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B CELL DEPLETION THERAPY EXACERBATES MURINE PRIMARY BILIARY CIRRHOSIS

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Abstract

Primary biliary cirrhosis (PBC) is considered a model autoimmune disease due to the clinical homogeneity of patients and the classic hallmark of anti-mitochondrial antibodies (AMAS). Indeed, the presence of AMAS is the most highly directed and specific autoantibody in autoimmune diseases. However, the contribution of B cells to the pathogenesis of PBC is unclear. Thus, although AMAs appear to interact with the biliary cell apotope and contribute to biliary pathology, there is no correlation of disease severity and titer of AMA. The recent development of well characterized mAbs specific for the B cell populations, anti-CD20 and anti-CD79, and the development of a well defined xenobiotic induced model of autoimmune cholangitis, prompted us to utilize these reagents and the model to address the contribution of B cells in the pathogenesis of murine PBC. Prior to the induction of autoimmune cholangitis, mice were treated with either anti-CD20, anti-CD79, or isotype matched control mAb and followed for B cell development, the appearance of AMAs, liver pathology and cytokine production. Results of the studies reported herein show that the *in vivo* depletion of B cells using either anti-CD20 or anti-CD79 led to the development of a more severe form of cholangitis than control mice which is in contrast with results from a number of other autoimmune models which have documented an important therapeutic role of B cell specific depletion. The anti-CD20/CD79 treated mice have increased liver T cell infiltrates and higher levels of pro-inflammatory cytokines. In conclusion, our results

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reflect a novel disease protective role of B cells in PBC and suggest that B cell depletion therapy in humans with PBC should be approached with caution.

INTRODUCTION

Although the role of B cells in autoimmunity has historically been associated with the ability to produce autoantibodies (1), it is now clear that B cells are involved in multiple mechanisms beyond antibody secretion, including regulatory function (2–3). Indeed, B cells efficiently present antigens (4), act as co-stimulators during the initiation of immune responses (5–7), and secrete cytokines (3,8–10). Not surprisingly, this increased awareness of the importance of B cells in the pathogenesis of autoimmunity has led to the development of novel B cell-targeted biological therapies (11–15).

Primary biliary cirrhosis (PBC) is considered a model autoimmune disease highlighted by the presence of high titers of anti-mitochondrial antibodies (AMA) against the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) found in 95% of patients (16–20), and considered the most specific autoantibody in human autoimmune disease. Interestingly, there is no correlation of disease severity with the titer of AMA (21–22) and recent studies in the TGF- β receptor II dominant negative (dnTGF- β RII) murine model of PBC (23) have suggested that while depletion of B cells in adult mice worsens liver disease (24), similar depletion of B cells in young dnTGF- β RII mice has a marginal beneficial clinical effect (24).

Until recently our understanding of PBC has been limited by the absence of appropriate animal models. Based upon a rigorous quantitative analysis of the epitope of PDC-E2, our laboratory has identified several organic compounds which resemble the immunodominant epitope of PDC-E2. In particular, 2-octynoic acid (2OA), a compound found in perfumes, lipstick, and many common food flavorings reacts equally or even better than lipoic acid to AMAs (25–26). Importantly, immunization with 2OA when coupled to BSA, induces high titer AMAs and portal inflammation strikingly similar to human PBC (27–29). We report herein that treatment of this xenobiotic induced murine model of human PBC with either anti-CD20 or anti-CD79 monoclonal antibodies (mAbs) exacerbates liver pathology, even though it successfully depletes B cells and diminishes the production of AMAs. These findings have important clinical implications for the treatment of PBC and other autoimmune diseases in which B cell regulatory function may be critical.

MATERIALS AND METHODS

Mice. Experimental protocol

Female C57BL/6J (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in ventilated cages under specific pathogen-free conditions at the animal facilities of the University of California at Davis. The Animal Care and Use Committee in University of California Davis approved all studies.

To deplete B cells *in vivo*, four independent groups of 6-week-old mice were injected intraperitoneally (i.p.) weekly with either sterile murine IgG2a anti-mouse CD20 antibody (n=8) (250 μ g/250 μ L in PBS), hamster IgG2 anti-mouse CD79b antibody (n=10) (1 mg/100 μ L in PBS), or isotype-matched control monoclonal antibodies (mAbs). The anti-mouse CD20 IgG2a (Biogen Idec, San Diego, CA) and the Armenian hamster anti-mouse CD79b IgG utilized herein have been described elsewhere (30–31). The non cross reactive mouse anti-human CD20 antibody (250 μ g/250 μ L in PBS) and an Armenian hamster normal IgG (1 mg/mL) (Innovative Research, Novi, MI), were used respectively, as controls.

One week after the beginning of B cell depletion therapy, autoimmune cholangitis was induced as previously described (24). Briefly, a mixture of 2-octynoic acid BSA conjugated (2OA-BSA; 100 µg/100 µL in PBS) was injected i.p. with Complete Freund's Adjuvant (CFA; Sigma-Aldrich, St. Louis, MO) containing 1 mg/mL of *Mycobacterium tuberculosis* strain H37RA, and subsequently boosted every two weeks with 2OA-BSA in incomplete Freund's Adjuvant (IFA; Sigma-Aldrich, St. Louis, MO). Additionally, mice received 100 ng of pertussis toxin (List Biological Laboratories, Campbell, CA) at the time of initial immunization with 2OA-BSA in Complete Freund's Adjuvant.

Peripheral blood samples from individual mice were obtained from the tail vein prior to the initiation of treatment with mAbs (baseline) and then at 2-week intervals. Sera was collected prior to mAb treatment, a week after, and thereafter every 4 weeks, and stored at -70°C until use. Animals were sacrificed at 15 weeks of age.

Detection of anti-PDC-E2 antibodies

Serum titers of anti-PDC-E2 autoantibodies were measured by enzyme-linked immunosorbent assay (ELISA) using our well standardized recombinant autoantigens (32).

Mononuclear Cell Preparation and Flow Cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized murine blood using Accupaque (Accurate Chemical & Scientific Company, Westbury, CT) to assess levels of B cells. Cells were pre-incubated with anti-mouse FcR blocking reagent and then incubated at 4°C with a predetermined optimum concentration of APC-conjugated anti-TCRβ (BioLegend), PE-conjugated anti-mouse IgM (Caltag), and FITC-conjugated anti-CD19 (BioLegend); B cell frequency was then determined by flow cytometry. The liver and spleens were collected from mice immediately following sacrifice and single cell mononuclear cell suspensions prepared for multicolor flow analysis as previously described (23).

Histopathology and Immunohistochemistry

Immediately after sacrifice, liver and spleen tissues were harvested and fixed in 10% buffered formalin, embedded in paraffin, and cut into 4 µm sections for routine hematoxylin (DakoCytomation, Carpinteria, CA) and eosin (American Master Tech Scientific, Lodi, CA) (H&E) staining. Evaluation under light microscopy and scoring of liver inflammation was performed on coded H&E-stained sections of liver using a set of three indices by a “blinded” pathologist (KT); indices included degrees of portal inflammation, parenchymal inflammation, and bile duct damage (33). Phenotypic analysis of bile duct damage was performed as described (34).

Serum liver enzyme quantification

Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphates (ALP) were measured using the Roche Diagnostics COBAS INTEGRA 400 Plus (Indianapolis, IN).

Cytokine Detection

Serum levels of the pro-inflammatory cytokines, interleukin 6 (IL-6), IL-10, monocyte chemotactic protein-1 (MCP-1), interferon gamma (INF-γ), tumor necrosis factor alpha (TNF-α), and IL-12p70 were quantified using the BD Cytometric Bead Array (CBA) Mouse Inflammatory Kit (BD Biosciences) as described (35). The serum samples were loaded onto the plate neat. The samples were then analyzed using the commercial protocol utilizing a FACScan flow cytometer (BD Immunocytometry, San Jose, CA) and BD CBA Software.

Statistical Analysis

The statistical difference between two groups was determined using a two tailed Mann-Whitney non-parametric test with 95% confidence interval (CI). All results were expressed as means $\pm$ standard error (SEM). The Prism statistical package (GraphPad Software Inc, La Jolla, CA) was used. Values of $p < 0.05$ were deemed statistically significant.

RESULTS

Anti-CD20 and anti-CD79 antibodies deplete B cells

To assess the efficacy of the B cell depletion, the frequency of CD19⁺ cells in peripheral blood was quantified by flow cytometry. The vast majority of B cells were depleted one week after the beginning of antibody administration in mice treated with either anti-CD20 (Figure 1A–C) or anti-CD79 (Figure 1B–D), while control mice exhibited no detectable changes in B cell frequency. Indeed, the frequency of CD19⁺ cells in PBMC from anti-CD79 treated mice was 9.60% versus 46.89% in controls ($p < 0.001$) after one week of treatment, and 0.34% in anti-CD20 treated mice versus 32.63% in controls ($p < 0.001$). Importantly, both treated groups consistently displayed marked depletion of B cells after 8 weeks of therapy. B cell depletion was also assessed in the liver and spleen of the anti-CD20 and anti-CD79 treated mice (Table 1). Again, the anti-CD20 and anti-CD79 treated mice demonstrated a decrease in B cells as compared to the control mice.

B cell depletion abolishes PDC-E2 specific antibody production

The effect of B cell depletion on serum reactivity against PDC-E2 was assessed at weeks 4 and 8 after the first immunization with 2OA. As seen in Fig. 2, whereas mice treated with control antibodies produced high titers of PDC-E2 specific antibodies, the sera from mice depleted of B cells showed undetectable levels of PDC-E2 reactive antibodies ($p < 0.0001$).

B-cell depleted mice develop more aggressive PBC-like liver disease

Liver sections from anti-CD20 (Figure 3A) and CD79 (Figure 4A) treated mice demonstrated a marked increase of cellular infiltrates in the portal tract and around the interlobular bile ducts, and an overall marked increase in liver inflammation compared to the respective controls (*data not shown*). Increased infiltration of lymphocytes or mononuclear cells surrounding damaged bile ducts was frequently observed in portal areas. The degrees of portal tract inflammation plotted individually are shown in Figures 3B and 4B. Furthermore, bile duct damage was observed, and epithelioid granulomas were scattered within some portal tracts and also in hepatic parenchyma. Bile duct damage was studied by immunostaining with anti-CK22 (Figure 5). Histological findings characteristic of PBC-like disease including interlobular bile duct damage and non suppurative destructive cholangitis were readily noted in the liver tissues from the B cell depleted mice.

B cell depletion by anti-CD20 and anti-CD79 increases the number of CD4⁺ and CD8⁺ T cell infiltrates in the liver

To clarify whether T cell infiltration was affected by B cell depletion with anti-CD20 and anti-CD79, total T cells numbers in the liver and spleen were quantified by flow cytometric analysis (Table 1). The number of CD3⁺ T cells, as well as absolute number of liver CD4⁺ and CD8⁺ T cells, was significantly increased in livers of B cell depleted mice compared to the control groups. Importantly, the absolute number of both CD4⁺ and CD8⁺ T cells that co-expressed CD44 but lacked CD62L, the phenotype of mouse effector memory T cells, was increased over controls in the liver: CD4⁺ CD62L[−] CD44⁺ cells 0.136 ± 0.05 versus 0.09 ± 0.02 ($\times 10^6$) ($p = 0.0262$) in CD20 treated mice versus controls, and 0.64 ± 0.11 versus 0.27 ± 0.03 ($\times 10^6$) ($p = 0.004$) in CD79 treated mice versus controls; CD8⁺ CD62L[−] CD44⁺

cells 0.14 ± 0.06 versus 0.05 ± 0.01 ($\times 10^6$) ($p=0.0348$) in CD20 treated mice versus controls, and 0.362 ± 0.06 versus 0.158 ± 0.029 ($\times 10^6$) ($p=0.007$) in CD79 treated mice versus controls. As expected, there was no difference in the phenotypic distribution of MNC in spleens (*data not shown*).

B-cell depleted mice have elevated levels of liver enzymes

ALT, AST, and ALP levels were measured to assess the correlation between serum biochemical values and histopathological abnormalities. As seen in Table 2, mice treated with anti-CD79 produced higher levels of ALT, AST, and ALP compared to that of the control treated mice ($p<0.05$). Mice treated with anti-CD20 demonstrated a significant increase in AST levels only ($p<0.05$).

Cytokine profiles

The levels of serum inflammatory cytokines in B cell depleted mice were higher compared to control treated mice starting at 4 weeks of 2OA immunization (Figure 6A). In particular, serum levels of IFN- γ in the CD20 and the CD79 treated mice were significantly higher as compared with sera from control mice ($p=0.046$ and $p=0.011$, respectively). Similarly, serum levels of MCP-1 in CD20 and CD79 treated mice were also significantly higher than sera from the control mice ($p=0.0017$ and $p=0.042$, respectively) at 8 weeks after cholangitis induction. As expected, IL-10, in part secreted by B cells, was lower in B cell depleted mice (Figure 6B).

DISCUSSION

We demonstrate herein that acute B cell depletion in mice with otherwise normal immune system exacerbates cholangitis induction. Liver cell infiltrates from B-cell-depleted mice contained increased populations of CD4⁺ and CD8⁺ T cells and activated counterparts, and exhibited increased serum levels of IFN- γ and monocyte chemotactic protein-1 (MCP-1), but lower levels of IL-10, compared to B-cell-sufficient mice. These findings should be considered in the context of recent studies on the role of B cells in a genetic animal model of PBC, the dnTGF- β RII mouse (23). Anti-CD20 therapy in young dnTGF- β RII mice attenuates liver damage but exacerbates inflammatory bowel diseases (34); however, B cell depletion in older mice did not modify the course of liver disease (34). Importantly, double transgenic mice with PBC like disease and B cell depletion, Igu^{-/-}dnTGF- β BII mice, developed a more severe form of cholangitis (24). The present work demonstrates that B cell depletion immediately before disease induction in the xenobiotic induced murine model enhances disease severity in the absence of AMAs, and consequently suggests that B cells play a critical regulatory role in the breakdown of tolerance against PDC-E2. These studies demonstrate that B cells play both positive and negative regulatory roles in the pathogenesis of PBC, and that the balance of these effects is likely to determine the severity of disease. In the study reported here, we deplete B cells before induction of cholangitis by xenobiotics. However, future studies on different timings of B cell depletion after induction of cholangitis by xenobiotics will be helpful to better define the role of B cells in the natural history of established disease.

A beneficial effect of anti-CD20 therapy has been reported in animal models of T cell mediated disease including experimental autoimmune encephalomyelitis (EAE) (2), type 1 diabetes (36) and systemic sclerosis (37); it has been mainly attributed to reduced T cell activation, however the reduction of antibody production may also have a beneficial effect (12). The importance of B regulatory cells (Bregs) has been suggested in several autoimmune diseases (2,38–39) and may reflect a role of IL-10 producing B cells as suppressors of autoimmune and inflammatory diseases (40–41). A recent study reports that

Bregs predominantly control disease initiation in the EAE model, whereas Tregs reciprocally inhibit late-phase disease (42). A proposed model for EAE may explain the role of Bregs in PBC (43). Fillatreau and colleagues suggest that following immunization with autoantigen in complete Freund's adjuvant, activation of antigen presenting cells (APC) through toll-like receptor 2 (TLR2), TLR4 and TLR9 (by mycobacterial ligands) stimulates the production of high levels of cytokines (i.e. IL-6, IL-12, IL-23) and drives the expansion of two auto-antigen-reactive CD4⁺ T-cell populations: an auto-aggressive population and a regulatory T (Treg) cell population. Concomitant stimulation of B cells through TLR2 or TLR4 early in the response induces production of IL-10, which has an inhibitory effect on the cytokine production by APCs', and eventually limits the initial expansion of the auto-aggressive cohort, ultimately leading to resolution of the disease. In the absence of B cells (or B-cell-derived IL-10), the early expansion of the auto-aggressive population dominates, and the Treg cohort is unable to control this population. In accordance with this theory, we have found lower levels of IL-10 in B cell depleted mice

The mechanisms responsible for exacerbation of cholangitis in B cell depleted mice remains enigmatic but the following data is relevant. First, B cell depleted mice generate high levels of IFN- γ , a potent activator of the innate immune system (44). The innate immune system of patients with PBC demonstrates a higher reactivity than controls (45). Indeed, the frequency and absolute number of blood and liver resident natural killer cells are increased in patients with PBC, as is their cytotoxic activity and perforin expression (46); moreover peripheral monocytes from patients with PBC secrete higher levels of cytokines (47). Second, B cell depletion in the gut lymphoid tissue may increase portal venous concentrations of pathogen-associated molecular patterns with consequent activation of the innate immune system through TLR recognition. Third, evidence suggests that antigen-naïve B cells exert anti-inflammatory properties (48), which may inhibit APC maturation and proinflammatory differentiation (49); in this regard, it has been demonstrated that dendritic cells from B cell deficient mice produce higher levels of IL-12 and promote pro-inflammatory T cell differentiation (8).

Thus, our findings suggest that B cell depletion therapy may be contraindicated in PBC. Indeed, we know that B cell depletion in dnTGF- β BII mice exacerbates inflammation of bile ducts (24). Although a biochemical benefit of anti-CD20 therapy in PBC patients refractory to ursodeoxycholic acid has been reported (50), we suggest that more data regarding the role of B cells in human mucosal autoimmune diseases, such as PBC, is needed. Finally, the findings reported here underscore the fact that unanticipated problematic issues can arise from the use of biologics in humans and thus the importance of trials in murine models and rigorous post marketing surveillance of such agents in humans.

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ABBREVIATIONS

PBC	Primary Biliary Cirrhosis
AMAs	anti-mitochondrial antibodies
mAbs	monoclonal antibodies
PDC-E2	E2 subunit of the pyruvate dehydrogenase complex
ELISA	enzyme-linked immunosorbent assay

TCRβ	T cell receptor β
IL	interleukin
INF-γ	interferon gamma
TNF-α	tumor necrosis factor alpha
MCP-1	monocyte chemotactic protein-1
dnTGF-βRII	TGF- β receptor II dominant negative

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Figure 1.

Time course of anti-CD20 (A) and anti CD-79 (B) induced B cell depletion in peripheral blood. Representative MNC gated plots are shown for both CD20 mAb (C) and CD79 mAb (D) and respective control mAbs after 4 weeks of treatment. B cell frequencies were determined by immunofluorescence staining with flow cytometry analysis. Line and dot graphs indicate mean $\pm$ SEM. Significant differences were determined with Mann-Whitney test and are indicated as *** $p < 0.0001$.

Figure 2.

Serum reactivity to PDC-E2 was quantified by ELISA before immunization with 2OA in both anti-CD20 (A) and anti CD79 treated mice (B). All values were corrected with baseline to eliminate background noise; significant differences between treatment antibodies and their controls were evaluated with Mann-Whitney test and are indicated as *** $p < 0.001$.

Figure 3. (A) Hematoxylin-eosin-stained tissue sections of livers from anti-CD20 -mAb-treated mice (right) demonstrate greater levels of lymphoid infiltration in the portal tract (black arrows) than controls (left). Bile duct damage is indicated with red arrows and portal space lacking bile ducts with an asterisk; two asterisks highlight a granuloma. Black scale bars = 100 μ m. (B) The portal inflammation score, percentage of portal granulomas and parenchymal granulomas are presented for each sample. ** $p < 0.01$, * $p < 0.05$ in Mann-Whitney test.

Figure 4.

(A) Histological evidence of cholangitis in representative hematoxylin-eosin-stained liver sections; tissue sections from anti-CD79 -mAb-treated mice demonstrate increase of cellular infiltrates around portal spaces (black arrows) and interlobular bile duct damage (red arrows). Portal space lacking bile ducts are indicated with an asterisk; two asterisks highlight a granuloma. Black scale bars = 100 μ m. (B) The portal inflammation score, percentage of portal granulomas and parenchymal granulomas are presented for each sample. ** $p < 0.001$, * $p < 0.05$ in Mann-Whitney test.

Figure 5. (A, C) Bile duct damage was studied by immunostaining with anti-CK22. Black arrow indicates interlobular bile duct damage, and the red arrow chronic non suppurative destructive cholangitis. Portal spaces with bile duct loss are indicated with an asterisk. Scale bars = 100 μ m. (B, D) A quantitative evaluation of bile duct damage (percentage of portal spaces without interlobular ducts) is presented for each sample. * $p < 0.05$ in Mann-Whitney test

Figure 6.

(A) Serum concentration of IFN- γ and MCP-1 was significantly higher in B cell depleted mice than controls, whereas (B) IL-10 was lower. The bar graphs indicate mean $\pm$ SEM. Significant differences are indicated as **p < 0.01, *p < 0.05.

Table 1

Phenotypic analysis of mAb treated mice

A.

Tissue	CD19 ⁺ TCRβ ⁺ cells (x10 ⁶)			
	control mAb	CD20 mAb	control mAb	CD79 mAb
Liver	1.07 ± 0.11	0.01 ± 0.01 **	1.68 ± 0.41	0.11 ± 0.02 ***
Spleen	32.01 ± 3.65	0.06 ± 0.01 ***	53.05 ± 4.91	2.92 ± 0.32 ***

B.

	Control mAb	CD20 mAb	<i>p</i>
CD3 ⁺ T cells (x10 ⁶)	0.68 ± 0.53	1.04 ± 0.12	0.011
CD3 ⁺ CD4 ⁺ (x10 ⁶)	0.26 ± 0.02	0.38 ± 0.02	0.017
CD3 ⁺ CD8 ⁺ (x10 ⁶)	0.14 ± 0.04	0.25 ± 0.08	0.030
CD4 ⁺ CD62L ⁺ CD44 ^{hi} (x10 ⁶)	0.09 ± 0.01	0.13 ± 0.02	0.026
CD8 ⁺ CD62L ⁺ CD44 ^{hi} (x10 ⁶)	0.05 ± 0.01	0.14 ± 0.06	0.034
CD3 ⁺ NK1.1 ⁺ (x10 ⁶)	0.33 ± 0.04	0.39 ± 0.08	ns
CD3 ⁺ NK1.1 ⁺ (x10 ⁶)	0.24 ± 0.02	0.27 ± 0.04	ns

C.

	Control mAb	CD79 mAb	<i>p</i>
CD3 ⁺ T cells (x10 ⁶)	1.97 ± 0.18	4.32 ± 0.50	0.001
CD3 ⁺ CD4 ⁺ (x10 ⁶)	0.71 ± 0.07	1.23 ± 0.13	0.005
CD3 ⁺ CD8 ⁺ (x10 ⁶)	0.63 ± 0.07	0.91 ± 0.08	0.027
CD4 ⁺ CD62L ⁺ CD44 ^{hi} (x10 ⁶)	0.27 ± 0.03	0.64 ± 0.11	0.004
CD8 ⁺ CD62L ⁺ CD44 ^{hi} (x10 ⁶)	0.15 ± 0.02	0.36 ± 0.06	0.007
CD3 ⁺ NK1.1 ⁺ (x10 ⁶)	0.46 ± 0.06	1.00 ± 0.14	0.002
CD3 ⁺ NK1.1 ⁺ (x10 ⁶)	0.43 ± 0.05	0.50 ± 0.04	ns

Values are expressed as mean ± SEM;

p < 0.001,**
p < 0.005.

Values are expressed as mean ± SEM; ns, not significant.

Table 2

Serum levels of liver enzymes in mAb treated mice.

Enzyme	Control mAb	CD20 mAb	Control mAb	CD79 mAb
ALT	35.5±2.9	36.6±3.1	26.7±2.1	43.4±5.8*
AST	108.9±8.7	136.2±9.3*	122.0±9.9	160.5±11.6*
ALP	97.4±2.9	97.6±4.6	92.4±2.6	106.2±5.0*

Values are expressed as mean ± SEM;

*
p < 0.05.