their functional properties as well as describe a critical role for B cells in fibrotic liver disease models.

Results

B cells represent a major lymphocyte population in the liver. B cells have been extensively studied in embryonic liver, the major site of

Nonstandard abbreviations used: ALT, alanine aminotransferase; ANIT, α-naphthylisothiocyanate; BAFβ, B cell activating factor; CCl4, carbon tetrachloride; coll, collagen I; DAB, 3,3′-diamobenzidine; IF, intrahepatic; IHB, IH B (cell); PC, peritoneal cavity.

Conflict of interest: The authors have declared that no conflict of interest exists.

hematopoiesis in the developing embryo. However, little is known about hepatic B cells in the adult liver. We set out to phenotypically and functionally characterize IHB cells.

We quantified the proportion of IHB cells in a lymphocyte-enriched population from PBS-perfused liver by staining for CD19, a B lineage–specific marker. In both BALB/c and C57BL/6 mice, B cells represent about 50% of intrahepatic (IH) lymphocytes (range 30–60%; Figure 1A and data not shown). The absolute number of B cells isolated from a liver was approximately $2 \times 10^6$. CD19+ IHB cells were shown to express IgM, IgD, B220, MHCII, and CD62L at levels similar to their splenic counterparts (Figure 1, A and B, and data not shown). IHB cells do not express the CD43 and Mac-1 markers typical for B-1 or immature B cells (data not shown). IHB cells express CD5 at a level higher than that detected on blood B cells but lower than observed on PC B cells (Figure 1B). Higher CD5 levels are indicative of conventional B cell activation (10). IHB cells express CD23 but at a lower level than splenic or blood B cells. CD21 surface expression is also slightly lower for IHB than for splenic B cells but higher than for blood B cells (Figure 1B). Taken together, with regard to expression of these markers, liver B cells are most similar to follicular splenic B cells.

**Hepatic B cells are functionally competent.** As liver is often regarded as a destination for dying lymphocytes (11), we tested whether IHB cells are proapoptotic using annexin V, which binds to phosphatidylserine (PS) that translocates from the inner to the outer layer of the cellular membrane as cells undergo apoptosis. Annexin V bound up to 30% of hepatic B cells compared with approximately 15% of splenic B cells (Figure 1C and data not shown). Thus, most liver B cells do not show a predisposition to apoptosis, and the higher number of apoptotic cells in liver compared with spleen might be related to differences in lymphocyte isolation.

The proliferative capacity of B lymphocytes in response to mitogenic and B cell receptor cross-linking stimuli is an important functional characteristic that differs substantially for B cell subsets (12–14). We compared hepatic and splenic B cells for their extent of proliferation and upregulation of costimulatory molecules, such as CD86 (B7.2) and MHCII, in response to various stimuli. The proliferative response of IHB cells was very similar to that of splenic B lymphocytes (Figure 1D); the response to Toll-like receptor 4, RP105, and CD40 stimulation is the same whereas response to IgM cross-linking is greater in the absence but not in the presence of IL-4. The difference noted may reflect better survival of IHB cells in culture without an exogenous survival factor like IL-4 and is consistent with an activated status of IHB cells suggested by CD5 upregulation (Figure 1B). A similar extent of upregulation of MHCII, CD86, and CD5, induced by tested stimuli, was demonstrated for liver and splenic B cells (Figure 1, B and D, and data not shown).
**Table 1**

Sequences of VD1HJH junctional regions of Ig gene rearrangements in B cells from neonatal spleen

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<th>D</th>
<th>P,N</th>
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The VD1HJH joints are shown from the codon encoding the second cysteine (position 104) of the Vδ1 gene and extending to the conserved glycine of the JH region. The sequences were analyzed using DnaPlot. Nucleotides not encoded in the germline, called either N nucleotides (55, 56) or P nucleotides (57), are listed in the P.N column. Sequences of the Dγ1 family, Dε element, and Jα elements. Vδ sequences were assigned to published Dδ segments (58) if there was homology of at least 4 nucleotides. D7 indicates that the respective D element could not be unambiguously assigned to a particular gene. Bolded text indicates P,N nucleotides.

**IHB cells resemble splenic B2 cells and are not of embryonic liver origin.** B cells in adult liver may represent residual hepatic B cell generation from embryonic liver. Alternatively, IHB cells may be BM derived as are splenic B cells in an adult organism. To address the origin of IHB cells, we performed genetic analyses of their VDJ rearrangements. Few insertions of nontemplated (N,P) nucleotides are seen in the VDJ junctions of neonatal B cells generated in the embryonic liver, which is similar to what has been reported for B1 cells (15–17). In contrast, adult splenic and blood B cells have extensive nontemplated nucleotide additions (18, 19). We compared CDR3 sequences derived from pooled adult liver lymphocytes with those derived from splenic cells of 2-day-old mice or adult mouse blood B cells. Adult IHB cells markedly differed from neonatal B cells and resembled splenic B2 cells or recirculating blood B cells in their VDJ joint sequence (Tables 1 and 2). The average number of N,P nucleotides in neonatal B cells was 0.5 for the VD junction and 0.1 for the DJ junction (Table 1). This is notably different from 3.5 (or 4.5) for the VD and 4.4 (or 3.4) for the DJ junctions of B cells in the adult liver (or blood) (Table 2). Interestingly, adult liver and blood B cells also appeared different in the length of their VD and DJ junctions; this difference is on the border of being statistically significant (P = 0.1, Student’s t test). IHB cells have fewer N,P nucleotides in their VDJ joints than in their DJ joints, the converse of what is reported for conventional adult B2 cells (18). The difference in the length of N,P insertions in the IHB and adult blood B cells might be a result of IHB cell selection. In addition, the difference strengthens the notion that liver B cells represent a true IH population with no significant contamination by peripheral blood B cells (see also Methods).

**B cell role in hepatic fibrosis.** Every CCl4 administration causes a pronounced necroinflammatory liver injury that is followed by a chronic repair response. This model has an advantage over many
### Table 2
Sequences of \( V_{\beta}D_{\beta}J_{\beta} \) junctional regions of Ig gene rearrangements in B cells from adult liver and adult blood

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The degree of liver fibrogenesis is proportionate to B cell numbers. Mice were gavaged with 3.5 ml/kg (A and B and C, left panel) or 1.75 ml/kg (C, right panel, and D) of CCl₄ or mineral oil. Each plot represents 1 experiment. (A) J₉/H–/– and BALB/c WT control mice were bled 24 hours after a single CCl₄ or oil administration, and ALT levels were measured. A representative experiment out of 3 is shown. (B–D) One week after the sixth weekly treatment of oil or CCl₄, mice were sacrificed, and collagen-specific Sirius red staining of liver sections was performed to quantify interstitial collagen deposition in J₉/H–/– and WT BALB/c mice (C) or in WT C57BL/6 and BAFF-Tg mice (D). Magnification, ×50. In each case, a representative experiment out of at least 2 is shown. A column of dots represents a series of sections from 1 animal; mean values are depicted as red bars.

α-SMA producing myofibroblasts were observed after 6 CCl₄ treatments (data not shown). Thus, B cells appear indispensable for the liver to develop fibrotic changes in response to CCl₄.

To test whether B cell function is limited to the specific case of CCl₄-induced injury or rather, plays a more general role in hepatic tissue repair, we induced hepatotoxicity with ANIT, as ANIT causes liver destruction by a mechanism distinct from that induced by CCl₄. The hepatotoxicity induced by ANIT is manifested as neutrophil-dependent necrosis of bile duct epithelial cells and hepatic parenchymal cells (21). After 8 weeks of ANIT treatment, we found that J₉/H–/– had about 7 times fewer collagen deposits than WT mice (data not shown). Thus, fibrosis is reduced in the absence of B cells in at least 2 model systems.

To examine whether increasing B cell numbers above normal leads to more pronounced fibrosis, we made use of B cell–activating factor–Tg (BAFF-Tg) mice that show a 20–30% increase in B cell numbers (22) compared with the corresponding C57BL/6 WT control mice. Following 6 CCl₄ treatments, fibrosis developed in the BAFF-Tg and C57BL/6 controls. This fibrosis was characterized by less collagen fiber deposition than noted in BALB/c mice (data not shown and ref. 23). However, the BAFF-Tg mice had about twice the amount of collagen deposits as their WT C57BL/6 counterparts (Figure 2D).

B cell–deficient and WT mice respond differently to a single CCl₄-induced injury. To understand what acute effects trigger changes in collagen deposition after 6 weeks of treatment, the kinetics of tissue changes were analyzed in liver sections of B cell–deficient and control mice 1, 3, and 5 days after a single CCl₄ challenge. Interestingly, TUNEL staining detecting apoptotic cells showed that despite similar initial injury at day 1, J₉/H–/– mice cleared apoptotic cells completely by day 3, whereas in WT mice, some dying cells were still detected even 5 days after injury (Figure 3 and Supplemental Figure 1). When staining sections for the tissue macrophage–specific marker F4/80, we found that as early as day 1, J₉/H–/– mice cleared dying hepatocytes more completely than WT mice, whereas dying cells were still detected 5 days after injury (Figure 3 and Supplemental Figure 1). Thus, it seems that in the absence of B cells, macrophages are better able to clear dying hepatocytes. As the major cellular source for collagen fibers is a population of myofibroblasts (24), we also monitored α-SMA that marks activated hepatic stellate cells/myofibroblasts in the injured liver. Myofibroblasts were first detectable at day 3 at levels similar to those in B cell–deficient and control mice. By day 5, however, WT mice showed many more myofibroblasts (Figure 3 and Supplemental Figure 1). Higher efficiency of macrophages to remove dying hepatocytes may have reduced myofibroblast activation and eventually resulted in the lower collagen deposition. In a recent study (25), macrophages were shown to play distinct,
opposing roles during liver injury and repair. It appears that in the absence of B cells, those macrophages that contribute to recovery from inflammatory scarring are preferentially activated.

**B cell–derived soluble factors have the potential to stimulate collagen production by activated hepatic stellate cells.** To investigate whether soluble factors produced by activated B cells can directly affect proliferation and collagen production by myofibroblasts, we cultured T6-HSC (26), a rat cell line derived from activated hepatic stellate cells, in the presence of supernatants from purified splenic rat B cells activated with LPS. We chose LPS as the most likely agent reaching the liver of CCl₄-gavaged mice through the portal system. LPS mimics polyclonal T cell–independent activation of B cells. We used human recombinant TGF-β, a known regulator of fibrogenesis, as a positive control. Proliferation was measured by \[^{3}H\]-thymidine and de novo collagen synthesis by \[^{3}H\]-proline incorporation (Figure 4). B cell supernatants induced increases in collagen synthesis similar to human TGF-β at 10 ng/ml with LPS having an intermediate effect (Figure 4). Thus, soluble factors produced by stimulated B cells can induce collagen synthesis by hepatic stellate cells. However, the magnitude of in vitro response of already activated hepatic stellate cells (T6-HSC) to a known profibrogenic cytokine, TGF-β, is substantially less than that observed in vivo (data not shown). For this reason, we do not believe that use of a cell line is the optimal approach to unravel in vivo mechanisms of B cell–dependent effects of fibrogenesis. It is noteworthy that in vitro data do not rule out alternative B cell–mediated effects on HSCs, such as cell-cell interaction or indirect influence.

**CD4⁺, CD8⁺, and γδ T cells do not influence hepatic fibrosis to a significant degree.** To assess whether mice deficient in T cells also have a defect in fibrogenesis, we performed a series of CCl₄-induced liver injury experiments with mice that lack both B and T cells (RAG2⁻/⁻), CD4⁺ T cells (Aβ⁻/⁻), CD8⁺ T cells (β2m⁻/⁻), and γδT cells (TCRδ⁻/⁻). For every mouse mutant strain, a control strain of the same genetic background was used (Table 3). Of these, only RAG2⁻/⁻ mice showed dissimilar amounts of collagen deposition following long-term treatment with CCl₄ compared with appropriate WT counterparts (Figure 5 and data not shown). RAG2⁻/⁻ mice, lacking all lymphocytes that require DNA rearrangement to assemble their receptors, showed a 3- to 4-fold reduction in...
Corresponding WT strains were kept in quarantine facilities. These animals. Both of different housing conditions and/or concurrent infection of liver fibrosis are antibody independent. It is noteworthy that the degree of CCl₄-induced liver fibrosis in these experiments was rated at the place of their serum or have Ig levels severely reduced. Mice expressing their WT BALB/c controls. Moreover, IgH locus (DₙLMP2ᵃ allele; ref: 27) lack both surface and circulating immunoglobulin whereas mice expressing surface but not secreted Ig (28) showed the same degree of fibrosis in WT BALB/c mice in these experiments as WT control BALB/c mice (Figure 6B). Thus, B cell effects on the pathology of CCl₄-induced liver fibrosis are antibody independent. It is noteworthy that the degree of fibrosis in WT BALB/c mice in these experiments was lower than in previous ones (Figures 2, 5, and 6), possibly because of different housing conditions and/or concurrent infection of these animals. Both LMP2ᵃ and mgM mouse colonies were positive for Helicobacter hepaticus; therefore, these mice as well as corresponding WT strains were kept in quarantine facilities.

Table 3

<table>
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<th>Control</th>
<th>Commercial source</th>
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<td>BALB/c (BALB)</td>
<td>Taconic</td>
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<tr>
<td>Lpm2a (27)</td>
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References or commercial source is provided for each strain.

Discussion

In this work we demonstrate that IHB cells represent a sizable population with phenotypic and functional characteristics resembling those of conventional B2 cells. IHB cells express CD5 at somewhat higher levels than conventional B2 cells, and they proliferate better in response to IgM cross-linking without supplementing IL-4 in vitro (Figure 1), implying activated status of IHB cells. Despite the fact that adult liver has been known to contain c-kit⁺ pluripotent hematopoietic stem cells that could give rise to multilineage leukocytes (29, 30), most B cells in the adult liver appear to be BM-derived, in contrast to self-propagating embryonic liver-derived B1 lineage cells (4). IHB cells are likely of BM origin; VDJ junctions of IHB cells contained extensive N nucleotide insertions, with total average length similar to conventional B2 cells (Tables 1 and 2). Notably, expression of terminal deoxyribonucleotidyl transferase (TdT), the enzyme responsible for N nucleotide insertion, has not been studied in adult liver (31). Thus, the formal but unlikely possibility exists that adult liver B cells are generated in the liver with N nucleotide insertions (perhaps in a TdT-dependent fashion).

Here we show that B cells play an important antibody-independent role in the development of liver fibrosis, adding another disease model likely dependent on local B cell function. In the presence of B cells, liver fibrosis develops to a much greater extent, indicating that B cells are non-redundant participants in liver disease progression. An imperative role of B cells has also been demonstrated for autoimmune diabetes in NOD mice. B cell–deficient NOD.Ignull and B cell–depleted NOD mice did not develop insulinis or insulin-dependent diabetes mellitus, supporting the idea that B cells are critical for the initiation and/or activation of autoreactive T cells (32, 33). B cells were also shown to be required for lupus nephritis in the polygenic, fas-intact, and fas-deficient MRL models of systemic autoimmunity (28, 34, 35). In both cases, an antibody-independent mechanism turned out to be crucial for B cell involvement (28, 36).

In our experiments, we used mice constitutively devoid of B cells to study B cell involvement in fibrotic pathology. Although normal in gross physiology, B cell–deficient mice lack follicular dendritic networks (37–39), follicle-associated epithelium in the intestinal Peyer’s patches (40), and a noncanonical subset of NKT...
cells (41). Mice lacking B cells also have defects in CD4+ T cell function (42) and perhaps some other as yet undescribed developmental/functional deficiencies. Thus, the results obtained with the B cell–deficient mice cannot alone be interpreted to indicate that B cells directly affect the pathogenesis of liver fibrosis. Since T cell–deficient mice do not show any difference in the development of liver fibrosis (data not shown), a CD4+ T cell defect is unlikely to account for the strongly attenuated liver fibrosis observed in the J_{H1}^{–/–} mice. However, a B cell–dependent NKT cell subset that expresses V_{α}19 containing invariant TCR (41), resident in murine liver (43), might contribute to the reduced fibrosis noted in the B cell–deficient mice. NKT cells are known for their ability to respond in a rapid manner and to produce both TH1- and TH2-type cytokines (44). Such qualities allow NKT cells to participate in immune response regulation (44). We did not find any pronounced differences in liver fibrosis development in CD1^{−/−} mice (data not shown) that lack conventional V_{α}14 TCR NKT cells. Unfortunately, there is no mouse mutant available that allows one to address the role of noncanonical V_{α}19 invariant NKT cells in liver fibrosis. Nonetheless, as RAG^{−/−} animals show inhibition of fibrosis to an extent similar to that of B cell–deficient mice, a role for cell types that require gene rearrangement for their development (B and T cells of various lineages) is implied. Thus, together the data suggest that either non-CD1 restricted NKT cells that require B cells for their development (41) or B cell autonomous function has a role in the fibrosis manifested in the CCl_{4}-induced hepatotoxicity model.

By using 2 previously generated mouse strains (LMP2a insertion and mIgM-Tg mice) deficient in immunoglobulin production, we have shown that antibodies are not required to develop CCl_{4}-induced liver fibrosis. LMP2a mice have normal B cell numbers and completely lack both secreted antibodies and surface expression of immunoglobulin (27). LMP2A does not only mimic BCR signaling, but triggers additional signaling pathways (45, 46). To eliminate the possibility that the effects observed in LMP2a mice are compensated for by LMP2a signaling, we assessed fibrogenesis in the mIgM-Tg (J_{H1}^{−/−}) mice. mIgM-Tg (J_{H1}^{−/−}) mice express a surface Ig receptor and have 300–to 500-fold reduced antibody titers compared with normal mice (28). Both mouse lines developed liver fibrosis to an extent similar to that of controls. Thus, the B cell role in liver fibrosis pathology appears to be antibody independent, suggesting that it is mediated by functions (e.g., cytokine secretion and/or cell–cell contact) of local B cells as opposed to potentially long-range effects mediated by B cells localized elsewhere in the organism. An antigen presentation role for B cells is unlikely to play a significant role in liver fibrosis as mice deficient in conventional T cells show fibrogenesis similar to that of their WT counterparts. Moreover, LMP2a B cells do not have the ability to bind, internalize, and present antigens because they lack B cell receptors on the surface. Yet LMP2a mice develop collagen deposits similar to those of WT mice. Together, these data suggest that liver tissue repair is affected by local B cell function, which may be mediated in part by the IHB

Figure 6
B cells mediate antibody-independent effects on CCl_{4}-induced liver fibrosis. (A) Mice expressing Epstein-Barr virus–derived protein LMP2a from a gene incorporated at the place of J elements of the IgH locus that lack both surface and circulating immunoglobulin, (B) mIgM-Tg (J_{H1}^{−/−}) mice expressing surface but not secreted Ig, and corresponding WT control mice were gavaged with 1.75 ml/kg of CCl_{4} or with mineral oil. One week after the sixth weekly treatment, mice were sacrificed, collagen-specific Sirius red staining of liver sections was performed, and interstitial collagen deposition was quantified. A column of dots represents a series of sections from 1 animal; mean values are shown as red bars.
cells defined herein. Formally, it is possible that B cells overwhelm clearance mechanism(s) in the liver. However, B cell numbers are very small compared with hepatocyte numbers.

Our results are in agreement with reports that the degree of hepatic damage in response to CCl₄ was significantly milder in splenectomized vs. sham-operated rats (47) and in SCID mice on BALB/c background compared with appropriate controls (23). However, liver fibrosis induced by the Schistosoma mansoni parasite is increased in B cell–deficient compared with control mice (48). The differences in the fibrosis induction mechanism by repeated hepatocyte damage (as is the case for CCl₄, and ANIT) or by low-level worm infection could explain the discrepancy.

B cell function has also been associated with fibrosis in human skin and lung. B lymphocyte gene signature characterizes skin biopsies from systemic sclerosis patients (49). Moreover, a B cell line established from the lung tissue of a patient with scleroderma exhibits augmented proliferation and inflammatory responses that are likely to lead to fibrotic changes (50).

In sum, this study describes the isolation and characterization of B cell populations in adult liver and directly demonstrates an indispensable role of B cells in tissue repair following hepatic injury.

Methods

Mice. Unless otherwise stated, mice were kept in a specific pathogen-free mouse facility at Biogen Idec. All animal procedures were approved by Biogen Idec’s Institutional Animal Care and Use Committee. Male mice of different strains (Table 3) had to weigh 20 g or more and be at least 6 weeks of age to be included in the study.

CCl₄ and ANIT injury models. A mix of CCl₄ (Sigma-Aldrich) with mineral oil (Sigma-Aldrich) was delivered by gavage in approximately 0.2 ml with a 20-gauge animal-feeding needle. Experiments were performed using a 3.5 ml/kg or 1.75 ml/kg dose of CCl₄. The latter dose was preferred because it reduced morbidity/mortality and still induced changes in serum ALT levels and collagen deposition comparable to the higher dose. For a long-term experiment, mice were gavaged once a week for 6 weeks. Short-term experiments included one CCl₄ administration. ANIT (Sigma-Aldrich) was dissolved in mineral oil (Sigma-Aldrich) at 30 mg/ml. Mice were gavaged with 50 mg/kg twice a week for 8 weeks.

Serum ALT levels were measured 24 hours after CCl₄ administration. One week after the 6th weekly gavage or on the indicated day after a single gavage, mice were sacrificed and 3 different liver lobes were taken from each mouse and incubated in 4% PFA in PBS for 2 days prior to embedding for immunohistochemical analysis.

Liver lymphocyte isolation. Mice were euthanized by CO₂ inhalation. The hepatic portal vein was cannulated with a 25-gauge needle and perfused with 10 ml of cold PBS. After removal of the gall bladder, the liver was cut into segments and passed through a 70-µm mesh cell strainer (BD Falcon; BD) in 50 ml of ice-cold RPMI, 5% FBS. The liver slurry was centrifuged at 300 g for 10 minutes at 4°C in a 50-ml tube per liver. The pellet was resuspended in 10 ml of 0.02% collagenase IV (Sigma-Aldrich) in RPMI 1640 and left for 45 minutes at 37°C. Ice-cold RPMI, 5% FBS (30 ml) was added to each tube, then centrifuged for 3 minutes at 30 g. The pellet was discarded. The supernatant was centrifuged for 10 minutes at 300 g at 4°C. The cell pellet was resuspended in 6 ml of ice-cold RPMI 1640 (or in 45% Percoll [Amersham Biosciences]) and underlaid with 24% metrizamide (Sigma-Aldrich) in PBS (or with 70% Percoll, respectively). A centrifugation at 1000 g for 20 minutes at 4°C followed. Lymphocytes at the interface were harvested, washed with RPMI, 5% FBS, and used for further analyses.

The degree of IH lymphocyte contamination by blood lymphocytes is likely minimal, as the results (Tables 1 and 2 and data not shown) indicate a liver-specific increase in NKT cells and a different ratio of N nucleotide insertions at the VD and DJ junctions in IHB lymphocytes (3.5 and 4.4) compared with blood B cells (4.5 and 3.4; see also Results).

Isolation of lymphocytes from spleen, blood, and PC. Spleens were minced through a nylon mesh (Cell Strainer, BD Falcon, BD) to obtain single cell suspensions in DMEM, 5% FCS, and 2 mM l-glutamine. Erythrocytes were lysed by incubating in lysis buffer (140 mM NaCl, 17 mM Tris-HCl, pH 7.65) for 3 minutes on ice. Blood was collected in EDTA-containing tubes (BD Biosciences — Pharmingen). To isolate blood lymphocytes, 200 µl of blood was underlaid with Ficoll-Paque (Amersham Biosciences) and centrifuged at 1000 g at room temperature for 20 minutes. Lymphocytes were collected from the interface. The PC was washed with 5 ml of DMEM, 5% FCS, and 2 mM l-glutamine to collect PC leukocytes. Following these procedures, lymphocytes were washed twice in DMEM, 5% FCS by 300 g centrifugation at 4°C and resuspended in PBS/BSA/azide for flow cytometric analysis or in cell culture medium for proliferation studies.

Purification of rat B cells and T6-HSC culture. Splenocytes from 4-month-old Sprague-Dawley rats were purified using negative selection with anti-rat CD43 MACS beads according to manufacturer’s protocol (Miltenyi Biotec). B cells (>95% purity, data not shown) were seeded at 3 × 10⁶ cells/ml in RPMI 1640, 5% FCS, β-mercaptoethanol, l-glutamine and cultured for 3.5 days with or without 4 µg/ml of Ultrapure LPS (InvivoGen).

T6-HSC cells were seeded at 25% confluence in a 96-well plate in RPMI, 5% FBS, penicillin, streptomycin 24 hours later and an equal volume of B cell–conditioned medium was added with [³H]-thymidine to measure proliferation or with [³H]-proline to measure collagen synthesis. After 18 hours, the amount of incorporated [³H] was quantified. Each treatment was performed in triplicate.

Flow cytometry. Fluorescence staining was performed as previously described (51). Annexin V, 7AAD, and antibodies specific for IgM, IgD, CD19, CD23, CD5, CD69, CD86, B220, MHCCI, CD43, Mac-1, CD4, CD8, CD9 (BD Biosciences — Pharmingen), or CD21 (eBioscience) were used. Antibodies were conjugated to FITC, PE, allophycocyanin (APC), peridinin chlorophyll protein (PerCP), Cy-Chrome, or biotin. Biotinylated antibodies were detected with streptavidin conjugated to PerCP. Stained cells were fixed and analyzed using FACScalibur (BD Biosciences).

In vitro stimulation of CSE-labeled B cells. To generate a stock solution, CSE (Invitrogen Corp.) was dissolved to 5 mM in DMSO and stored at –80°C. Splenic B cells were MACS purified by enrichment with MACS beads coupled to the anti-B220 antibodies (Miltenyi Biotec) on the LS magnetic columns (Miltenyi Biotec) according to the manufacturer’s instructions. Cells were then washed twice with RPMI 1640, resuspended at 5 × 10⁷ cells/ml in a 5-nM concentration of CSE in warm RPMI 1640 for 10 minutes at 37°C, washed 3 times in RPMI 1640, 5% FCS, resuspended in RPMI 1640, 5% FCS, β-mercaptoethanol, l-glutamine at 2 × 10⁶/100 µl, and transferred into a flat-bottom 96-well plate in 100 µl/well. Another 100 µl RPMI was added that contained stimulating reagents at 2 times final concentration. The stimuli used were pure F(ab’₂) fragment goat anti-mouse IgM (2.5 µg/ml; Jackson ImmunoResearch Laboratories Inc.), IL-4 (25 U/ml; R&D Systems), anti-mouse CD40 antibodies (0.25 µg/ml; eBioscience), anti-RIPO5 antibodies (10.5 µg/ml; ebioscience), or LPS (20 µg/ml; Sigma-Aldrich).

Immunohistochemistry. Antibodies specific for α-SMA (clone 1A4, Dako) and cytokeratin (clone 4A4, clone 1A4, Dako) were used in a 1:50 dilution with 30 minutes incubation. Heat-induced epitope retrieval pretreatment of tissue sections was performed in 10 mM citrate buffer, pH 6.0, for 30 seconds at 125°C, kept at 90°C for 10 seconds, and cooled to room temperature for an additional 20 minutes prior to immunostaining. Binding of primary antibodies to tissue elements was detected using an MM Biotinylation Kit (Biocare Medical), with 3,3′-diaminobenzidine (DAB) substrate. Slides were counterstained with Mayer’s hematoxylin for 1 minute.
research article

F4/80-specific antibodies (clone CLA3-1, Serotec Inc.) were used at 20 μg/ml. Tissue sections were pretreated with proteinase K (DakoCytomation) for 5 minutes at room temperature. Binding of primary antibodies was detected using a Vector Elite ABC kit (Vector Laboratories), using DAB substrate. Slides were counterstained with Mayer’s hematoxylin for 1 minute.

TUNEL staining was performed using an ApopTag In Situ Apoptosis Detection kit (Chemicon International) according to the manufacturer’s instructions. Labeled apoptotic cells were detected using DAB/nickel chloride as the substrate. Slides were counterstained for 5 minutes with methyl green (Vector Laboratories). Collagen fibers were detected using Sirius red stain (52); H&E staining was performed as previously described (53).

PCR and Ig gene rearrangement analysis. DNA was extracted from cells positively selected on CD193 magnetic beads (Miltenyi Biotec) according to the genomic DNA isolation kit (QIAGEN) manufacturer’s protocol. DNA (2 μl, equivalent of about 10^3 B cells) was used for amplification of the VDJ joints. Two rounds of amplification were performed using VHA, VHB, and VHE 3’ primers specific for J558L, Q52, and 7183 V_{H} families; JH4E 3’ primer (54) was used for the first and nested JH1 or JH4A 3’ primers for the second rounds. All primers were synthesized at Biogen Idec. Twenty cycles were performed for the first round (1 minute at 95°C, 1 minute at 60°C, and 1.5 minutes at 72°C); 30 cycles (1 minute at 95°C, 1 minute at 63°C, and 1.5 minutes at 72°C) were performed for the second round, using 2 μl of the first-round reaction as a template. The expected 0.4-kb fragment was purified from the gel and subcloned into the pcR4-TOPO vector (Invitrogen Corp.). DNA from individual colonies was prepared and sequenced using standard vector specific primers. Sequences were analyzed using the program DnaPlot (www.dnaplot.de).

Intersitial collagen quantification. A total of 3 sections from the liver (each from a different lobe) were stained for each animal. Black and white pictures of Sirius red staining were made in polarized light at ×50 magnification. Pictures were made so that liver tissue occupied the whole area captured by the camera to ensure that total image area was identical in each picture (4–10 pictures per animal). Vessels constitutively containing collagen were electronically removed from each image. Next, the amount of white staining (interstitial collagen) was quantified by MetaMorph image analysis software (Universal Imaging Corporation). Quantification is displayed in arbitrary units (1 correlates to 1000 pixels). The absolute amount of white area cannot be directly compared among different experiments because it varied with the intensity of Sirius red staining.

Statistics. Statistical analysis was performed using internal Microsoft Excel (Microsoft Corp.) statistical functions.

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30. Taniguchi, H., Toyoshima, T., Fukao, K., and...
32. Serrze, D.V., et al. 1996. B lymphocytes are essen-
tial for the initiation of T cell-mediated autoim-
immune diabetes: analysis of a new “speed con-
35. Chao, O.T., Madaio, M.P., and Shlomchik, M.J. 1999. B cells are required for lupus nephritis in the
polygenic, Fas-intact MRL model of systemic auto-
cells in type 1 diabetes in the NOD mouse. Dia-
betes. 53:2581–2587.
ular dendritic cell clusters in a lymphotoxin alpha-
38. Gonzalez, M., Mackay, F., Browning, J.L., Kosco-
Vilbois, M.H., and Noelle, R.J. 1998. The sequential
role of lymphoetoxin and B cells in the development of
rig networks depend on expression of lymphoetox-
in beta receptor by radioresistant stromal cells and
of lymphoetoxin beta and tumor necrosis factor by
40. Golovkina, T.V., Shlomchik, M., Hannum, L.,
and Chernovskiy, A. 1999. Organogenic role of
conserved mucosal-associated invariant T cells by
42. Baumgarth, N., Jager, G.C., Herman, O.C., and
Herzenberg, L.A. 2000. CD4+ T cells derived from
B cell-deficient mice inhibit the establishment of
43. Shimamura, M., and Huang, Y.Y. 2002. Presence of
a novel subset of NKT cells bearing an invariant
both ways: immune regulation via CD1d-depen-
45. Ikeda, A., Caldwell, R.G., Longnecker, R., and Ikeda,
M. 2003. Itchy, a Nedd4 ubiquitin ligase, downreg-
ulates latent membrane protein 2A activity in B-cell
virus LMP2A interferes with global transcription
factor regulation when expressed during B-lym-
splenectomy on CCl4-induced liver fibrosis in
48. Ferru, I., Roye, O., Delacre, M., Auriault, C., and
Wołowczuk, I. 1998. Infection of B-cell-deficient
mice by the parasite Schistosoma mansoni: dem-
onstration of the participation of B cells in granu-
specific gene expression patterns in scleroderma
50. Kondo, K., et al. 2001. Establishment and char-
acterization of a human B cell line from the lung
tissue of a patient with scleroderma; extraordinary
high level of IL-6 secretion by stimulated fibro-
51. Forster, I., and Rajewsky, K. 1987. Expansion and
functional activity of Ly-1+ B cells upon transfer
of peritoneal cells into allotope-congenic, newborn
52. Luna, L.G. 1992. Histopathologic methods and color
atlas of special stains and tissue artifacts. American His-
tolabs. Gaithersburg, Maryland, USA. 767 pp.
methods of the armed forces institute of pathology. McGraw-Hill
54. Ehlich, A., Martin, V., Muller, W., and Rajewsky, K.
1994. Analysis of the B-cell progenitor compartment
55. Alt, F.W., and Baltimore, D. 1982. Joining of immu-
noglobulin heavy chain gene segments: implications for
a chromosome with evidence of three D-JH
56. Strohl, R., Helmberg, A., Kroemer, G., and Kolker, R.
1989. Mouse VK gene classification by nucleic acid
sequence similarity. Immunogenetics. 30:475–493.
into heavy-chain genes is correlated with expres-
311:752–755.
58. Lafaille, J.J., DeClos, A., Bonneville, M., Takagaki,
Y., and Tonegawa, S. 1989. Functional sequences of T
cell receptor gamma delta genes: implications for
gamma delta T cell lineages and for a novel inter-