Protective and Pathogenic Roles for B Cells during Systemic Autoimmunity in NZB/W F1 Mice

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Delineating the relative contributions of B lymphocytes during the course of autoimmune disease has been difficult. Therefore, the effects of depleting all mature B cells using a potent CD20 mAb, or of depleting circulating and marginal zone B cells using a ligand-blocking CD22 mAb, were compared in NZB/W F1 mice, a model for human systemic lupus erythematosus. Single low-dose mAb treatments depleted B cells efficiently in both NZB/W F1 and C57BL/6 mice. Prophylactic B cell depletion by repeated CD20 mAb treatments prolonged survival during pristane-accelerated lupus in NZB/W F1 mice, whereas CD22 mAb had little effect. Despite effective B cell depletion, neither mAb treatment prevented autoantibody generation. In addition, CD20, CD22, and control mAb-treated NZB/W F1 mice developed anti-mouse IgG autoantibodies in contrast to parental NZB and NZW strains, which may have reduced the effectiveness of B cell depletion. Despite this, low-dose CD20 mAb treatment initiated in 12–28-wk-old mice, and administered every 4 wk thereafter, significantly delayed spontaneous disease in NZB/W F1 mice. By contrast, B cell depletion initiated in 4-wk-old mice hastened disease onset, which paralleled depletion of the IL-10–producing regulatory B cell subset in NZB/W F1 mice. Thus, B cell depletion had significant effects on NZB/W F1 mouse survival that were dependent on the timing of treatment initiation. Therefore, distinct B cell populations can have opposing protective and pathogenic roles during lupus progression.


Although B cells are generally thought to promote lupus and other autoimmune conditions by producing autoantibodies, B cells also have critical functions in regulating autoimmune disease pathogenesis that extend beyond Ab production (13). For example, B cells regulate disease development and pathogenesis by promoting pathogenic CD4+ T cell activation through their APC function and cytokine production (14–17). In support of this, mIgM.MRL/lpr mice that express transgenic membrane IgM, but do not secrete appreciable serum Ig, still develop nephritis and vasculitis in contrast to B cell-deficient MRL/lpr mice that exhibit comparatively less pronounced disease (18, 19). While informative, the use of genetically B cell-deficient mice to study autoimmunity is complicated by their multiple pre-existing immune system alterations (20–25). Furthermore, it is not possible to examine the role of B cells at different time points during disease in congenitally B cell-deficient mice. This is important to assess because B cells have opposing roles during the initiation and progressive stages of inflammation, experimental autoimmune encephalomyelitis (EAE), and potentially other autoimmune diseases (26–28).

B cell depletion using a therapeutic CD20 mAb has shown clinical efficacy in some patients with SLE, although the rituximab anti-human CD20 mAb has not had a significant therapeutic benefit in larger phase II/III and phase III randomized, placebo-controlled clinical trials for lupus nephritis and moderate to severe SLE without active nephritis (29–33). In part, this may be explained by the recent report that autoimmune B cells are inherently resistant to B cell depletion in MRL/lpr mice expressing a human CD20 transgene (34). Despite resistance to therapy, high-dose mouse anti-human CD20 mAb treatments reduced clinical disease and anti-nuclear Ab (ANA) levels, although it is unknown whether B cell depletion or high doses of exogenous IgGameliorated disease symptoms, because disease was not assessed in control IgG-treated mice. In contrast, CD22 represents another B cell-specific target for human and mouse B cell depletion (35, 36). A humanized CD22 mAb,
C57BL/6 and female NZB/W F1 mice for pristane-accelerated lupus and the analysis of short-term B cell depletion were from The Jackson Laboratory (Bar Harbor, ME). For accelerated lupus, 8-wk-old NZB/W F1 mice were injected i.p. with 200 μl pristane (Sigma-Aldrich, St. Louis, MO). NZB and NZW mice were purchased from Japan SLC (Shizuoka, Japan) for conventional lupus experiments, with female NZB/W F1 mice generated by mating female NZB and male NZW mice. All mice were housed under specific pathogen-free conditions. All procedures were approved by Duke University’s Animal Care and Use Committee and the Animal Committee of The International Medical Center of Japan.

**Abs**

Mouse anti-mouse CD20 mAb, MB20-11, and mouse anti-mouse CD22 mAb, MB22-11, were as described (36–38). MB20-11 and MB22-11 mAbs were purified using protein A Hi-Trap columns according to the manufacturer’s instructions (Amersham Pharma, Piscataway, NJ). MB20-11 and MB22-11 are IgG2a mAbs because of their C57BL/6 origins (49, 50). Nonetheless, IgG2a-specific Ab reagents (Southern Biotechnology Associates, Birmingham, AL) reacted with IgG2a Abs. Other reagents included: FITC-, PE-, and PE-cy5–conjugated anti-mouse B220 (CD45R, RA3-6B2), CD5 (53-7.3), CD19 (1D3), CD21 (B38), CD24 (M1/69), CD43 (ST), and CD93 (AA4.1) mAbs (BD Biosciences, San Jose, CA); CD11b (M170) and CD1d (1B1; eBioscience, San Diego, CA) mAbs; and polyclonal anti-IgM and -IgD Abs (Southern Biotechnology Associates). IgG1, IgG2a, and IgG2b mAb isotype standards were either generated in-house or purchased from Southern Biotechnology Associates.

**B cell depletion and phenotype analysis**

Sterile MB20-11, MB22-11, or IgG2a isotype control (generated in house) mAbs (10–100 μg in 100–200 μl PBS) were given to NZB/W F1 or C57BL/6 mice. For pristane-accelerated lupus, each mAb (100 μg) was given i.p. 2 wk after pristane injection, with repeated mAb treatment every 10 d for a total of 11 doses. For the conventional lupus model, mAb (10 μg i.v.) was given between 4 and 32 wk old, with treatment repeated every subsequent 4 wks. After mAb treatment, blood leukocyte numbers were quantified by hemocytometer following red cell lysis, with cell frequencies determined by immunofluorescence staining with flow cytometry analysis. Single-cell leukocyte suspensions from spleen, bone marrow (bilateral femur), peritoneal lavage, and peripheral lymph nodes (bilateral inguinal) were isolated, with erythrocytes lysed in Tris-buffered 100 mM ammonium chloride solution. Leukocytes were then stained at 4°C using predetermined optimal concentrations of each Ab for 30 min. For whole blood, erythrocytes were lysed after staining using FACS Lysing Solution (BD Biosciences, San Jose, CA). Ab binding was analyzed on a FACSscan flow cytometer (BD Biosciences) by gating on cells with the forward and side light scatter properties of lymphocytes. Nonreactive, isotype-matched Abs (Caltag Laboratories [Burlingame, CA], eBioscience, and BD Biosciences) were used as controls for background staining.

**ANA and proteinuria assays**

Serum ANA reactivity with human HEP-2 cells (HEP-2 Cell Substrate Slide; MBL, Japan) was examined by incubating sera (diluted 1:100 in PBS) with fixed HEP-2–cell–coated slides for 30 min at room temperature. The slides were then washed three times with PBS, with ANA detected using FITC-conjugated goat anti-mouse IgG (H+L) Ab at optimal concentrations for 30 min at room temperature. The slides were evaluated by fluorescence microscopy at ×40 magnification. ANA staining exhibiting homogeneous, speckled, or nucleolar patterns was considered positive. Proteinuria was assessed using Nephrosticks L (Bayer Medical, Tokyo, Japan). NZB/W F1 mice demonstrated glomerulonephritis and immune complex deposition on glomerular basement membranes when their proteinuria values exceeded 3 mg/ml (data not shown). Therefore, mice were considered positive for proteinuria when readings were >3 mg/ml.

**B10 cell enumeration and analysis**

Intracutaneous IL-10 production by B cells was examined by flow cytometry as described (26). Briefly, isolated leukocytes or purified cells were re-suspended (2 × 10⁶ cells/ml) in complete medium (RPMI 1640 media containing 10% FCS, 200 μg/ml penicillin, 200 U/ml streptomycin, 4 mM L-glutamine, and 5 × 10⁻⁵ M 2-ME [Life Technologies, Carlsbad, CA])
with LPS (10 μg/mL, Escherichia coli serotype O111: B4; Sigma-Aldrich), PMA (50 ng/mL, Sigma-Aldrich), ionomycin (500 ng/mL, Sigma-Aldrich), and monensin (2 μM; eBioscience) for 5 h at 37 °C. After surface-staining, the cells were washed, fixed, and permeabilized using a Cytofix/Cytoperm Kit (BD Pharmingen, San Diego, CA) according to the manufacturer’s instructions. Cells were then stained with PE-conjugated mouse anti-IL-10 mAb (JES5-16E3; eBioscience). A PE-conjugated rat IgG2b isotype control mAb was used as a negative control to establish background staining levels. Dead cells were cultured for 48 h with agonistic CD40 mAb (1 μg/mL, clone HM40-3; BD Biosciences), with LPS, PMA, ionomycin, and monensin added during the final 5 h of culture. Leukocytes from IL-10−/− mice served as negative controls to verify reagent specificity and to establish background IL-10-staining levels.

Statistical analysis

ANA, proteinurea, and survival data were analyzed using Kaplan-Meier curves and the Log rank test. Unless indicated otherwise, data are shown as mean values ± SEM. Comparisons between groups were made using Steel-Dwass analysis or Student t test; p < 0.05 was considered statistically significant.

Results

CD20 and CD22 mAb-induced B cell depletion in C57BL/6 and NZBW F1 mice

The efficacy of CD20 and CD22 mAb-induced B cell depletion was first assessed in 8- to 10 wk-old female NZB/W F1 mice. CD20 mAb (100 μg) depleted comparable numbers of mature B cells in NZB/W F1 and C57BL/6 mice 7 d after treatment (Fig. 1A). In NZB/W F1 and C57BL/6 mice, CD20 mAbs depleted mature B cells from the bone marrow (96 versus 94%), blood (97 versus 99%), spleen (90 versus 84%), spleen marginal zone (99 versus 83%), and lymph nodes (86 versus 84%). Thus, significant intrinsic differences in CD20 mAb-induced B cell depletion were not found between NZB/W F1 and C57BL/6 mice.

Mouse anti-mouse CD22 mAb rapidly depletes mature recirculating bone marrow, blood, and marginal zone B cells through ADCC-independent mechanisms, but only ~20% of mature CD22+ follicular B cells in C57BL/6 mice (36). The MB22-11 mouse anti-mouse CD22 mAb (100 μg) depleted mature recirculating bone marrow (49 versus 58%), blood (75 versus 84%), and marginal zone (77 versus 82%) B cells to the same extent in NZB/W F1 and C57BL/6 mice (Fig. 1B). Although the CD20 and CD22 mAbs deplete different mature B cell subsets through different mechanisms, there do not appear to be intrinsic differences in CD20 or CD22 mAb-induced B cell depletion between NZB/W F1 and C57BL/6 mice.

B cell depletion improves survival during accelerated lupus with minimal effects on Ig and autoantibody levels

NZB/W F1 mice normally develop ANA by 16–24 wk old, nephritis from 28–36 wk, and die at ~36 wk. However, pristane treatment of NZB/W F1 mice accelerates the course of lupus-like disease (52, 53). Therefore, the role of B cells in pristane-accelerated mortality in 8-wk-old female NZB/W F1 mice was evaluated following low-dose pristane administration with subsequent CD20, CD22, or control mAb (100 μg) treatments every 10 d for 16 wk. From the initiation of mAb treatment up until 34 wk old, survival was improved in CD20 mAb-treated mice compared with control mAb-treated mice (29–34 wk; p < 0.05, Log rank test; Fig. 2A). Median (50%) survival was also increased 23%, from 26 wk in control mAb-treated mice to 32 wk in CD20 mAb-treated mice. However, CD20 mAb-treated mice began to succumb to disease more rapidly after this point such that differences in overall survival curve comparisons at 52 wk were not found to be significant (p = 0.09; Fig. 2A). In contrast to the improved survival during early disease with total mature B cell depletion, survival following CD22 mAb treatment was more comparable to IgG2a control throughout the course of disease (Fig. 2B). Thus, the depletion of most mature B cells improved survival in pristane-accelerated lupus.

Prophylactic B cell depletion using CD20 mAb significantly reduces isotype switching and IgG immune responses after Ag challenge, and autoantibody generation in autoimmune prone mice, whereas therapeutic B cell depletion after autoimmune disease initiation has no effect (39, 40, 54). Similarly, total serum IgM was not dramatically altered by CD20 or CD22 mAb-induced B cell depletion from low-dose pristane-treated NZB/W F1 mice between 8 and 25 wk old (Fig. 2C). Unexpectedly, total serum IgG levels increased significantly with age (8 versus 16 wk; p < 0.05) despite CD20 or CD22 mAb treatment when compared with control mAb-treated NZB/W F1 littermates. Anti-dsDNA and anti-histone IgM and IgG autoantibodies also increased significantly between 8 and 16 wk old in all treatment groups (p < 0.05; Fig. 2D, 2E). Thus, mature B cell and B cell subset depletion did not dramatically affect serum Ig levels or autoantibody production in low-dose pristane-treated NZB/W F1 mice.

Because B cell depletion delayed mortality, but did not cure disease, in pristane-accelerated lupus (Fig. 2), the efficiency of B cell depletion during disease was assessed. First, the ability of CD20 and CD22 mAbs to deplete blood B cells was examined in pristane-treated NZB/W F1 mice given CD20, CD22, or control mAbs during 16 wk of treatment. The level of B cell depletion observed at 16 wk for both CD20 and CD22 mAb-treated mice was decreased compared with depletion in 8-wk-old mice that did not receive pristane (Figs. 1, 2F). Whereas CD20 and CD22 mAbs reduced blood B cell numbers by 75–97% in 8-wk-old mice (Fig. 1), blood B cell numbers were reduced only by 28–43% in 16-wk-old NZB/W F1 mice (Fig. 2F). NZB/W F1 mice, like other lupus-prone mice, can generate serum IgM and IgG rheumatoid factors as disease progresses (55, 56). Therefore, circulating anti-IgG Ab levels were quantified before and during mAb treatment. All NZB/W F1 mice had significantly higher levels of IgG Abs that were reactive with IgG2c, IgG2a, and IgG1 Abs at 16 wk old, compared with levels at 8 wk old (Fig. 2G). Sixteen-week-old mice given control, CD20, or CD22 mAb four times had ≥2.5-fold higher levels of serum IgG (IgG2a Abs not measured in the assay) reactive with the mAb that they were given when compared with 8-wk-old mice. Sixteen-week-old mice given CD20 or CD22 mAbs had even higher levels of serum IgG (IgG2a Abs not measured in the assay) reactive with IgG2c (MB20-11 and MB22-11) and IgG2a (control and unrelated mAb) compared with 16-wk-old mice that received control mAb. There were no significant differences among age-matched treatment groups in IgG1-reactive serum IgG levels (IgG1 Abs not measured in the assay). Thus, B cell depletion by CD20 or CD22 mAb treatment did not abrogate the generation of anti-IgG levels in NZB/W F1 mice, which may have contributed to the diminished effectiveness of B cell depletion over time. Moreover, NZB/W F1 mice were unique in that CD20 mAb treatment did not prevent the development of isotype-switched autoantibodies (i.e., anti-DNA and anti-histone IgG), whereas IgG autoantibodies do not increase in other strains of autoimmune mice after CD20 mAb treatment (39, 40, 42, 54).

Development of a therapy model for B cell depletion in NZB/W F1 mice

The effects of B cell depletion using lower and less frequent mAb doses in NZB/W F1 mice without pristane pretreatment was next assessed. Mice were given a 10-fold lower mAb treatment dose at 4-wk intervals. One 10-μg dose of CD20 mAb depleted >95% of
blood B cells for ∼4 wk in 8-wk-old NZB/W F1 mice (Fig. 3A) as published for C57BL/6 mice (37, 45). However, when 8-wk-old NZB/W F1 mice were given CD20 mAb every 4 wk, five times, circulating B cells were depleted for <1 wk after the last treatment in these 28-wk-old mice. When mice were repetitively given a higher dosing regimen of CD20 mAb (100 μg), the period of B cell depletion following the fifth mAb treatment was equivalent to that observed in mice given 10 μg of mAb repetitively (Fig. 3B). Circulating B cells from depletion-resistant NZB/W F1 mice given CD20 mAb five times every 4 wk expressed normal levels of CD20 as assessed by CD20 mAb staining in vitro (Fig. 3C). Rather, CD20 mAb was cleared rapidly in vivo because circulating B cells from depletion-resistant NZB/W F1 mice did not have residual CD20 mAb bound to their cell surface, as detectable by anti-mouse IgG2a mAb staining (Fig. 3C). Furthermore, serum from depletion-resistant mice contained significantly less circulating CD20 mAb 7 d after treatment than from mice that had been treated with only CD20 mAb once, as determined using serum from mAb-treated mice to stain normal B cells (data not shown). In contrast to NZB/W F1 mice, no difference was observed in B cell clearance in parental NZB, parental NZW, or C57BL/6 mice given CD20 mAb repeatedly in comparison with mice treated once with mAb (Fig. 3D). Therefore, resistance to mAb-induced B cell depletion was unique to NZB/W F1 mice, developed independent of pristane treatment, and was not mAb dose dependent or due to reduced CD20 expression.
To determine whether resistance to mAb-induced B cell depletion was associated with the production of anti-IgG Abs, NZB/W F₁ mice were given 200 μl pristane i.p. Two weeks later, mice were given either CD20 (A), CD22 (B), or control mAb (100 μg) i.p., with the same mAb dose administered every 10 d over a 16-wk period (11 doses total; arrowheads). Kaplan-Meier survival curves are shown with statistical differences assessed at 52 wk old using the Log-rank test. C–G. Effects of B cell depletion on serum Ig, autoantibody, and anti-treatment Ab levels in pristane-accelerated lupus. Total serum (C), anti-dsDNA (D), and anti-histone (E) Ab levels in 8–25-wk-old mice treated with CD20 (n = 10), CD22 (n = 18), or control (n = 18) mAbs. F–G, Resistance to B cell depletion in pristane-accelerated lupus in female NZB/W F₁, F. Representative plots show blood B220⁺ B cell depletion 7 d following the fifth dose of CD20, CD22, or control mAb. Bar graphs show mean (± SEM) blood B cell depletion in each mAb treatment group (n = 10). G. Reactivity of serum IgG obtained from mice treated with MB20-11 (CD20 mAb), MB22-11 (CD22 mAb), treatment control mAb, or irrelevant IgG2a and IgG1 mAbs as determined by ELISA. C–G. Significant differences between mean (± SEM) OD values for control mAb and CD20 or CD22 mAb-treated mice are indicated; °p < 0.05. Significant differences between mean (± SEM) OD values for 8- and 16-wk-old mice are indicated; †p < 0.05, (n ≥ 10 mice per group).
both control and CD20 mAb-treated NZB/W F1 mice, but not NZB, NZW, or C57BL/6 mice, with higher levels observed in depletion-resistant NZB/W F1 mice that had received CD20 mAb.

**B cell depletion accelerates or delays disease depending on the timing of depletion**

Because total mature B cell depletion improved survival in pristane-accelerated lupus, the efficacy of B cell depletion during conventional (nonaccelerated) lupus development was assessed. NZB/W F1 mice were given CD20 mAb (10 μg) starting at different time points, with repeated treatment every subsequent 4 wk. Unexpectedly, survival was significantly decreased when B cells were depleted beginning at 4 wk old, in comparison with control mAb treated littermates (Fig. 4A). By contrast, survival was significantly enhanced when B cells were first depleted between 12 and 28 wk old. CD20 mAb treatment initiated at 32 wk had no effect on survival. The development of proteinuria was also accelerated when treatment was initiated at 4 wk old, whereas proteinuria was significantly delayed when CD20 mAb was first given at 16 wk old (Fig. 4B). CD20 mAb treatment did not significantly alter the frequency of ANA development when compared with control mAb-treated littermates (Fig. 4C). Differences

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**FIGURE 3.** NZB/W F1 mice generate anti-IgG Abs and exhibit reduced clearance after multiple treatments with B cell-depleting mAbs in conventional lupus. **A–C**, Generation of anti-IgG Abs in conventional NZB/W F1 lupus. **A**, Blood B cell depletion in 8-wk-old female NZB/W F1 mice that were given one 10-μg dose of CD20 or control mAb i.p., or five mAb treatments at 4-wk intervals. B220+ blood B cell numbers were analyzed immediately before and after the final CD20 mAb dose at days 1 and 3 and then weekly thereafter. Significant differences in B cell clearance between mice receiving one and five doses of CD20 mAb are indicated. *p < 0.05; **p < 0.01; n = 25 mice per group. **B**, Blood B cell depletion in NZB/W F1 mice given CD20 mAb (either 10 or 100 μg) every 4 wk, five times (n = 5 mice per group). Blood B cell numbers were determined after the last dose of CD20 mAb as in **A. C**, CD20 expression on B cells from NZB/W F1 mice after five doses of CD20 mAb. Blood was harvested from CD20 or control mAb-treated mice 7 d after the fifth mAb treatment and stained with PE-conjugated B220 mAb and CD20 mAb, followed by FITC-conjugated anti-mouse IgG2a Ab. Blood samples were also stained separately with FITC-conjugated anti-mouse IgG2a Ab alone to detect residual surface CD20 mAb that had been bound in vivo. **D**, Blood B cell depletion in C57BL/6, NZB, and NZW mice given one or five doses of CD20 mAb. Eight-week-old mice were given 10 μg CD20 mAb either one time or five times at 4-wk intervals, with depletion assessed as in **A. E**, NZB/W F1 mice given CD20 or control Ab five times produce IgG2a mAb reactive with both IgG2a and IgG2c Abs. NZB/W F1 mice were given CD20 or control mAb (10 μg) five times as in **A**, with serum IgG reactivity with MB20-11 mAb (IgG2c), control treatment mAb (IgG2a), or an irrelevant IgG2c mAb (clone 6.3) measured by ELISA. Mean results (horizontal lines) are significantly different between groups; *p < 0.0001. **F**, Only NZB/W F1 mice produce IgG Abs following CD20 mAb (10 μg) treatment five times as in **A. Reactivity of C57BL/6, NZB, NZW, and NZB/W F1 mouse serum with MB20-11 mAb was measured by ELISA. Mean results (horizontal lines) are significantly different between groups as indicated; **p < 0.0001.
Kaplan-Meier survival curves are shown with statistical differences assessed using the Log-rank test. 

In disease parameters among untreated NZB/W F1 mice in comparison with littersmates that had received control IgG2a mAb were not observed (data not shown). Furthermore, differences in disease parameters among NZB/W F1 mice that had received control IgG2a mAb beginning at 4 versus 32 wk old were not observed (Fig. 4). Therefore, B cell depletion had remarkable yet opposing effects on disease course in NZB/W F1 mice, with early treatment (begun at 4 wk) exacerbating disease and later treatment (begun at 12–28 wk) improving disease.

**Regulatory B10 cells expand in young NZB/W F1 mice and are depleted by CD20 mAb**

As in NZB/W F1 mice, early B cell depletion by CD20 mAb promotes inflammation (26) and exacerbates EAE (27). This condition results from the depletion of B10 cells, a unique IL-10–producing regulatory B cell subset found within the spleen CD5<sup>+</sup>CD1<sup>d</sup><sup>hi</sup> subpopulation (26). Sixteen-week-old NZB/W F1 mice have significantly increased B10 cell numbers in comparison with C57BL/6 mice (48). IL-10–producing cell frequencies and numbers were also increased 4-fold in 10-wk-old NZB/W F1 mice when compared with C57BL/6 mice (Fig. 5A, 5B). IL-10–producing spleen B cells in NZB/W F1 mice expressed CD5 and higher levels of CD19 in comparison with other B cells (Fig. 5C) as reported for B10 cells in C57BL/6 mice (26). However, NZB/W F1 mice exhibited two splenic CD5<sup>+</sup> B cell populations that could be differentiated by their CD1<sup>d</sup> and B220 expression densities (Fig. 5D). The CD1<sup>d</sup><sup>hi</sup>/CD5<sup>-</sup> B cell subset expressed high density B220, comparable to that expressed by CD1<sup>d</sup><sup>hi</sup>/CD5<sup>+</sup> B cells. In contrast, the remaining CD5<sup>-</sup> B cells expressed intermediate CD1<sup>d</sup> levels (CD5<sup>-</sup>/CD1<sup>d</sup><sup>int</sup>) and low-density B220, characteristics of B-1a cells. The frequencies and numbers CD1<sup>d</sup><sup>hi</sup>/CD5<sup>-</sup>B220<sup>-</sup> cell were not expanded in 10-wk-old NZB/W F1 mice relative to C57BL/6 mice (Fig. 5D), although this subset increases significantly by 16 wk old (48). However, marginal zone (CD21<sup>hi</sup>/CD1<sup>d</sup><sup>hi</sup>) B cell and CD5<sup>-</sup> B220<sup>-</sup> B-1a cell frequencies and numbers were increased in 10-wk-old NZB/W F1 mice relative to age-matched C57BL/6 mice (Figs. 1, 5E) (4–9). Thus, in addition to MZ and B-1a B cells, the IL-10 competent B10 cell subset was significantly expanded in NZB/W F1 mice prior to disease onset.

As in C57BL/6 mice, CD20 mAb was able to deplete IL-10–producing B cells in NZB/W F1 mice. Seven days following CD20 or control mAb treatments, mouse splenocytes were cultured with LPS, PMA, ionomycin, and monensin, and assessed for cytoplasmic IL-10 production. Remarkably, CD20 mAb treatment reduced both the frequency and number of IL-10–producing B cells in NZB/W F1 mice by 99% (Fig. 5F). Thus, CD20 mAb treatment efficiently depletes both regulatory B10 cells and mature B cells in NZB/W F1 mice.

**B10 cell phenotype and development in NZB/W F1 mice**

Phenotypically unique spleen B cells that are competent to express cytoplasmic IL-10 following 5 h stimulation with LPS, PMA, and ionomycin are predominantly found within the CD1<sup>d</sup><sup>hi</sup>/CD5<sup>+</sup>/CD19<sup>hi</sup> subset in wild type C57BL/6 mice (26, 27, 48). We have previously shown that 5 h of LPS, PMA, and ionomycin stimulation does not significantly influence the cell surface phenotype of spleen B cells (26). Therefore, the phenotypes of IL-10<sup>+</sup> CD19<sup>+</sup> B10 cells were characterized in 8-wk-old NZB/W F1 mice in comparison with C57BL/6 mice. IL-10<sup>+</sup> B cells were predominantly IgM<sup>hi</sup>, IgD<sup>lo</sup>, CD1<sup>d</sup><sup>hi</sup>, CD5<sup>hi</sup>, CD19<sup>hi</sup>, CD21<sup>int/hi</sup>, CD23<sup>lo</sup>, CD24<sup>hi</sup>, CD24<sup>hi</sup>, CD11b<sup>-</sup>, CD43<sup>-</sup>, and CD93<sup>-</sup> (AA4.1) in both NZB/W F1 and C57BL/6 mice (Fig. 6A). Therefore, spleen B10 cells in NZB/W F1 mice share overlapping phenotypic markers with the B-1a, MZ, and T2-MZ precursor B cell subsets, but are nonetheless phenotypically distinct as observed in C57BL/6 mice (26, 27, 48).

B10 cell development and numbers in 8-wk-old NZB/W F1 and C57BL/6 mice were also compared. B10 cells were identified by their capacity to express cytoplasmic IL-10 after 5 h of LPS, PMA, and ionomycin stimulation (26, 27). Previous studies have also identified B10pro cells that acquire the capacity to express cytoplasmic IL-10 following 48 h of culture with either CD40 mAb or LPS in vitro, with LPS, PMA, and ionomycin stimulation during the final 5 h of culture (48). NZB/W F1 and C57BL/6 mice

**FIGURE 4.** Therapeutic B cell depletion by CD20 mAb either accelerates or improves conventional NZB/W F1 lupus depending on the timing of treatment. CD20 or control mAb (10 µg) were first given i.v. from 4–32 wk old as indicated and repeated every 4 wk with survival (A), proteinuria (B), and (C) ANA production evaluated at least weekly. All data were obtained from 15 mice in each treatment group with significant differences indicated. A, Kaplan-Meier survival curves are shown with statistical differences assessed using the Log-rank test. *p < 0.05; **p < 0.01.
had similar frequencies and numbers of B10 cells within bone marrow and peripheral lymph nodes (Fig. 6B; Table I). In contrast, the B10 cell subset was significantly expanded within the blood, spleen, mesenteric lymph nodes, and peritoneum of NZB/W F1 mice compared with C57BL/6 mice. This finding was also true for B10pro and B10 cells, in which 12–13% of blood and spleen B cells in NZB/W F1 mice had the capacity to mature and express cytoplasmic IL-10 with stimulation. Remarkably, almost half of the peritoneal cavity B cells in NZB/W F1 mice had the capacity to mature and express cytoplasmic IL-10 with stimulation. Thus, the B10pro and B10 subsets are rare in both NZB/W F1 and C57BL/6 mice, but these regulatory cells are already significantly expanded in NZB/W F1 mice at the time that B cell depletion exacerbates lupus development (Fig. 4).

Discussion
This study revealed both protective and pathogenic roles for B cells in murine lupus. Prophylactic depletion of mature B cells in NZB/W F1 mice by CD20 mAb prolonged survival in pristane-accelerated lupus up until ∼32 wk old (Fig. 2). By contrast, CD22 mAb depletion of the mature recirculating bone marrow, blood, and marginal zone B cell subsets resulted in only a slight improvement in survival. Therapeutic low-dose CD20 mAb treatment also successfully prolonged survival and delayed the appearance of proteinuria during spontaneous lupus in NZB/W F1 mice when administered during the onset of disease symptoms between 12 and 20 wk old (Fig. 4). In contrast, CD20 mAb-induced B cell depletion at 4 wk old significantly shortened survival time and accelerated proteinuria development. B cell depletion during late-stage disease did not affect survival or disease symptoms in NZB/W F1 mice. Therefore, mature B cell depletion by CD20 mAb resulted in strikingly different disease outcomes depending on the timing of B cell depletion, with B cell depletion during disease progression preferentially reducing the pathogenic effects of B cells on disease. Mature B cell depletion in young NZB/W F1 mice prior to disease symptoms led to accelerated mortality, nephritis, and ANA production (Fig. 4). Similarly, total B cell depletion during contact hypersensitivity induction or EAE initiation dramatically exacerbates inflammation and disease, whereas B cell depletion during EAE progression significantly inhibits disease manifestations (26, 27). Early B cell depletion exacerbates both hypersensitivity responses and EAE because of the depletion of splenic B10 cells, a recently characterized IL-10–producing regulatory B cell subset (26–28, 57). IL-10–producing B10 cells and potentially...
other regulatory B cell subsets also have critical suppressive roles in arthritis, colitis, and autoimmune diabetes in mice (40, 42, 58–60). B10 cells in NZB/W F1 mice were phenotypically similar, if not identical, to their IL-10 competent B cell counterparts in C57BL/6 mice (Fig. 6). However, B10 and B10pro cell frequencies and numbers were significantly expanded along with the marginal zone and B1a subsets in 8–10-wk-old NZB/W F1 mice (Figs. 1, 5, 6; Table I), a time point prior to disease onset (4–9). Importantly, CD20 mAb treatment depleted 99% of IL-10–producing B cells in both NZB/W F1 and C57BL/6 mice (Fig. 5). IL-10–producing B cell elimination by early CD20 mAb treatment may thereby explain exacerbated lupus-like disease in young NZB/W F1 mice. Proof of this concept awaits the production of CD20-deficient NZB/W F1 mice that will allow the adoptive transfer of IL-10–producing CD1dhiCD5+ B cells from CD20−/− NZB/W F1 mice into CD20 mAb-treated NZB/W F1 mice. The generation of B cell-specific IL-10−/− NZB/W F1 mice will also determine whether exacerbated disease is due exclusively to B cell IL-10 production. Nonetheless, as currently demonstrated in the companion manuscript, the pathologic manifestations of nephritis appear significantly earlier, and survival is significantly reduced in NZB/W F1 mice that lack B10 cells because of constitutive CD19-deficiency (61). Moreover, the transfer of splenic CD1dhiCD5+ B cells from wild type NZB/W F1 mice into CD19−/− NZB/W F1 recipients significantly prolongs their survival. Thus, B10 cells in NZB/W F1 mice appear to be functionally similar to their phenotypically identical counterparts in C57BL/6 mice (Fig. 6), where they are known to regulate acute inflammation. Therefore, both early- and late-stage lupus represent a balance between regulatory B10 cells and pathogenic B cell functions in combination with regulatory and pathogenic T cells.

B cell depletion during autoimmunity in NZB/W F1 mice is likely to have beneficial effects beyond reducing autoantibody production (13). Specifically, B cell depletion had protective effects in NZB/W F1 mice, but did not substantially reduce anti-histone or dsDNA autoantibody production, isotype switching to pathogenic IgG subclasses (Fig. 2, data not shown), or ANA generation (Fig. 4C). B cell depletion using the MB20-11 CD20 mAb normally blocks humoral immune responses and the serum Ig and autoantibody increases typically found in aging autoimmune mice (39, 40, 54). However, CD20 mAb-induced B cell depletion does not have significant effects on pre-existing (CD20−negative) long-lived plasma cells and produces only small reductions in serum Ig or autoantibody levels in adult mice (54).

**FIGURE 6.** B10 cell phenotype, development, and distribution in 8-wk-old NZB/W F1 and C57BL/6 mice. A, IL-10–producing B cells were predominantly found within the CD1dhiCD5+ B cell subset in NZB/W F1 and C57BL/6 mice. Splenocytes were cultured with LPS, PMA, ionomycin, and monensin for 5 h, and then stained for cell surface molecules before permeabilization and cytoplasmic IL-10 staining. Cell surface staining is shown for IL-10+ (heavy lines) and IL-10− (thin lines) CD19+ cells. Gray histograms represent isotype-matched control mAb staining. Results are representative of those obtained with B cells from at least three mice as determined by flow cytometric analysis. B, B10 and B10pro cell distributions. To identify B10 cells, B20+ cells were isolated from tissues and blood, with in vitro stimulation, staining, and analysis as outlined in A. To identify B10pro cells, the cells were cultured with agonistic CD40 mAb for 48 h with LPS, PMA, ionomycin, and monensin added during the last 5 h of each culture. The cultured cells were isolated, stained with CD19 mAb, permeabilized, and stained using IL-10 mAb with flow cytometric analysis as in A. Values within representative histograms indicate the percentage of IL-10–producing cells among CD19+ B cells within the gates shown. Results are representative of those obtained from at least three mice as determined by flow cytometric analysis.
Autoantibody production and increases in total serum IgG in NZB/W F1 mice might therefore reflect the inability of even potent CD20 mAbs to deplete long-lived plasma cells that could have been generated prior to mAb treatment and/or disease onset (54). Furthermore, B1a and conventional B cells in the peritoneal cavity are depleted at slower rates, which can provide a niche in NZB/W F1 mice that is protected from ADCC (45). Importantly, successful CD20 mAb treatment in patients with SLE does not always correlate with reduced autoantibody levels (30, 33, 62–64), consistent with pathogenic B cells contributing to autoimmunity via mechanisms independent of autoantibody production. Nonetheless, CD20 mAb treatment near the onset of disease initiation successfully enhanced survival in NZB/W F1 mice in both pristane- and collagen-induced lupus models.

Consistent with autoantibody and potential rheumatoid factor production by autoimmune NZB/W F1 mice (55, 56), serum IgG Abs reactive with IgG2a, IgG2c, and IgG1 were present in control, CD20, and CD22 mAb-treated NZB/W F1 mice at 16 wk old. Autoimmune patients treated with rituximab (anti-human CD20) or other mAb therapies frequently produce anti-therapeutic mAb Ab responses, and this is often correlated with decreased treatment efficacy (65–67). Anti-IgG Ab production was likely to also contribute to the reduced effectiveness of B cell depletion in older NZB/W F1 mice (Figs. 2F, 4), particularly in mice given CD20 or CD22 mAbs multiple times. It is possible that CD20 or CD22 mAbs bound to the surface of B cells form immune complexes that enhance the production of anti-mAb treatment Abs. It is possible that the therapeutic CD20 and CD22 mAbs or their idiotypes are recognized as foreign proteins, because the NZB and NZW mouse strains express IgG2a e and n haplotypes, respectively, whereas the MB20-11 and BALB/c-derived IgG2a isotype control mAbs are IgG2c (also known as IgG2b) and IgG2a (a haploype), respectively. Thus, it will be necessary to repeat and validate the current results in this study using properly isotype-matched IgG2a e or n haplotype mAbs to determine whether the current control and treatment mAbs had any effects beyond B cell depletion that might have ultimately contributed to the development of resistance to therapy in mice that received multiple mAb doses. However, it is interesting that in contrast to NZB/W F1 mice, parental NZB, NZW, and C57BL6 mice did not develop measurable resistance to CD20 (MB20-11) mAb depletion or generate anti-CD20 Ab responses after repeated treatments (Fig. 3). Anti-therapeutic Ab production and their consequential negative effects on B cell depletion were therefore unique to NZB/W F1 mice. Undoubtedly, the production of anti-therapy Ab and increased Ig and autoantibody production in NZB/W F1 mice following B cell depletion reflects the effects of their potent autoimmune susceptibility genes, which can contribute to the inability of total B cell depletion to provide effective long-term disease treatment.

B cells are inherently resistant to B cell depletion in autoimmune mice expressing a human CD20 transgene (34), and this has provided a potential explanation for the small therapeutic benefit from rituximab in recent clinical trials for lupus nephritis and SLE (29–33). However, CD20 and CD22 mAbs efficiently depleted blood and tissue B cells or B cell subsets, respectively, equally in both NZB/W F1 and C57BL6/6 mice (Fig. 1). One 10- or 100-μg dose of CD20 mAb (MB20-11) cleared ≥84% of spleen B cells in NZB/W F1 and C57BL/6 mice (Fig. 1). However, the lack of CD20 mAb in NZB/W F1 mice significantly improved survival and delayed proteinuria. These results contrast with those of Ahuja et al. (34), in which B cells from autoimmune strains, including NZB/W F1 and MRL/lpr mice, were found to be inherently refractive to high dose (3 mg/wk) CD20 (18B12, IgG1) mAb-induced depletion compared with nonautoimmune-prone BALB/c mice in which ~65% of spleen B cells were depleted. The reason for the striking discrepancies in B cell clearance between studies may be due in part to the particular CD20 mAbs or mAb isoforms used, because individual CD20 mAbs do not elicit comparable B cell depletion, and IgG2c CD20 mAbs are superior to IgG1 mAbs in eliciting B cell depletion by ADCC in vivo (37, 47, 68). Although B cell depletion using 10–100 μg MB20-11 CD20 mAb did not significantly decrease total ANA, IgG autoantibody levels, or serum IgG in the current study (Figs. 2, 4), Ahuja et al. (34) demonstrated that treatment of human CD20 transgenic-MRL/lpr mice with high doses of anti-human CD20 mAb (10 mg/wk given twice weekly for 7–10 wk) reduced disease manifestations including proteinuria, nephritis, and ANA levels, although survival was not assessed. It is unknown whether these high doses of exogenous IgG or B cell depletion ameliorated disease symptoms, because disease in mice treated with control IgG was not reported. Alternatively, differences in CD20 mAb-mediated alterations in serum Ig, autoantibody, and ANA levels between our study and study by the Ahuja et al. (34) may be related to differences in disease between NZB/W F1 mice versus human CD20 Tg-MRL/lpr mice.

B cells from NZB/W F1 mice were efficiently depleted at the onset of CD20 and CD22 mAb treatment, with total B cell depletion effectively improving survival without eliminating IgG

### Table 1. Tissue distribution of B10 and B10pro cells in 8-wk-old NZB/W F1 and C57BL6/6 mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percentage</th>
<th>Number</th>
<th>Tissue</th>
<th>Percentage</th>
<th>Number</th>
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<tr>
<td></td>
<td>C57BL6</td>
<td>NZB/W</td>
<td>C57BL6</td>
<td>NZB/W</td>
<td>C57BL6</td>
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<tr>
<td>Bone marrow</td>
<td>2.8 ± 0.6*</td>
<td>3.1 ± 0.2*</td>
<td>0.19 ± 0.03</td>
<td>0.15 ± 0.02</td>
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<tr>
<td>Blood</td>
<td>1.3 ± 0.1</td>
<td>5.1 ± 0.4**</td>
<td>0.03 ± 0.01</td>
<td>0.08 ± 0.02*</td>
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</tr>
<tr>
<td>Spleen</td>
<td>2.0 ± 0.2</td>
<td>6.5 ± 0.6*</td>
<td>1.15 ± 0.24</td>
<td>3.38 ± 0.38*</td>
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</tr>
<tr>
<td>Peripheral lymph nodes</td>
<td>0.6 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>0.03 ± 0.01</td>
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<tr>
<td>Mesenteric lymph nodes</td>
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<td>1.9 ± 0.2*</td>
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<td>0.05 ± 0.01*</td>
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<tr>
<td>Peritoneum</td>
<td>24.4 ± 3.0</td>
<td>38.2 ± 1.8**</td>
<td>0.50 ± 0.02</td>
<td>1.57 ± 0.05**</td>
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Numbers include both the B10pro and B10 cell subsets, as in Fig. 6B, with at least three mice per value.

*Values represent the mean number of IL-10+ cells within the B220+ B cell compartment isolated from each tissue. Significant differences between mean values from C57BL6 versus NZB/W F1 mice are indicated.

**Values represent the mean number of IL-10+ B220+ B cells (∗10^6) within each tissue, except when blood values represent 10^6 cells/ml.

Numbers include both the B10pro and B10 cell subsets, as in Fig. 6B, with at least three mice per value.

*p < 0.05; **p < 0.01.
autoantibodies in NZB/W F1 mice. Therefore, B cells in NZB/W F1 mice may contribute significantly to autoimmunity through their significant role during CD4+ T cell activation in response to autoantigens and low-dose Ag challenge (16). B cell-depleting therapies, such as CD20 and CD22 mAbs, may also elicit effects that extend beyond depleting B cells. Although premature B cell depletion in NZB/W F1 mice had deleterious effects, the results of this study suggest that mature B cell depletion can be an effective treatment for patients with SLE, when initiated shortly after disease onset. Although rituximab has variable effects on autoantibody levels in patients with SLE, treatment can improve the clinical manifestations of SLE for some patients in the absence of significant changes in serum anti-DsDNA Ab levels (30, 33, 62–64). This finding suggests the combined use of immunosuppressive therapies to inhibit anti-IgG Ab development that can antagonize B cell depletion during CD20 mAb treatment and inhibit T cell activation in human autoimmune disease. Because the effects of rituximab and other B cell-depleting therapies on human regulatory B10 cells are unknown, caution should be used when administering B cell-depletion therapies, because regulatory B10 cell depletion may potentially initiate and/or accelerate the development of autoimmunity in some cases.

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Disclosures

T.F.T. is a shareholder and paid consultant for Angelica Therapeutics, Inc. and is a consultant for MedImmune. K.M.H was a paid consultant for Angelica Therapeutics.

References


