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Dual B-cell targeting therapy ameliorates autoimmune cholangitis

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Abstract

Objective: The ability to regulate B cell development has long been recognized to have therapeutic potential in a variety of autoimmune diseases. However, despite the presence of a classic autoantibody in primary biliary cholangitis (PBC), B cell depleting therapy and indeed therapy with other biologic agents has been disappointing. Unsuccessful treatment using Rituximab is associated with elevation of B-cell activating factor (BAFF) level. Indeed, therapies for PBC remain directed at modulating bile salt biology, rather than targeting effector pathways. With these data in mind, we proposed that targeting two major stages of B cell development, namely long-lived memory B cells and short-lived peripheral autoreactive plasma cells would have therapeutic potential.

Methods: To address this thesis, we administrated anti-BAFF and anti-CD20 monoclonal antibody to ARE-Del mice, a well-characterized murine model of human PBC. We evaluated and compared the therapeutic efficacy of the two agents individually and the combination of anti-BAFF and anti-CD20 in female mice with well-established disease.

Results: Our data demonstrate that there was an increased level of B cell depletion that resulted in a significantly more effective clinical and serologic response using the combination of agents as compared with the use of the individual agents. The combination of anti-BAFF and anti-CD20

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Author statement

Weici Zhang: Conceptualization, Data acquisition, visualization and curation, Investigation, Writing-Original draft preparation. Tihong Shao: Data acquisition, Investigation. Patrick S. Leung: Investigation, Writing-reviewing. Koichi Tsuneyama: Pathology data acquisition and visualization, Methology. Luke Heuer: Data acquisition. Howard A. Young: Writing-reviewing and Editing. William M. Ridgway: Supervision, Writing-reviewing and Editing. M. Eric Gershwin: Conceptualization, Supervision, Writing-reviewing and Editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2022.102897.

treatment was more effective in reducing serum levels of antimitochondrial antibody (AMA), total IgM and IgG compared to mice treated with the 2 individual agents. Combination treatment efficiently depleted B cells in the peripheral blood, peritoneal cavity and spleen. Importantly, we identified a unique IgM^+ $FCRL5^+$ B cell subset which was sensitive to dual B-cell targeting therapy and depletion of this unique population was associated with reduced portal infiltration and bile duct damage. Taken together, our data indicate that dual B cell targeting therapy with anti-BAFF and anti-CD20 not only led to the efficient depletion of B cells both in the peripheral blood and tissues, but also led to significant clinical improvement. These findings highlight the potential application of combination of anti-BAFF and anti-CD20 in treating patients with PBC. However, additional studies in other animal models of PBC should be undertaken before considering human trials in those PBC patients who have incomplete responses to conventional therapy.

Keywords

B-cell targeting therapy; Primary biliary cholangitis; Antigen-specific B cells; Antimitochondrial antibody; Autoimmunity

1. Introduction

Primary biliary cholangitis (PBC) is a chronic progressive autoimmune mediated liver disease of small-to medium sized intrahepatic bile ducts predominantly affecting women. PBC patients develop cholestasis, fibrosis, and are at risk for cirrhosis; there is a major void in therapeutic choice [1]. Current therapy for PBC is relatively limited and a number of clinical trials with newer biologics have failed [2–5]. Although ursodeoxycholic acid (UDCA) is the first line therapy for PBC globally, it is often insufficient to prevent the progression of disease to hepatic decompensation, but the discontinuation of UDCA often leads to elevation of serum enzymes. Importantly, 20–30% of PBC patients have incomplete responses to conventional therapy associated with significant risk of disease progression. Obeticholic acid, an FXR agonist, has been approved for the treatment of PBC but has the side effect of pruritus and is neither novel nor a breakthrough. New strategies for patients with PBC who do not respond to conventional therapy is therefore an urgent priority [6].

PBC is characterized by the presence of highly specific anti-mitochondrial antibodies (AMA) in over 95% of patients and is often present long before clinical disease [7]. Our previous study has demonstrated the pathological significance of AMA in PBC [8]. For example, AMA enhances the generation of PBC specific cytotoxic T cells in the presence of mitochondrial autoantigen pulsed antigen specific cells [9]. IgA-AMA transcytoses through biliary epithelial cells with the formation of IgA-AMA-mitochondrial protein complexes that leads to the induction of biliary epithelial cells apoptosis [10]. We also have evidence that the activation of AMA generating plasma cells is ongoing process, indicating the sustained rigorous response of autoantigen-specific B cells in PBC [11]. A recent study on paired analysis of the liver infiltrating and circulating B cell repertoire in PBC patients provides supportive evidence of an oligoclonal expanded infiltrating B cell repertoire in PBC and further reflects the importance of B cell immunity in the pathogenesis of PBC [12]. Clonal

exchange of autoreactive B cell clones between the infiltrating and peripheral repertoire may account for the elevated frequency of AMA producing plasmablasts in PBC [11].

The B cell activating factor of the TNF-family (BAFF) acts through BAFFR to promote B cell survival, facilitating Ig class switch and recombination. BAFFR expression is acquired at the immature stage maintained through the memory stage in B cells [13]. In addition to supporting B cell survival, BAFF delivers co-stimulatory signals to T cells and promotes inflammation via the Th1/Th17 pathways [14]. Belimumab, an anti-BAFF monoclonal antibody, was approved by the FDA for the treatment of systemic lupus erythematosus (SLE) and Lupus nephritis [15,16]. In PBC, there is a positive correlation between serum BAFF levels, AMA titer and frequency of circulating plasmablasts, suggesting BAFF may contribute to induction and development of PBC [17].

CD20 is a molecule expressed on the surface of B cells at almost all stages of B cell development before plasmablast and plasma cell differentiation, which functions to optimize B cell responses, especially to T cell dependent antigens [18]. CD20 does not have known natural ligands. Anti-CD20 monoclonal antibodies are effective in depleting peripheral short-lived autoreactive plasma cells [19] but much less so in solid tissues [20]. Anti-CD20 has been used to deplete B cells in multiple autoimmune disorders in both humans and murine models but with mixed results [21]. A major factor to consider in the mixed responses to anti-CD20 is BAFF. Rituximab treatment leads to elevations of serum BAFF level and increases in number of plasmablasts in patients with SLE, Sjogren syndrome and pemphigus vulgaris [22–25]. Interestingly, baseline serum levels of BAFF is inversely correlated with the duration of B cell depletion [26]. Recent studies demonstrate that the administration of anti-CD20 with anti-BAFF effectively reduces the number of long-lived splenic plasma cells in a murine model of autoimmune cytopenia and also in lupus prone NZB/W mice [27]. Phase III BLISS-BELIEVE trial findings presented at the ACR Convergence 2021 virtual meeting indicated that a combination of B cell-targeting therapy with anti-BAFF and a single cycle of anti-CD20 did not improve disease control/remission in patients with SLE [28].

In the present study, we evaluated and compared the therapeutic efficacy of a combination of two presumably effective agents that included anti-BAFF and anti-CD20 monoclonal antibodies and for comparison tested the two single-agents alone in a female dominant murine model of PBC with established disease [29,30]. Our data indicate that anti-BAFF in combination with anti-CD20 are effective therapeutic agents that lead to improved immunological characteristics accompanied by marked improvement in clinical features of autoimmune cholangitis in the ARE-Del model. These data have potential for not only UDCA refractory patients with PBC but for other anti-CD20 refractory autoimmune diseases.

2. Materials and methods

2.1. Animal group and study design

Female heterozygous ARE-Del $^{+/-}$ mice were generated by crossing male homozygous ARE-Del $^{-/-}$ mice [29,30] with female wild type B6 mice. Mice were housed in

specific pathogen free conditions at the University of California, Davis vivarium. 12 weeks old female ARE--Del$^{+/-}$ were randomly divided into four experimental groups and intraperitoneally (IP) administrated with either phosphate buffered saline (PBS, controls), anti-BAFF alone, anti-CD20 alone or anti-BAFF in combination with anti-CD20 mAbs. Anti-BAFF (10F4, GlaxoSmithKline, GSK), a neutralizing recombinant hybridoma monoclonal antibody raised against a mouse BAFF protein produced in Armenian Hamster cell line (No Hamster material present). Anti-BAFF was administered at 100 mcg (5 mg/kg) three times a week, anti-CD20 (Ultra-LEAF™ purified anti-mouse CD20, BioLegend) was administered at 250 mcg (12.5 mg/kg) once every 4 weeks for a total of 12 weeks. Serum immunoglobulin (Ig) G, IgA, IgM, AMA levels and inflammatory cytokine levels were monitored every 4 weeks. All studies were conducted in accordance with the University of California, Davis and the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals and were reviewed and approved by the Institutional Animal Care and Use Committee at GSK and the University of California Animal Care and Use Committee.

2.2. Detection of immunoglobulin (Ig) G, IgA, IgM and AMA levels

Standard enzyme-linked immunosorbent assay (ELISA) was performed to detect serum levels of IgG, IgA, IgM and AMA. Serum IgG, IgM, IgA were measured using the standardized panel of mouse IgG, IgM and IgA ELISA quantitation kits (Bethyl Laboratories). Serum AMA levels were detected using our well-established ELISA against recombinant proteins of the pyruvate dehydrogenase complex-E2 subunit (PDC-E2) [31]. Briefly, 96-well ELISA plates were coated with recombinant PDC-E2 proteins (5 mcg/mL) in carbonate coating buffer at 4 °C overnight. A 3% non-fat dry milk in PBS was dispensed into each well followed by incubation with 1:250 dilution of the serum samples for 1 h. The plates were then washed with PBS containing 0.05% Tween 20 and incubated for 1 h with a predetermined optimized dilution of horse-radish peroxidase (HRP) conjugated anti-mouse IgG, IgM and IgA, respectively (Invitrogen, Carlsbad, CA), washed and developed with BD OptEIA Substrate (BD Biosciences, San Diego, CA).

2.3. Measurement of cytokine production

Serum cytokine levels including IL-6, IL-10, MCP-1, interferon-gamma (IFN-γ), TNF, and IL-12p70 were quantified using the Mouse Inflammatory Kit, BD™ Cytometric Bead Array (CBA) (BD Biosciences) [31]. Data were acquired and analyzed using a FACScan flow cytometer with CBA software (BD Biosciences).

2.4. Cell isolation and flow cytometry analysis

Peripheral blood samples were collected in an anti-coagulant tube containing 20 USP units/ml sodium heparin. RBC lysis buffer (eBioscience) was used to lyse the red blood cells. The resulting white blood cells were pre-incubated with anti-mouse CD16/CD32 FcR blocking antibodies, and aliquots then incubated with a pre-determined optimum concentration of antibody cocktails. Mononuclear cells (MNCs) from spleen and liver tissue were harvested as previously described [32]. Monoclonal antibodies for cell surface markers CD4, CD8a, B220, CD19, IgM, NK1.1, F4/80, TCRβ were purchased from BioLegend (BioLegend, San Diego, CA). FCRL5 was purchased from R&D systems. Optimal dilutions of antibodies were used throughout and appropriate positive and negative

controls included with each analysis. Data were acquired either on a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA) upgraded by Cytec Development (Fremont, CA) or on a LSRII flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Treeland, Ashland OR).

2.5. Histopathology

A portion of liver and spleen tissue were excised immediately upon sacrifice, and fixed in 4% paraformaldehyde (PFA) solution for 2 days at room temperature, embedded in paraffin, cut into 4-μm sections and deparaffinized for routine hematoxylin and eosin (H&E) staining [33, 34]. Portal inflammation was examined and scored based on two parameters: severity and frequency as follows: severity; 0, normal liver histology; 1, minimal inflammation; 2, mild inflammation; 3, moderate inflammation; and 4, severe inflammation. Frequency; 0, normal liver histology; 1, 1%–10%; 2, 11%–20%; 3, 21%–50%, 4, more than 50%. The bile duct damage was graded based on severity and frequency as follows: severity; 0, no significant changes of bile duct; 1, epithelial damage with cytoplasmic changes; 2, epithelial damage with nuclear changes; 3, chronic non-suppurative destructive cholangitis (CNSDC); 4, bile duct loss. All sections were evaluated by a pathologist who was blind to the design of the study.

2.6. Statistical analysis

Data sets between treatment and control group were analyzed for normality and log normality test. The statistical significance was evaluated by one-way ANOVA for data with a normal distribution and Kruskal-Wallis test for non-normally distributed data using Graphpad Prism software (GraphPad Software Inc., La Jolla, CA). Data are shown as mean ± SD; statistical significance is indicated with asterisks as follows, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

3. Results

3.1. B-cell targeting treatments reduced B cell subsets in periphery

The current study was designed to evaluate the therapeutic potential of a combination of B cell-directed treatment for PBC. We took advantage of the recently developed female dominant PBC murine model named ARE-Del $^{+/-}$ mice [29] and compared the serological alteration and clinical characteristics after the various treatment regimens. The study included a total of 59 female mice and groups of 13 each were administrated with anti-BAFF alone, anti-CD20 alone or anti-BAFF in combination with anti-CD20. The remaining mice were treated with PBS and used throughout as controls. In ARE-Del $^{+/-}$ mice with established disease, the frequency of peripheral blood CD19^{+} B cells were significantly reduced in all three treatment groups 4 weeks after treatment. This decline was more evident in anti-BAFF in combination with anti-CD20 group (combination group) 12 weeks after treatment as compared to the two single treatment groups and PBS controls (Fig. 1A). During B cell differentiation, IgM^{+} immature B cells start receiving BAFF signals through BAFFR to maintain and prolong their survival. Fc receptor-like 5(FCRL5) expression on IgM + B cells was utilized as a marker of long-lived antigen-specific memory B cells [35]. Therefore, we monitored the alterations of IgM^{+} B cells (Fig. 1B) and IgM^{+} FCRL5^{+} B cells

(Fig. 1C) in the peripheral blood in the 3 groups of antibody treated and for comparison the control mice. Our data demonstrated that the frequency of IgM^+ B cells in the periphery decreased in all three treatment groups as compared to PBS controls starting from 4 weeks following treatment and throughout the 12-week treatment period. Interestingly, peripheral IgM^+ $FCRL5^+$ B cells significantly and transiently increased in the anti-BAFF alone group (18.40 ± 4.82% of total B cells; Mean ± SD) compared to the PBS controls (13.62 ± 2.42%) at 4 weeks following treatment, and remained at comparable levels with PBS controls at 12 weeks after treatment. However, peripheral IgM^+ $FCRL5^+$ B cells significantly decreased in the group treated with the combination antibodies (9.41 ± 4.13%) as compared to the PBS controls (13.62 ± 2.42%) starting from 4 week and throughout the treatment period. By the end of the 12-week treatment period, the group receiving the combination antibodies showed a significantly reduced frequency of IgM^+ $FCRL5^+$ B cells in peripheral blood (5.29 ± 0.67%) as compared to the two single-agent treated groups and the PBS group (Fig. 1C).

3.2. B-cell targeting treatments reduced serum levels of autoantibody AMA, total IgM and IgG

To examine the effects of antibody treatment on the autoimmune profile serum AMA, total IgM and IgG levels were measured by ELISA. Anti-BAFF and combination treatment led to a significant reduction of serum AMA (Fig. 2A) and IgM (Fig. 2B) levels following 4 weeks of treatment that lasted through 12 weeks. However, the serum IgG levels were not significantly altered until 12 weeks of treatment. After 12-weeks of treatment, although anti-BAFF therapy alone reduced the serum levels of AMA and IgM it had no significant effect on serum IgG level. The anti-CD20 alone therapy group did not result in any significant reduction of serum AMA, IgM and IgG levels. However, at 12 weeks the group that received combination treatment showed a significant reduction in the serum AMA, IgM and IgG levels as compared to the PBS control and the anti-CD20 alone treatment group (Fig. 2C). The levels of serum inflammatory cytokines IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 did not differ among the antibody treatment groups as compared to PBS control (data not shown). Taken together, combination treatment with anti-BAFF and anti-CD20 antibodies is more efficient in reducing serum levels of AMA, total IgM and IgG than the groups treated with single agents alone.

3.3. Anti-BAFF and anti-CD20 combination treatment significantly reduced the frequencies of IgM + FCRL5+ B cells in the peritoneal cavity

It has been previously reported that peritoneal cavity B cells are more resistant to mAb-induced depletion than splenic B cells [36]. Therefore, we assessed the frequencies of peritoneal B cells upon single-agent and combination treatment. Although treatment with single agents alone led to a significant reduction of B cells (from 68% to 31%), approximately 30%–40% of $B220^+$ B cells still remained in the peritoneal cavity at 12 weeks post treatment (Fig. 3A and B). By contrast, B220+ B-cell depletion was successfully achieved in the group that received the combination therapy (<3%). Since the kinetic analysis of peripheral B cell in response to the mAb-mediated B cell depletion has previously indicated the resistance of peripheral IgM^+ $FCRL5^+$ B cells to depletion using anti-BAFF and anti-CD20 single reagents (Fig. 1C), we evaluated the frequency of this specific B subset in peritoneal cavities. Our data demonstrated that anti-CD20

significantly reduced IgM^+ $FCRL5^+$ B cells residing in peritoneal cavity by 2-fold (10.49 ± 6.65%) whereas anti-BAFF treatment (17.67 ± 9.49%) had no significant depletion effect as compared to the PBS controls (23.96 ± 8.14) (Fig. 3B). Consistent with the data obtained on values of the peripheral blood, IgM^+ $FCRL5^+$ B cells in peritoneal cavity were significantly reduced following combination treatment when compared to anti-BAFF alone and the PBS control groups.

3.4. Anti-BAFF and anti-CD20 combination treatment significantly ameliorates autoimmune cholangitis

We further assessed the impact of B-cell targeted treatments on the B and non-B cell lineages in spleen and liver tissues. There was a significantly reduced mononuclear cell (MNC) count detected in the spleen and liver tissue from anti-BAFF alone and combination treatment groups compared to that from the PBS control group at 12 weeks after treatment (Fig. 4A and B). To examine the depletion of B cells by the three treatment strategies, the B cells frequency was analyzed by flow cytometry. The frequencies of B cells in spleen and peritoneal cavity were greatly reduced in anti-BAFF alone and anti-BAFF and anti-CD20 combination treatment. Although anti-CD20 alone and anti-BAFF alone groups had a comparably reduced B cells in the peritoneal cavity, the frequencies of splenic B cells from anti-CD20 alone group were not significantly reduced when compared to PBS controls. A dramatic and significant reduction of B cells was achieved in anti-BAFF and anti-CD20 combination treatment with 5.53 ± 6.87% of B cells in spleen when compared with the frequencies in spleen 72.35 ± 5.16% from PBS controls, respectively (Fig. 4C, left graph). Our previous study [29] suggested that adoptive transfer of splenic CD4 T cells enhances GC responses and TBA secretion, an initial step in the pathogenesis of disease in ARE-Del mice. We examined the frequency of CD4 T cells in spleen, which were reduced in both anti-BAFF alone and combination treatment groups as compared to PBS controls (Fig. 4C, right graph). Most importantly, liver histological analysis was performed to assess the impact of B-cell targeting treatment on the clinical improvement of autoimmune cholangitis. Our results demonstrate that only the combination treatment with anti-BAFF and anti-CD20 led to a significantly improved liver pathology as reflected by reduced portal inflammation and bile duct damage as compared to PBS controls (Fig. 5A and Fig. 5B). The data indicate that B cell targeting therapy with a combination of anti-BAFF and anti-CD20 not only led to the reduced B cells in peripheral blood and tissues, but also led to reduced portal infiltration and a marked improvement in bile duct damage.

4. Discussion

Loss of B cell tolerance and production of autoantibodies are the primary features of autoimmune disease. It has been clear that, rather than merely bystanders, B cells are active participants in autoimmune disease through multiple mechanisms. Therapeutic strategies targeting B cells such as B cell depletion (anti-CD20, anti-CD19) and inhibition of B cell survival signaling (anti-BAFF) are currently employed in autoimmune diseases including SLE, multiple sclerosis (MS), rheumatoid arthritis (RA) and primary Sjogren Syndrome (pSS).

However, therapeutic strategies for PBC are very limited, and treatment of PBC patients using anti-CD20 have exhibited limited biochemical and clinical efficacy. Thus, finding an effective therapy for PBC is an important priority. Given the disease and patient heterogeneity as well as the diversity of B cell subsets and their involvement in the disease pathology, preclinical animal models are essential to provide important information on how B cell-targeting treatment affects B cell and non-B cell populations in the circulation and tissue, and eventually disease progression. B cell depletion therapies using anti-CD20 and anti-CD79 tested in two different murine models of PBC had variable results. B cell depletion using anti-CD20 ameliorated autoimmune cholangitis, but exacerbated colitis in TGF-β receptor II dominant negative (dnTGFβRII) mice [37]. While anti-CD20 and anti-CD79 therapy exacerbated autoimmune cholangitis in 2-octynoic acid (2OA) induced PBC mouse model [38]. The exacerbated disease may be due to a depletion of B regulatory cells by anti-CD20 administration, whereas the regulatory B cell function remains intact following anti-BAFF treatment [39]. In the current study, we compare the natural history of PBC by administering anti-BAFF alone, anti-CD20 alone, or anti-BAFF in combination with anti-CD20 in the ARE-Del mice with established disease. Our data demonstrate that what appears to be important is the degree of B cell depletion achieved, the alteration of serological features and clinical efficacy using these two single-agents and the combination agents.

The basic premise of the combination B cell directed therapy with anti-CD20 and followed by inhibition of B cell survival with anti-BAFF is that the production of BAFF induced by anti-CD20 might promote the maturation of autoreactive B cells [40,41]. Previous lupus studies demonstrated that an improvement of the efficacy of B cell depletion therapy was achieved using a combination of anti-CD20 and BAFF blockade with B lymphocyte stimulator receptor 3 fusion protein (R-3-Fc) in murine models of spontaneous or accelerated lupus [42]. In agreement with these studies, anti-BAFF in combination with anti-CD20 led to sustained B-cell depletion and improved immunological and clinical features of autoimmune cholangitis in the ARE-Del model presented herein.

It is a general feature that B cells in secondary lymphoid organs and target tissues are resistance to anti-CD20 mediated B cell depletion [26, 43–45]. For example, in patients with rheumatoid arthritis, rituximab is effective in depleting circulatory B cells but not B cells in synovial tissues [46–48]. The loss of CD20 results in a transient B cell activation inducing a B cell-to-plasma cell differentiation [49]. Anti-CD20 decreased the expression of IgM at the surface of B cells [50], but had no effect on the basal IgG levels in a mouse model of Graves' hyperthyroidism [45]. In agreement with these observations, anti-CD20 single agent treatment led to an approximal 60% reduction of B lineage cells in circulation, and 50% reduction of surface IgM expression on B cells. However, the frequency of splenic B cells did not differ from the control group. This partly explains why the AMA and total serum IgM and IgG levels in anti-CD20 treated mice were not significantly different than PBS controls. In contrast to anti-CD20, anti-BAFF treatment reduced autoantibody AMA production. The reduction of serum IgM levels and surface expression of IgM on peripheral B cells were detected throughout the entire study.

Anti-BAFF treatment in mice resulted in no change in plasma cell numbers in the bone marrow but a substantial reduction in the numbers of mature and memory B cells [51]. Similar results have been reported in anti-BAFF treatment study with patients with SLE that resulted in no reduction of the plasma cells and switched memory B cells during the treatment [52]. However, findings from 2 phase III trials, the Study of Belimumab in patients with SLE demonstrated significant sustained reductions in IgG, autoantibodies and decreased plasma cells [53]. In our study, the serum IgG levels were reduced but did not significantly differ from PBS control by the end of the treatment period implicating that class-switched and antibody-producing B cells were not efficiently depleted by anti-BAFF treatment.

FCRL5 known as CD307 and FCRH5, is a novel IgG binding protein expressed on plasma cells and mature B cells [54,55]. FCRL5 expression is induced by sustained BCR stimulation and upregulated in patients with SLE, RA and common variable immunodeficiency. FCRL5 was implicated as a key molecule for the generation of atypical memory B cells and driving naïve B cell proliferation [56]. A study reported that FCRL5 is expressed on the majority of antigen-specific germinal-center-derived memory B cells. The expression of FCRL5 on IgM^+ B cells marked long-lived, antigen-specific memory B cells [35]. Synergizing IFN- γ with TLR7/8 and IL-21 signals drive atypical memory B cells into plasma cells with upregulated activation markers and FCRL5 following infection [57]. ARE-Del mice utilized in this study have posttranscriptional dysregulation of IFN-γ through deletion of the IFN-γ 3′ untranslated region (3' UTR) AU-rich element (ARE-Del). ARE-Del mice exhibit prolonged and chronic over-expression of IFN-γ and more importantly, develop a female predominant autoimmune cholangitis, indicating that dysregulation of IFN-γ signaling triggers sex biased autoimmunity similar to human PBC [29]. TLR7, an X-chromosome-associated gene is significantly upregulated in female ARE-Del compared with males [29,30]. Therefore, these data imply that upregulated IFN-γ and TLR7 could drive FCRL5 expression for generating dysfunctional antigen-experienced memory B cells in ARE-Del mice. Indeed, the disease progress is associated with a significantly increased IgM^+ $FCRL5^+$ B cells (significantly increased from 13.61 ± 2.45% of total B cell at age of 12 week–16.62 ± 3.54% of total B cells at age of 24 week in periphery of ARE-Del mice treated with PBS (Supplementary Fig. 1)). Upon anti-BAFF alone treatment, the majority IgM^+ $FCRL5^-$ B cells were depleted in circulating and peritoneal cavity, however, the relative abundance of IgM^+ $FCRL5^+$ B cells remained at similar level as noted in the PBS controls. This suggests that IgM^+ $FCRL5^+$ B cells are resistant to anti-BAFF alone, but are relatively non-resistant to a combination of anti-CD20 and anti-BAFF therapy. Given the fact that improved disease manifestations were achieved in combination anti-body treated mice implicating the pathogenic function of IgM^+ $FCRL5^+$ B cells and depletion of this unique population could contribute to the clinical improvement.

In summary, our data revealed dynamic treatment-associated alterations of both known and unforeseen B cell phenotypes and the impact of B-cell directed treatments on the natural history of autoimmune cholangitis. Present data confirm that a combination of anti-BAFF and anti-CD20 is clinically beneficial in the murine model of human PBC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

Abbreviations

PBC	Primary Biliary Cholangitis
AMA	antimitochondrial antibody
UDCA	ursodeoxycholic acid
ELISA	standard enzyme-linked immunosorbent assay
Ig:	*immunoglobulin*
ARE	Del deletion of the IFN-γ 3′ untranslated region (3′ UTR) AU-rich element
PDC-E2	pyruvate dehydrogenase complex-E2 subunit
HRP	horse-radish peroxidase
PFA	paraformaldehyde
H&E	hematoxylin and eosin
MNCs	mononuclear cells
CNSDC	chronic non-suppurative destructive cholangitis
BAFF B	cell activating factor of the TNF-family
3′UTR:	*3′ untranslated region*
SLE	systemic lupus erythematosus
MS	multiple sclerosis
RA	rheumatoid arthritis
pSS	primary Sjogren Syndrome

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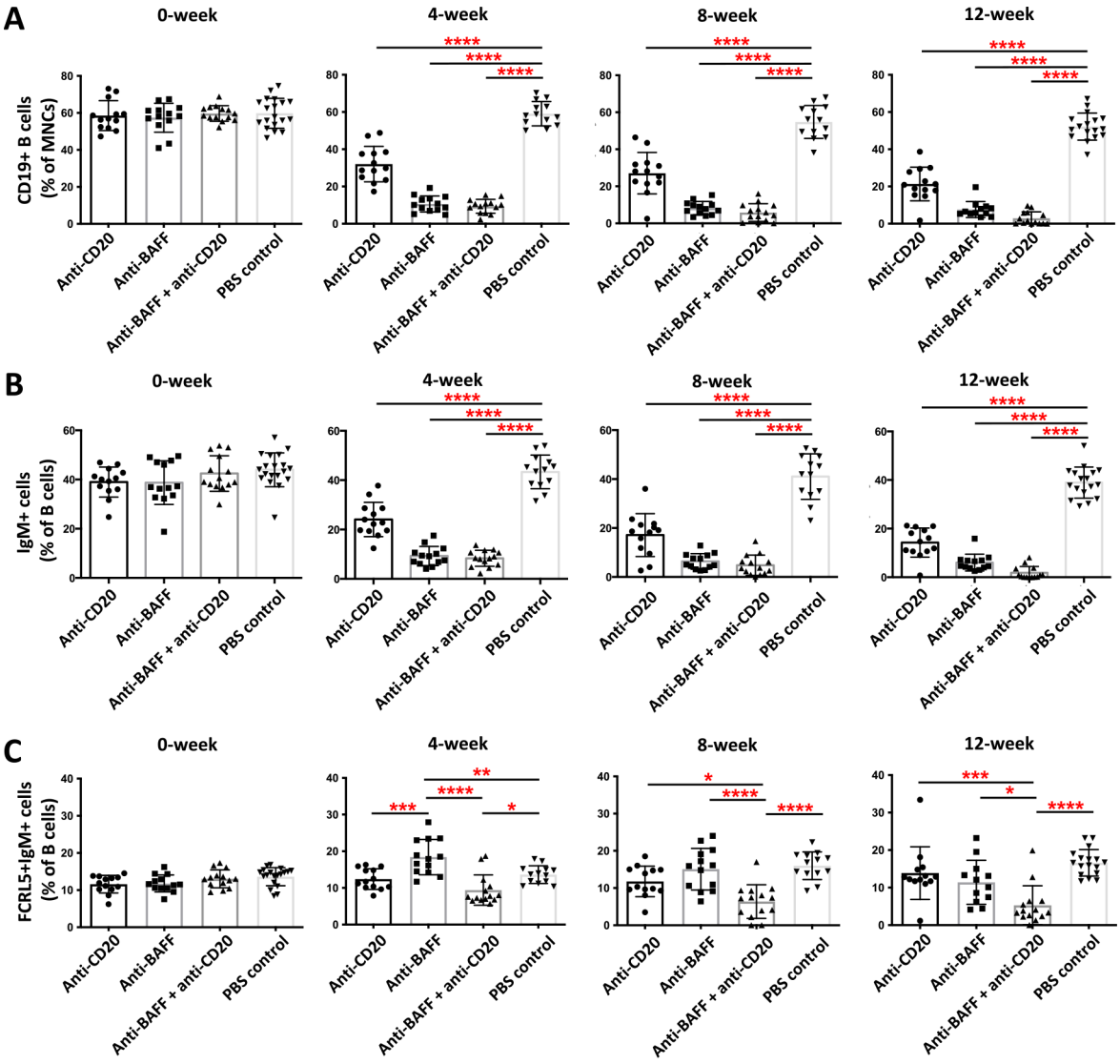

Fig. 1.

Kinetics of B cell depletion in periphery upon B-cell targeting treatments. B cell subsets in peripheral blood were analyzed using flow cytometry before and after treatment at 4-week intervals. A. The frequencies of $CD19^+$ B cells; B. IgM^+ B cells; and C. IgM^+ $FCRL5^+$ in peripheral blood.

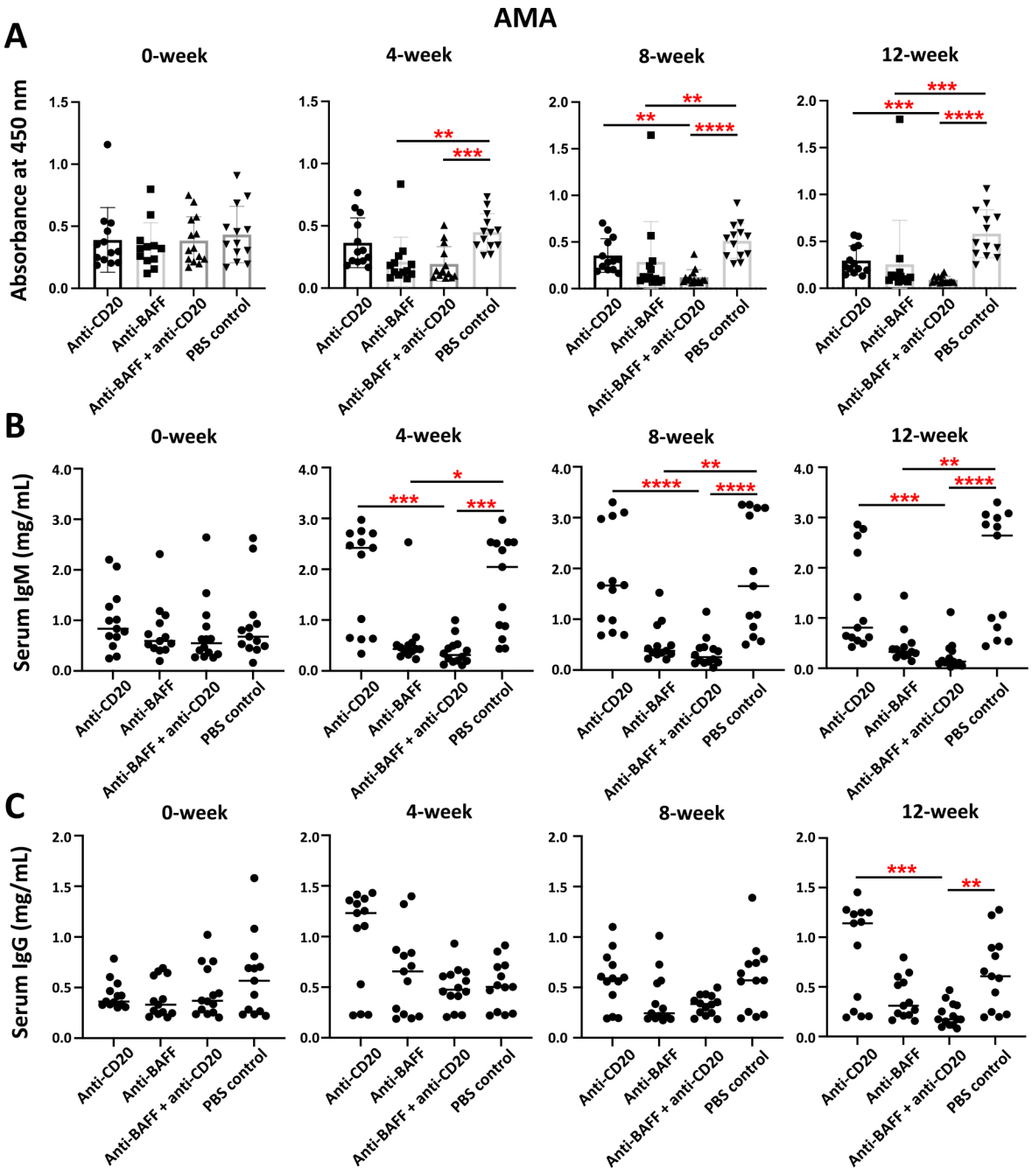

Fig. 2.

Alteration of serologic features upon B-cell targeting treatment. A. serum AMA (anti-PDC-E2 antibody) levels; B. total IgM; and C. total IgG levels.

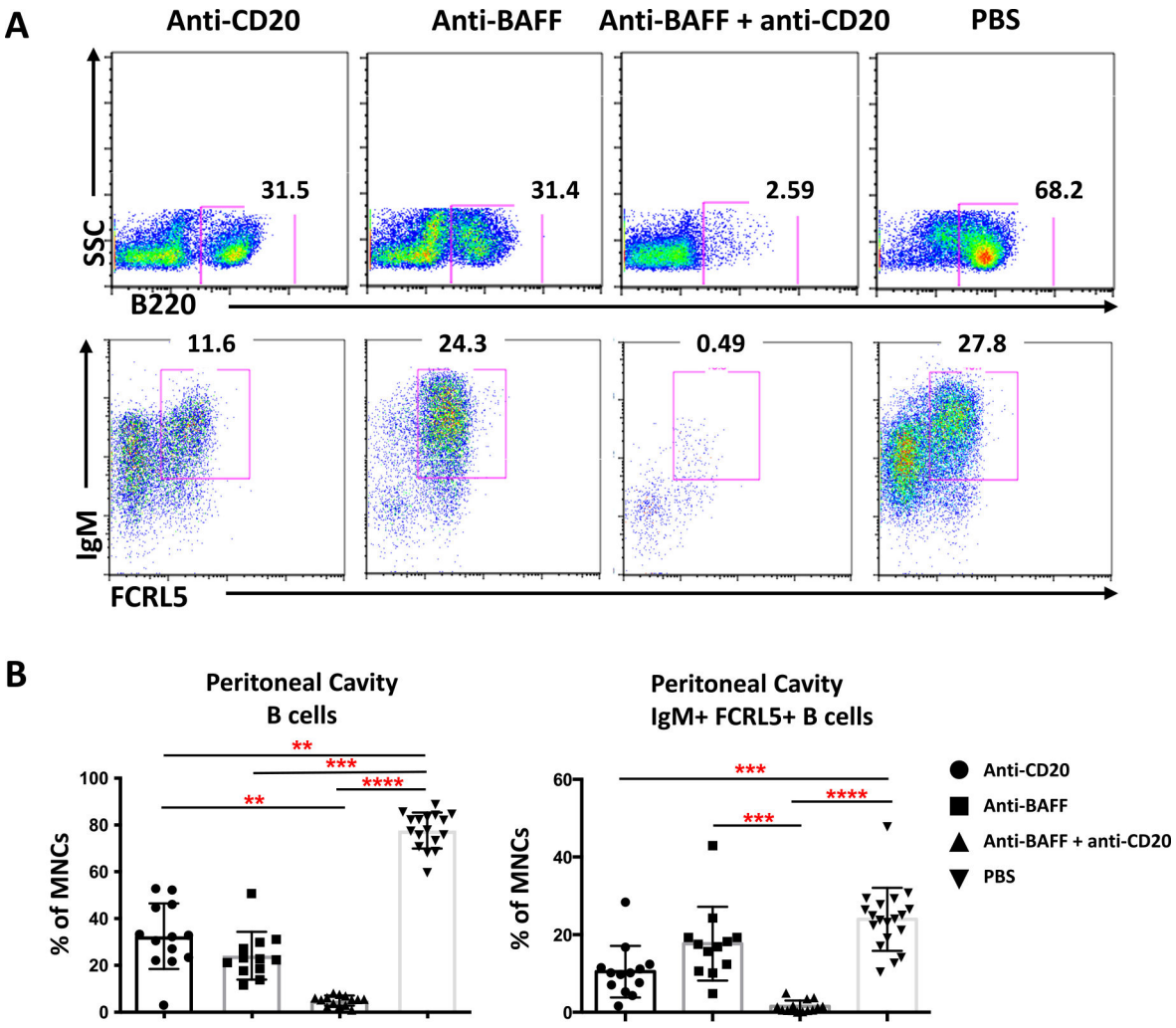

Fig. 3.

The degree of B cell depletion in peritoneal cavity from treated ARE-Del mice.A. The representative dot plot images showing the gating strategies on $B220^+$ B cells and IgM^+ $FCRL5^+$ B cells. The values shown in the dot plots were percentage of MNCs. B. Comparison of $B220^+$ B cells and IgM^+ $FCRL5^+$ B cells in peritoneal cavity among three treatment and control groups after 12 weeks of treatment.

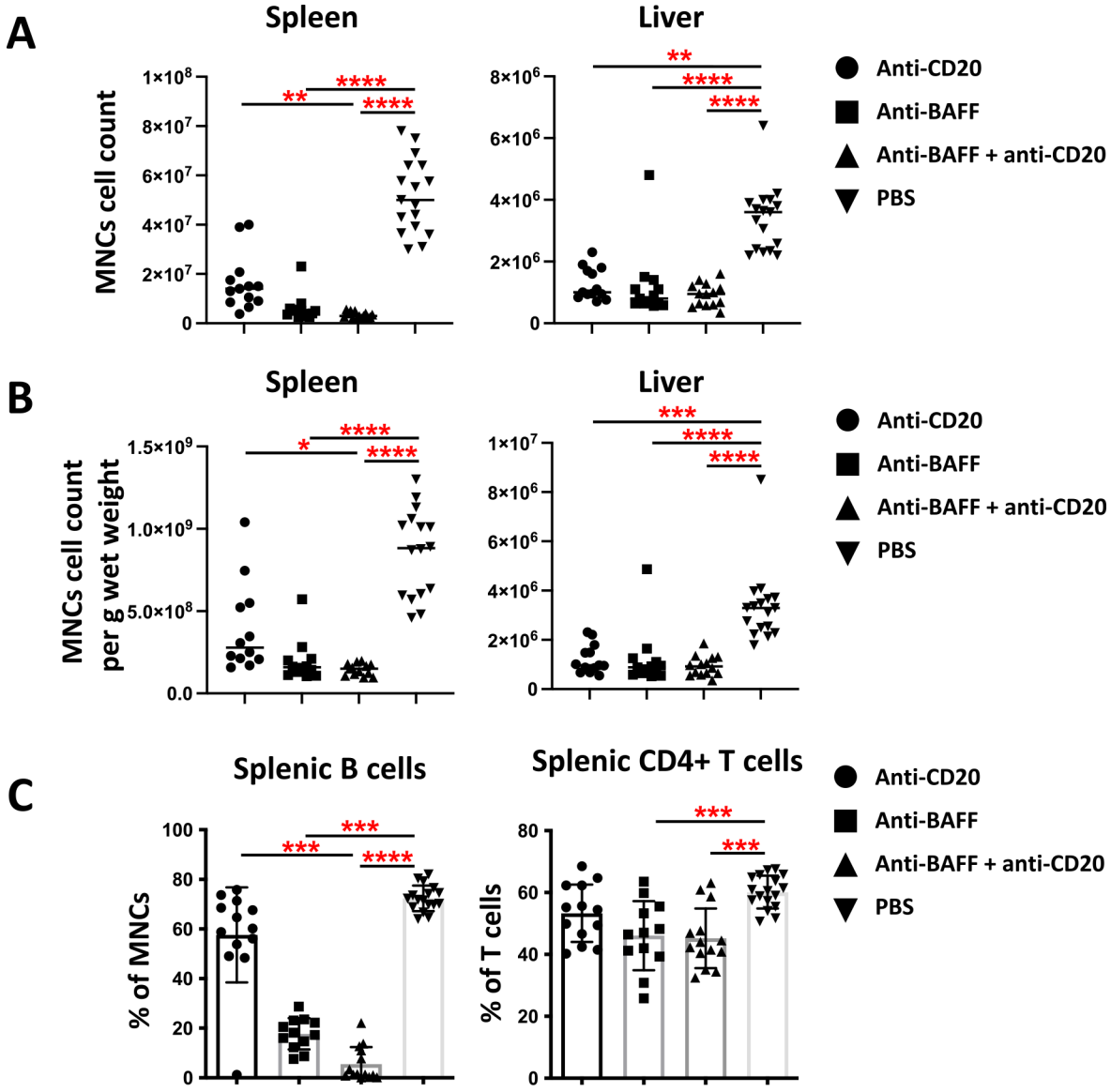

Fig. 4.

The impact of B-cell targeting treatment on the resident MNCs in spleen and liver from ARE-Del mice. A. The MNCs cells from spleen and liver tissues were isolated and quantified at 12 weeks post treatment. B. The MNCs count was expressed as per wet tissue weight. C. B cell and CD4 T cells were analyzed by flow cytometry and expressed as percentage of total MNCs and $NK1.1^{-}TCR\beta^{+}$ T cells, respectively.

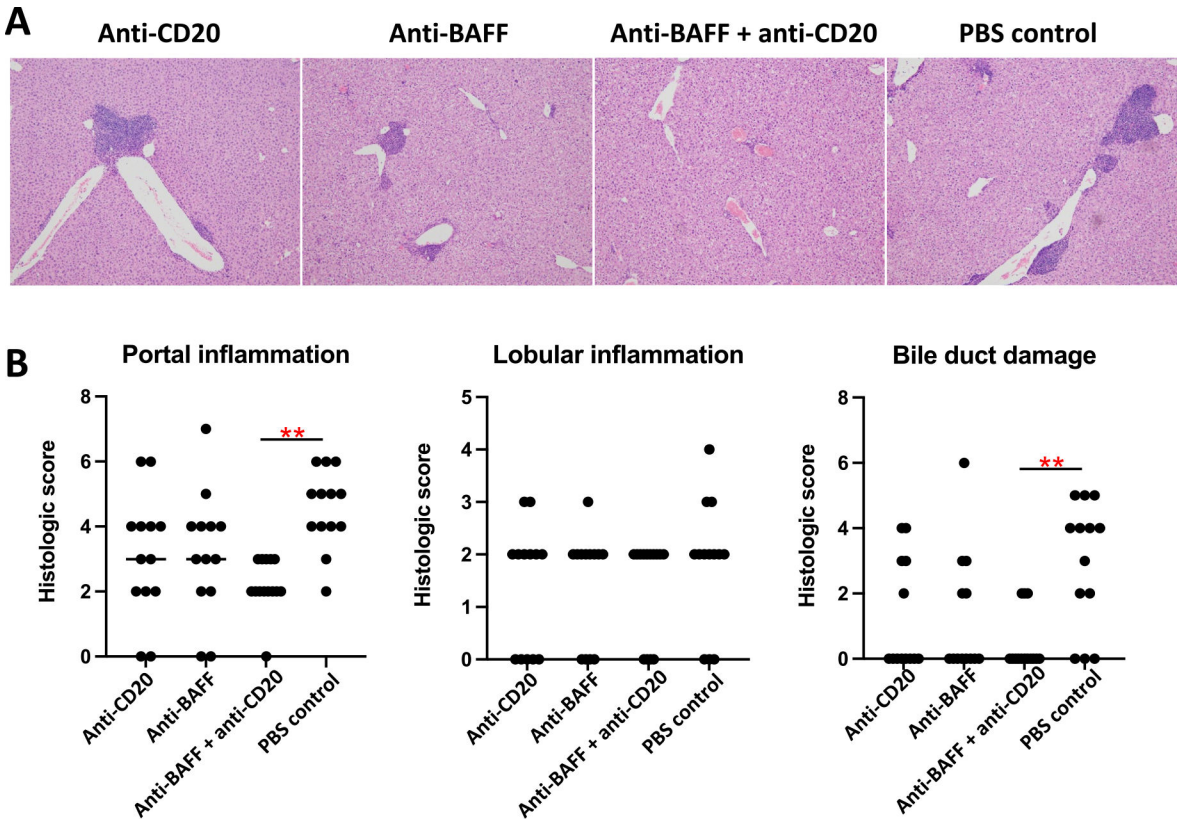

Fig. 5.

Hepatic histological features in ARE-Del mice upon three B-cell targeting treatments. A. Representative H&E staining images of liver sections from three treatment groups and PBS control. B. Histological scores of portal inflammation, lobular inflammation and bile duct damage.