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Regulatory B Cells (B10 Cells) Have a Suppressuve Role in Murine Lupus: CD19 and B10 Cell Deficiency Exacerbates Systemic Autoimmunity

Rei Watanabe,*† Nobuko Ishiura,*† Hiroko Nakashima,*,† Yoshihiro Kuwano,† Hitoshi Okochi, † Kunihiko Tamaki,*, Shinichi Sato,*, Thomas F. Tedder, ‡ and Manabu Fujimoto,*†,§

B cells play critical roles in the pathogenesis of lupus. To examine the influence of B cells on disease pathogenesis in a murine lupus model, New Zealand Black and New Zealand White F1 hybrid (NZB/W) mice were generated that were deficient for CD19 (CD19−/−; NZB/W mice), a B cell-specific cell surface molecule that is essential for optimal B cell signal transduction. The emergence of anti-nuclear Abs was significantly delayed in CD19−/− NZB/W mice compared with wild type NZB/W mice. However, the pathologic manifestations of nephritis appeared significantly earlier, and survival was significantly reduced in CD19−/− NZB/W mice compared with wild type mice. These results demonstrate both disease-promoting and protective roles for B cells in lupus pathogenesis. Recent studies have identified a potent regulatory B cell subset (B10 cells) within the rare CD1dhi CD5+ B cell subset of the spleen that regulates acute inflammation and autoimmunity through the production of IL-10. In wild type NZB/W mice, the CD1dhiCD5+B220+ B cell subset that includes B10 cells was increased by 2.5-fold during the disease course, whereas CD19−/− NZB/W mice lacked this CD1dhiCD5+ regulatory B cell subset. However, the transfer of splenic CD1dhiCD5+ B cells from wild type NZB/W mice into CD19−/− NZB/W recipients significantly prolonged their survival. Furthermore, regulatory T cells were significantly decreased in CD19−/− NZB/W mice, but the transfer of wild type CD1dhiCD5+ B cells induced T regulatory cell expansion in CD19−/− NZB/W mice. These results demonstrate an important protective role for regulatory B10 cells in this systemic autoimmune disease. The Journal of Immunology, 2010, 184: 4801–4809.

S ystemic lupus erythematosus (SLE) is a prototypic multisystem autoimmune disease characterized by the production of autoantibodies and the involvement of most organ systems (1). Recent studies have demonstrated a critical role for B cells in SLE pathogenesis (2–4). In addition to autoantibody production, abnormal B cell activities or functions, such as cytokine production and Ag presentation, are likely to contribute to SLE development. Indeed, B cell-targeted therapies including mAbs to CD20, CD22, and BAFF are currently under evaluation in the treatment of human SLE (5–8).

B cell activation depends on BCR-generated signals during immune responses to self and foreign Ags (9). Cell surface and intracellular molecules that inform B cells of their microenvironment, such as CD19, CD22, Fc receptors, and TLRs, also play critical roles in controlling B cell responses (10). Among these molecules, CD19 serves as a positive response regulator that amplifies the strength and duration of BCR and other signaling events by regulating Src-family protein tyrosine kinases, and other effector molecules (11–19). CD19 is a 95-kDa member of the Ig superfamily and is expressed on B cells and potentially follicular dendritic cells. CD19−/− mice are hyporesponsive to a variety of transmembrane signals (20, 21), whereas B cells from transgenic mice that overexpress CD19 are hyperresponsive to transmembrane signals and generate autoantibodies spontaneously (22, 23), suggesting that altered CD19 function or expression can influence B cell susceptibility to autoimmunity (24). Therefore, selective targeting of CD19 might be a less invasive B cell-directed strategy for treating SLE rather than total B cell depletion.

As a well-established murine lupus model, New Zealand Black (NZB) and New Zealand White (NZW) F1 hybrid mice (NZB/W mice) spontaneously develop as SLE-like disease in which IgG anti-dsDNA autoantibody production is associated with immune complex-mediated glomerulonephritis (25). Aged NZB/W mice have increased numbers of splenic CD23−CD21hi marginal zone B cells as well as increased numbers of peritoneal B220intCD5+ B1 cells, although their significance in the pathogenesis has been unclear (26–29). Recent studies have identified a phenotypically unique subset of spleen regulatory B cells that share phenotypic markers with both B-1 and marginal zone B cells (30–33). A portion of these rare CD1dhiCD5+ B cells are competent for IL-10 production and are therefore called B10 cells (34). B10 cells and potentially other regulatory B cell subsets negatively regulate inflammation and autoimmune disease in mice, including contact hypersensitivity, experimental autoimmune encephalomyelitis, inflammatory bowel diseases, and arthritis (30–40). Both contact
hypersensitivity responses and experimental autoimmune encephalomyelitis are augmented in CD19<sup>−/−</sup> mice because of the absence of B10 cells (34, 41, 42). Whereas B cells and autoantibodies play major pathogenic roles in NZB/W mice, B cells can also contribute to the suppression of the disease. In this context, we assessed the effect of CD19 deficiency on disease initiation and progression in NZB/W mice.

Materials and Methods

Mice

NZB, NZW, and C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). CD19<sup>−/−</sup> mice were generated as described (21) and backcrossed onto a C57BL/6 background ≥12 times. CD19<sup>−/−</sup> mice were also backcrossed 12 times onto the NZB or NZW genetic backgrounds to obtain CD19<sup>−/−</sup> NZB mice and CD19<sup>−/−</sup> NZW mice. Female NZB/W mice were generated by mating female NZB and male NZW mice. CD19<sup>−/−</sup> NZB/W mice were generated by mating female CD19<sup>−/−</sup> NZB and male CD19<sup>−/−</sup> NZW mice. Mice were housed in a specific pathogen-free barrier facility. All procedures were approved by the Animal Committee of International Medical Center of Japan.

Measurement of serum autoantibodies

Serum samples were obtained from NZB/W mice and CD19<sup>−/−</sup> NZB/W mice every 2 wk for determining serum IgG anti-nuclear Ab (ANA) levels. To determine ANA positivity, serum was diluted 1:100 and added to fixed HEp-2 cell ANA slides (MBL International Medical Center of Japan). Sera were diluted 1:100, added to the ELISA plates (Mesacup; MBL). Sera were diluted 1:100, added to the ELISA plates (Mesacup; MBL). Sera were diluted 1:100, added to the ELISA plates (Mesacup; MBL). Sera were diluted 1:100, added to the ELISA plates (Mesacup; MBL). Sera were diluted 1:100, added to the ELISA plates (Mesacup; MBL). Sera were diluted 1:100, added to the ELISA plates (Mesacup; MBL). Sera were diluted 1:100, added to the ELISA plates (Mesacup; MBL). Sera were diluted 1:100, added to the ELISA plates (Mesacup; MBL). Sera were diluted 1:100, added to the ELISA plates (Mesacup; MBL).

Measurement of intracellular calcium concentration

Spleen cells (1 × 10<sup>7</sup>/ml) in RPMI 1640 medium containing 5% BSA and 10 mM HEPES buffer were loaded with 1 μM Fluo-4 (Molecular Probes, Eugene, OR) at 37°C for 30 min. The cells were washed and stained with PE-Cy5-conjugated anti-B220 Ab for 20 min on ice and washed with PBS (10 min, 37°C). The fluorescence ratio (525/405 nm) of B220<sup>+</sup> cells was determined using an Epics Altra flow cytometer (Beckman Coulter, Miami, FL) with fluorescence intensity shown on a four-decade log scale. Fluorescence contours are shown as 50% log density plots. Positive and negative populations of cells were determined using nonreactive isotype-matched Abs (Southern Biotechnology Associates, Birmingham, AL) as controls for background staining. Baseline fluorescence ratios were collected in real time for 1 min before goat anti-mouse IgM (Fab<sub>2</sub>) Ab fragments (Cappel) were added. The results were plotted as fluorescence ratios at 10-s intervals, increasing fluorescence ratios indicating increased intracellular calcium concentration.

Evaluation of nephritis

Proteinuria was evaluated using Nephrosticks L (Bayer Medical, Tokyo, Japan). Kidneys were harvested from NZB/W and CD19<sup>−/−</sup> NZB/W mice and then bisected. The specimens were stained for interstitial and glomerular disease, as described previously (43), in a blinded manner. Crystall-tissue sections from frozen samples were fixed in acetone for 5 min, and were incubated with 10% normal rabbit serum in PBS (10 min, 37°C) to block nonspecific staining or flash-frozen in OTC compound (Sakura Fineteck, Torrance, CA) for the detection of glomerular immune-complex deposits. The H&E- and PAS-stained sections were scored for interstitial and glomerular disease, as well as the presence of protein in each lane, the blots were stripped and reprobed with anti-ERK2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

Measurement of Src-family kinase activity

After spleen B cells had been stimulated with goat anti-mouse IgM Fab<sub>2</sub> and lysed as described above, the lysates were analyzed using ProFlour Src-Family Kinase Assays (Promega) according to manufacturer’s protocol. The lysates were mixed with Src-family kinase R110 substrate, with ATP added to initiate the kinase reaction. After incubating the plate at room temperature for 60 min, protease solution was added to each well and incubated for 60 min at room temperature. After terminating the protease reaction, the fluorescence of the liberated R110 was read at a wavelength of 525 nm. The fluorescence of each well inversely relates to kinase activity within the cell lysate. The kinase activity of wild type (WT) B cells stimulated for 3 min was defined as 100%.

Measurement of intracellular cytokine expression

PBMCs were stained with PE-anti-Thy1.2 (30-H12; BD Pharmingen) mAbs, PE-conjugated CD5 (53-7.3; BD Pharmingen), and FITC-conjugated anti-CD25 (3B6; BD Pharmingen), and then bisected. The specimens were either fixed in 4% formalin for routine histologic analysis with H&E and periodic acid Schiff (PAS) staining or flash-frozen in OTC compound (Sakura Fineteck, Torrance, CA) for subsequent optimal concentration of FITC-conjugated goat anti-mouse IgM Ab (Cappel) as controls for background staining.

Immunization and isotype-specific ELISA

Eight-week-old mice were immunized i.p. with 100 μg 2,4-dinitrophenylated keyhole limpet hemocyanin (DNP-KLH; LSL, Tokyo, Japan) in CFA and were boosted 21 d later with 100 μg DNP-KLH in IFA. The mice were bled before and after immunizations. Serum DNP-specific Ab titers were measured by adding diluted sera to ELISA plates coated with DNP-BSA (5 μg/ml) for 1 h at room temperature. After washing the plates 5 times, bound Abs were detected using HRP-conjugated goat anti-mouse IgM or anti-mouse IgG1 Ab (Southern Biotechnology Associates) at predetermined optimal concentrations. The ELISA plates were developed using TMB substrate (Bethyl Laboratories, Montgomery, TX), stopped with 1N H<sub>2</sub>SO<sub>4</sub>, and read at a wavelength of 450 nm. A high-titer serum was plated in serial dilutions on each plate for quantification. The OD units were determined arbitrarily by taking a ratio between the OD values obtained for the test sample and for the high-titer sample at the same dilution.

Evaluation of nephritis

Proteinuria was evaluated using Nephrosticks L (Bayer Medical, Tokyo, Japan). Kidneys were harvested from NZB/W and CD19<sup>−/−</sup> NZB/W mice and then bisected. The specimens were stained for interstitial and glomerular disease, as described previously (43), in a blinded manner. Crystall-tissue sections from frozen samples were fixed in acetone for 5 min, and were incubated with 10% normal rabbit serum in PBS (10 min, 37°C) to block nonspecific staining. The tissue sections were incubated sequentially (20 min, 37°C) with predetermined optimal concentration of FITC-conjugated goat anti-mouse IgM Ab (ICN Biomedicals) Ab (ICN Biomedical). The stained sections were scored on a fluorescence microscope at x400 magnification, and images were captured with a constant exposure time of 0.5 s. Mean fluorescence was calculated from captured images. Three representative glomeruli per mouse were outlined, and mean pixel intensity was calculated with Adobe Photoshop (Adobe Systems, San Jose, CA).

B cell activation and Western blot analysis

B cells were purified from single cell splenocyte suspensions by removing T cells with anti-Thy-1.2 Ab-coated magnetic beads (Dynal, Lake Success, NY). B cell suspensions were always >95% B220<sup>+</sup>, as determined by flow cytometric analysis. B cells were resuspended (2 × 10<sup>6</sup>/ml) in RPMI 1640 medium containing 5% FCS at 37°C. The cells were stimulated with goat anti-mouse IgM Ab (Fab<sub>2</sub>) fragments (40 μg/ml; Cappel) and subsequently lysed in buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM Na orthovanadate, 2 mM EDTA, 50 mM NaF, and protease inhibitors. Protein concentrations were determined by light absorbance at 280 nm. The obtained lysates were subjected to SDS-PAGE with subsequent electrophoretic transfer to nitrocellulose membranes. These membranes were incubated with anti-phospho Akt Ab (Ser473; Cell Signaling, Beverly, MA), anti-active ERK Ab (Promega, Madison, WI), or anti-active JNK Ab (Promega), followed by incubation with HRP-conjugated donkey anti-rabbit IgG Abs (Jackson Immunoresearch Laboratories, West Grove, PA). These blots were developed using an ECL kit (Pierce, Rockford, IL). To verify the presence of equivalent amounts of protein in each lane, the blots were stripped and reprobed with anti-ERK2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence analysis

The following mAbs were used: FITC-, PE-, and PE-Cy5–conjugated anti-mouse B220 (CD45R, RA3-6B2; BD Pharmingen, San Diego, CA), FITC-conjugated anti-CD19 (MBI Pharmingen), FITC-conjugated CD14 (1B1; BD Pharmingen), PE-Cy5–conjugated CD4 (H129.19; BD Pharmingen), PE-conjugated CD5 (53-7.3; BD Pharmingen), and FITC-conjugated anti-Thy1.2 (30-H12; BD Pharmingen) mAbs.

Single-cell spleen suspensions were stained for two/three-color immunofluorescence analysis at 4°C using Abs at predetermined optimal concentrations for 20 min as described (14). Cell numbers were counted using a hemocytometer, with relative lymphocyte percentages among viable cells (based on scatter properties) determined by flow cytometric analysis. Erthrocytes were lysed after staining using FACS Lysing Solution (BD Biosciences, San Jose, CA). A PE-conjugated anti-mouse/rat/human FOXP3 Flow Kit (clone 150D; Biolegend, San Diego, CA) was used to detect intracellular Foxp3 expression by regulatory T (Treg) cells according to the manufacturer’s protocol. The labeled cells were analyzed on an Epics flow cytometer (Beckman Coulter, Fullerton, CA) with fluorescence intensity shown on a 4-decade log scale. Positive and negative populations of cells were identified using nonreactive isotype-matched Abs (Southern Biotechnology Associates) as controls for background staining.
Quantitative RT-PCR

Spleen B cells and T cells were purified with B220 mAb- and Thy1.2 mAb-coated microbeads (Miltenyi Biotech, Auburn, CA) by positive selection following the manufacturer’s instructions. In addition, CD1d<sup>+</sup>CD5<sup>+</sup> B cells were isolated from purified B cell preparations using an Epics Altra flow cytometer (Beckman Coulter) with purities of 85–95%. These cells were homogenized in Isogen S (Wako, Tokyo, Japan), with total RNA isolated according to the manufacturer’s instructions. Total RNA was reverse transcribed to cDNA using a RevertAid First Strand cDNA Synthesis Kit (ThermoFisher). Relative expression of the real-time PCR products was determined using the ΔΔC<sub>T</sub> technique. B cells from 28-wk-old WT NZB/W mice were used as the calibrator. Each set of samples was normalized using the difference in threshold cycle (C<sub>T</sub>) between the target gene and housekeeping gene (GAPDH): ΔC<sub>T</sub> = (C<sub>T target gene</sub> - C<sub>T GAPDH</sub>). Relative mRNA levels were calculated by the expression 2<sup>−ΔΔCT</sup> sample, where ΔΔC<sub>T</sub> = ΔC<sub>T</sub> sample − ΔC<sub>T</sub> calibrator. Each reaction was performed in triplicate at the least.

Measurement of IL-10 concentrations

Serum IL-10 levels were measured using mouse IL-10 ELISA kits (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Diluted sera were added to a 96-well plate precoated with anti-mouse IL-10 Abs, incubated for 90 min at 37°C, and washed with buffer four times. After the addition of biotin-conjugated anti-IL-10 Ab, the plate was incubated for 45 min at 37°C. The plates were then washed and incubated with HRP-conjugated streptavidin for 45 min at 37°C. The ELISA was developed using stabilized chromogen, terminated with stop solution, and read at a wavelength of 450 nm.

IL-10 present in tissue culture supernatant fluid was also quantified using the same assays. Splenic B cells from WT NZB/W mice were purified with B220 mAb-coupled microbeads (Miltenyi Biotech). CD1<sup>+</sup>CD5<sup>+</sup> B cells were isolated using an Epics Altra flow cytometer (Beckman Coulter). Isolated CD1<sup>+</sup>CD5<sup>+</sup> B cells as well as CD5<sup>−</sup> B cells (3 × 10<sup>5</sup>) were cultured in 200 μl RPMI 1640 medium containing 10% FBS, 10 mM HEPE, 55 μM 2-ME, 200 μg/ml penicillin, and 200 U/ml streptomycin (Life Technologies, Carlsbad, CA) in 96-well flat-bottom tissue culture plates at 37°C with 5% CO<sub>2</sub> in the presence of LPS (10 μg/ml). Each plate was then harvested and incubated with human IL-10 (Promega) for 45 min at 37°C. The plates were then washed and incubated with HRP-conjugated streptavidin for 45 min at 37°C. The ELISA was developed using stabilized chromogen, terminated with stop solution, and read at a wavelength of 450 nm.

Adoptive cell transfers

Spleen B cells were purified using B220 mAb-coupled microbeads (Miltenyi Biotech) from 20-wk-old WT NZB/W mice. Spleen CD1d<sup>+</sup>CD5<sup>+</sup> B cells were transferred i.v. into 20-wk-old WT NZB/W mice (n = 15), with nephritis and survival monitored.

Statistical analysis

ANA, proteinuria, and survival data were analyzed using Kaplan-Meier curves and the log-rank test. Unless indicated otherwise, comparisons between groups were made using the Mann-Whitney U test; p < 0.05 was considered statistically significant.

Results

**CD19 deficiency inhibits ANA development in NZB/W mice**

The age of ANA production in WT and CD19<sup>−/−</sup> NZB/W mice was compared using a fluorescent ANA assay with HEp-2 cells as substrates. Serum ANA was first detected in NZB/W mice between 16 and 24 wk old. However, the appearance of serum ANA was significantly delayed in CD19<sup>−/−</sup> NZB/W mice (p < 0.001; Fig. 1A). ANA titers were also significantly lower in CD19<sup>−/−</sup> NZB/W mice than in WT NZB/W mice at all the ages examined (Fig 1B). ANA staining had a homogenous to speckled nuclei staining pattern, with no difference observed between WT and CD19<sup>−/−</sup> mouse sera (data not shown). The development of autoantibodies to dsDNA was also significantly delayed in CD19<sup>−/−</sup> NZB/W mice as determined by ELISA (Fig. 1C).

**CD19 deficiency accelerates renal disease and shortens survival in NZB/W mice**

To assess renal disease in NZB/W mice, the relationship between proteinuria and IgG deposition in the basement membranes of glomeruli were first investigated. Protein levels >300 mg/dl in urine correlated with histologic nephritis based on H&E and PAS staining in both WT and CD19<sup>−/−</sup> NZB/W mice (Fig. 2A). Therefore, urinary protein excretion >300 mg/dl was defined as proteinuria onset. Proteinuria was monitored every 2 wk in WT and CD19<sup>−/−</sup> NZB/W mice. Proteinuria developed slightly but significantly earlier in CD19<sup>−/−</sup> NZB/W mice than WT NZB/W mice (p < 0.05 from 23 to 32 wk; Fig. 2C). Pathologic examination of the kidneys from 32-wk-old mice revealed that glomerulonephritis and interstitial nephritis...
developed both in WT and CD19−/− NZB/W mice. Glomerulonephritis and interstitial nephritis tended to be even more severe in CD19−/−/NZB/W mice than WT mice (Fig. 2B). The deposition of IgG in the basement membrane of glomeruli was also observed in both WT and CD19−/− mice. The fluorescence intensity of glomerular IgG staining was also slightly higher in CD19−/− mice, although the difference was not statistically significant (Fig. 2B, right panel). Glomerular IgG deposition was even detected in the kidneys of CD19−/−/NZB/W mice that had been found to be ANA negative (data not shown). Therefore, CD19−/− NZB/W mice developed glomerulonephritis earlier than did WT NZB/W mice, despite their low frequency and titers of anti-dsDNA Abs (Fig. 1).

WT NZB/W mice begin to succumb to disease at ~25 wk old (Fig. 2C), following the development of nephritis. In contrast, CD19−/− NZB/W mice begin to succumb to disease at ~20 wk old, consistent with their accelerated proteinuria development. Median survival in CD19−/− NZB/W mice was significantly shorter in comparison with WT NZB/W mice (30 versus 35 wk; p < 0.05). Death in CD19−/− NZB/W mice followed the development of nephritis, although some mice did not have detectable ANA or minimal anti-dsDNA Abs (data not shown). Collectively, CD19 expression negatively regulates the development of renal disease, which accelerated mortality.

FIGURE 2. CD19 deficiency accelerates nephritis and shortens survival in NZB/W mice. A. The relationship between proteinuria levels and nephritis histopathology. Kidneys were harvested from NZB/W WT and CD19−/− mice with various levels of proteinuria and were fixed in 4% formalin for H&E and PAS staining. The sections were scored for interstitial (left panel) and glomerular (right panel) disease. Each group contained 20 mice. B. Kidneys from NZB/W WT and CD19−/− mice at 32 wk old were evaluated for interstitial and glomerular diseases (left panel) and glomerular IgG deposition (right panel). Mean glomerular fluorescence staining intensity (arbitrary units) was determined for quantification. Each group contained seven mice. C. Proteinuria (urinary protein excretion > 300 mg/dl) in WT and CD19−/− NZB/W mice was monitored every 2 wk. Each group contained 25 mice. D. Histopathologic analysis of nephritis. Representative kidney sections from WT and CD19−/− NZB/W mice were stained with H&E, PAS, or FITC-conjugated goat anti-mouse IgG (H+L) Ab for the detection of glomerular immune-complex deposits. Original magnification ×200. E. Survival of WT and CD19−/− NZB/W mice. Each group contained 25 mice. *p < 0.05.
The phenotype of CD19<sup>−/−</sup> mice on the NZB/W strain

Because CD19 deficiency generally leads to an immunodeficient B cell phenotype in both mice and humans (20, 21, 44), the finding that CD19 deficiency accelerated disease progression in NZB/W mice was paradoxical. Therefore, it was determined whether strain differences might result in an unanticipated phenotype for B cells from CD19<sup>−/−</sup> NZB/W mice. Cell surface CD19 expression on B cells from the blood, spleen, and lymph nodes was identical between C57BL/6 and NZB/W mice (data not shown). In functional studies, IgM ligation generated augmented intracellular calcium responses by splenic B cells from NZB/W mouse relative to C57BL/6 mice (Fig. 3A), which is consistent with previous reports of polyclonal B cell activation in NZB/W mice (45–48). When WT and CD19<sup>−/−</sup> NZB/W B cells were compared, IgM-induced intracellular calcium responses were delayed in CD19<sup>−/−</sup> B cells (Fig. 3B), which is consistent with results obtained with CD19<sup>−/−</sup> B cells from C57BL/6 mice (49). IgM-induced Src-family kinase activation and Akt phosphorylation were also significantly reduced in CD19<sup>−/−</sup> NZB/W B cells compared with B cells from WT NZB/W mice (Fig. 3C, 3D), as previously reported for CD19<sup>−/−</sup> B cells from C57BL/6 × 129 mice (14, 50). Impaired ERK and JNK activation were also observed in CD19<sup>−/−</sup> NZB/W
B cells compared with WT NZB/W B cells (Fig. 3D). The proliferation of B cells cultured in the presence of F(ab')2 anti-IgM Abs was also reduced by CD19-deficiency in NZB/W mice (data not shown). In vivo, the influence of CD19 deficiency on humoral immune responses in NZB/W mice was assessed by immunizing mice with DNP-KLH, a T cell-dependent Ag. Following immunizations, the primary and secondary IgM and IgG1 responses in CD19−/− NZB/W mice were significantly lower than in WT NZB/W mice (Fig. 3E). Thus, CD19-deficiency in NZB/W mice results in B cell defects that are identical to those reported for CD19−/− mice on nonautoimmune backgrounds. This finding explains the impaired autoantibody production in CD19−/− NZB/W mice, but not the dissociated acceleration of nephritis progression.

Reduced CD1d+CD5+ B cells and IL-10 production in CD19−/− NZB/W mice

CD19 expression is critical for regulatory B10 cell development in C57BL/6 mice (34, 40, 41, 51). Therefore, the development of the spleen CD1d+CD5+ B cell subset, which includes B10 cells, was assessed in NZB/W mice. A spleen CD1d+CD5+B220+ B cell subset was identified in NZB/W mice that was increased in 28-wk-old WT NZB/W mice when compared with 12-wk-old mice (0.9 ± 0.2% at 12 wk and 2.3 ± 0.5% of B220+ cells at 28 wk). In contrast, splenic CD1d+CD5+ B cells were virtually absent in CD19−/− NZB/W mice at both 12 and 28 wk old (0.07 ± 0.03% at 12 wk and 0.13 ± 0.03% at 28 wk; p < 0.05 versus WT mice at each equivalent age; Fig. 4A, Table I). CD19−/− NZB/W mice also had reduced numbers of splenic marginal zone B cells with a CD23+CD1d+ phenotype as well as reduced numbers of peritoneal B1 cells with a B220+CD5+ phenotype (Table I), both of which increase with age in NZB/W mice (29).

Because IL-10 production is the hallmark of B10 cells, IL-10 secretion by CD1d+CD5+ B cells was investigated. At 12 wk old, IL-10 mRNA expression in splenic B cells was comparable between WT and CD19−/− NZB/W mice. IL-10 mRNA levels of splenic B cells from WT NZB/W mice were increased by 2.5-fold at 28 wk old compared with those at 12 wk (Fig. 4B, left). IL-10 mRNA levels in splenic B cells from CD19−/− NZB/W mice remained unaltered at 28 wk old. Whereas B10 cells are not only IL-10 secreting B cells in the spleen, increased numbers and enhanced activation of B10 cells can at least partially contribute to the increase of IL-10 expression in splenic B cells from WT mice, because CD1d+CD5+ B cells from WT NZB/W mice produced augmented IL-10 levels at 28 wk compared with CD1d−CD5− B cells (p < 0.05; Fig. 4B, right). IL-10 secretion from B cells was 11.2-fold higher in WT mice by 12 wk old and 11.4-fold at 28 wk old, respectively, than in CD19−/− mice (p < 0.05 for each; Fig. 4C, left). When splenic B cells from WT NZB/W mice were separated into CD1d+CD5+ cells and non-CD1d+CD5+ cells, CD1d+CD5+ cells secreted 4- to 5-fold more IL-10 compared with non-CD1d+CD5+ B cells (p < 0.05; Fig. 4C, right). Thus, CD1d+CD5+ B cells were increased in number and produced significant levels of IL-10 during disease, whereas these cells were severely reduced in CD19−/− NZB/W mice at all time points. In addition, serum IL-10 concentrations increased during disease progression in WT NZB/W mice, but remained significantly lower in CD19−/− NZB/W mice (p < 0.01 at 12, 20, and 28 wk; Fig. 4D). Therefore, modest IL-10 production and the absence of CD1d+CD5+ B10 cells offered an explanation for accelerated disease in CD19−/− NZB/W mice.

WT CD1d+CD5+ B cells delay disease in CD19−/− NZB/W mice

To determine whether the absence of regulatory B10 cells in CD19−/− NZB/W mice explains their accelerated disease progression, CD1d+CD5+B220+ B cells from 20-wk-old WT NZB/W mice were transferred into CD19−/− NZB/W mice of the same age. As a control, spleen CD5+B220+ follicular B cells were also transferred into CD19−/− NZB/W mice. The transfer of WT CD1d+CD5+B220+ cells into CD19−/− NZB/W mice resulted in 85% survival until 35 wk old (p < 0.05; Fig. 4B) to the extent seen in WT NZB/W mice. In fact, CD19−/− NZB/W mice that received WT CD1d+CD5+ B10 cells lived even longer than WT NZB/W mice (median survival, 37 versus 35 wk). Nephritis and survival were not significantly altered in CD19−/− NZB/W mice that received WT CD1d−CD5− B cells as compared with CD1d−CD5− NZB/W mice (106 cells) in WT CD1d−CD5− NZB/W mice. The adoptive transfer of CD1d+CD5+ B cells did not improve nephritis or survival significantly (data not shown). Therefore, spleen regulatory CD1d+CD5+ B cells can inhibit lupus progression when transferred into CD19−/− NZB/W mice, demonstrating that this subset normally inhibits disease initiation in NZB/W mice.

CD1d+CD5+ B cells induce Treg cells in CD19−/− NZB/W mice

CD4+ Foxp3+ Treg cell numbers increase in WT NZB/W mice during disease (Fig. 6A) as described (52). The CD4+Foxp3+ Treg cell subset comprised 2.4 ± 0.7% of splenic Thy1.2+ T cells (2.4 ± 0.8 × 10⁶ cells) in 12-wk-old WT NZB/W mice. Treg cell frequencies increased to 8.5 ± 1.7% (17.0 ± 1.9 × 10⁶ cells) in WT NZB/W mice that developed ANA and proteinuria at 28 wk old. Although spleen Treg cell frequencies were comparable between WT and CD19−/− NZB/W mice at 12 wk old (2.1 ± 0.8%; 1.8 ± 0.8 × 10⁶ cells in CD19−/− mice), there was not a significant increase in the Treg subset in CD19−/− mice at 28 wk old (2.8 ± 0.9%);

Table I. Frequency and number of splenic B cell subsets in NZB/W mice

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<th>Phenotype</th>
<th>12 wk</th>
<th>28 wk</th>
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<td>CD1d+CD5+ B cells</td>
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<td>CD21+CD23+</td>
<td>11.3 ± 2.5</td>
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<td>B220+CD5+</td>
<td>6.1 ± 1.1</td>
<td>7.1 ± 1.0</td>
</tr>
<tr>
<td>CD1d+CD5+</td>
<td>0.9 ± 0.2</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>Number of each B220+ cell subset</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD21+CD23+</td>
<td>5.7 ± 0.5</td>
<td>16.9 ± 3.3</td>
</tr>
<tr>
<td>B220+CD5+</td>
<td>3.1 ± 0.5</td>
<td>7.1 ± 1.7</td>
</tr>
<tr>
<td>CD1d+CD5+</td>
<td>0.5 ± 0.1</td>
<td>2.2 ± 0.2</td>
</tr>
</tbody>
</table>

Values represent means (± SEM) results obtained from three mice of each genotype. Values represent the percentage of lymphocytes expressing the indicated cell surface molecules out of total B220+ lymphocytes, or B cell numbers calculated based on the total number of spleen lymphocytes.

*p < 0.05; **p < 0.01.
WT CD1d<sup>hi</sup>CD5<sup>+</sup> B cells restore the Treg cell subset in NZB/W CD19<sup>−/−</sup> mice

To determine whether the absence of CD1d<sup>hi</sup>CD5<sup>+</sup> B cells in CD19<sup>−/−</sup> NZB/W mice influences Treg cell expansion, CD1d<sup>hi</sup>CD5<sup>+</sup> B cells from 20-wk-old WT NZB/W mice were transferred into CD19<sup>−/−</sup> NZB/W mice of the same age. Four weeks after cell transfers, the numbers and percentages of Treg cells in CD19<sup>−/−</sup> NZB/W mice given WT CD1d<sup>hi</sup>CD5<sup>+</sup> B cells were significantly higher than in age-matched CD19<sup>−/−</sup> NZB/W mice (5.0 ± 1.1 × 10<sup>5</sup> cells versus 1.9 ± 0.3 × 10<sup>5</sup> cells; p < 0.05; 6.1 ± 0.12% versus 2.3 ± 0.2% of Thy1.2<sup>+</sup> T cells, p < 0.05; Fig. 6C). Therefore, B10 cells or other CD1d<sup>hi</sup>CD5<sup>+</sup> regulatory B cells are likely to play a critical role in Treg cell expansion in NZB/W mice.

Discussion

Nephritis and death were accelerated in CD19<sup>−/−</sup> NZB/W mice relative to WT NZB/W mice (Fig. 2), despite B cell hyporesponsiveness and their immunodeficient phenotype (Fig. 3) of CD19<sup>−/−</sup> mice (20, 21). These unexpected findings were due to the virtual absence of B10 cells in CD19<sup>−/−</sup> NZB/W mice (Fig. 4) as described previously for C57BL/6 CD19<sup>−/−</sup> mice (34, 51). This was confirmed by the adoptive transfer of splenic CD1d<sup>hi</sup>CD5<sup>+</sup> B cells from WT NZB/W mice into CD19<sup>−/−</sup> NZB/W mice, which significantly prolonged their survival and demonstrated an important protective role for regulatory B10 cells in this systemic autoimmune disease. Consistent with these observations, B cell depletion by CD20 mAb treatment eliminated 99% of B10 cells and accelerated disease development in young NZB/W mice as demonstrated in the companion paper to these studies (54). These studies demonstrate protective roles for B cells in lupus pathogenesis.

CD19 expression had both protective and disease promoting roles in lupus pathogenesis in NZB/W mice. CD19 deficiency significantly delayed the generation of ANA, especially anti-dsDNA Abs, in this lupus-prone mouse strain (Fig. 1). However, the manifestation of nephritis was paradoxically accelerated by the loss of CD19, although the difference was modest (Fig. 2). This result paralleled enhanced mortality in CD19<sup>−/−</sup> NZB/W mice. Consistent with these observations, B cell depletion by CD20 mAb treatment eliminated 99% of B10 cells and accelerated disease development in young NZB/W mice as demonstrated in the companion paper to these studies (54). These studies demonstrate protective roles for B cells in lupus pathogenesis.

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FIGURE 5. WT CD1d<sup>hi</sup>CD5<sup>+</sup> B cells delay nephritis onset and prolong survival in CD19<sup>−/−</sup> NZB/W mice. CD1d<sup>hi</sup>CD5<sup>+</sup>B220<sup>+</sup> or CD5<sup>+</sup>B220<sup>+</sup> B cells (2 × 10<sup>6</sup>) from 20-wk-old WT NZB/W mice were transferred into CD19<sup>−/−</sup> NZB/W mice of the same age, with subsequent monitoring of (A) proteinuria and (B) survival. All groups contained 15 mice. Nontreated WT and CD19<sup>−/−</sup> NZB/W mice assessed in Fig. 2 are shown as controls. Each value indicates mean (± SEM) results from three mice.

FIGURE 6. Decreased Treg cells and IL-10 production in CD19<sup>−/−</sup> NZB/W mice. A. CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells in CD19<sup>−/−</sup> NZB/W mice. Splenocytes from 12- and 28-wk-old WT and CD19<sup>−/−</sup> NZB/W mice were stained with anti-Thy1.2 and CD4 mAbs, followed by intracellular staining for Foxp3 and flow cytometric analysis. These results represent those obtained from three independent experiments. B. IL-10 transcript expression by spleen T cells from WT and CD19<sup>−/−</sup> NZB/W mice at the indicated weeks of age. IL-10 mRNA levels were analyzed by quantitative RT-PCR and normalized relative to the internal GAPDH control. Each value indicates mean (± SEM) results from three mice. *p < 0.05. C. CD1d<sup>hi</sup>CD5<sup>+</sup> B cells from WT NZB/W mice increase CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in CD19<sup>−/−</sup> NZB/W mice. CD1d<sup>hi</sup>CD5<sup>+</sup>B220<sup>+</sup> B cells (2 × 10<sup>6</sup>) from 20-wk-old wild type NZB/W mice were transferred into CD19<sup>−/−</sup> NZB/W mice of the same age. Four weeks later, the numbers of spleen CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in CD19<sup>−/−</sup> NZB/W mice were assessed. WT and CD19<sup>−/−</sup> NZB/W mice of the same age were assessed as controls. Each value indicates mean (± SEM) results from seven mice. *p < 0.05.
autoimmunity, but did not accelerate mortality or clinical evidence of renal dysfunction. Consistent with this finding, B cells from these CD19-transgenic mice are hyper-responsive to transmembrane signals, but have significantly increased B10 cell numbers (21, 34, 51). Therefore, CD19 expression positively correlates with autoantibody production, but is likely to have opposing roles during autoimmune disease by regulating B10 cell development. That severe glomerulonephritis can occur in the absence of ANA, including anti-DNA Abs, and that autoreactive B cells can exert pathogenic effects independent of Ab secretion has also been demonstrated in other lupus-prone mouse strains (56–58). Thus, the severe renal disease observed in CD19\(^{-/-}\) NZB/W mice is likely to result from B cell functions other than autoantibody secretion. These studies demonstrate that this B cell function is attributable in part to the suppressive role of B10 cells that normally negatively regulate disease progression.

IL-10 is a pleotropic cytokine with both immunosuppressive and immunostimulatory properties (53, 59). The role of IL-10 in lupus pathogenesis is complex, including the effects of high serum IL-10 levels in human SLE and lupus-prone mouse strains (60–64). For example, serum IL-10 levels positively correlate with SLE disease activity scores and anti-dsDNA autoantibody titers, but negatively correlate with C3 and C4 levels and lymphocyte counts (60, 65, 66). Patients with SLE also have significantly more IL-10–secreting mononuclear cells in their peripheral blood than do normal controls, and disease severity correlates with increased numbers of circulating IL-10–secreting mononuclear cells (62). Furthermore, IL-10 production by B cells is higher for patients with SLE than in normal controls, and Ig production by SLE B cells is largely dependent on IL-10 (61). Therefore, IL-10 can be pathogenic for lupus acceleration, but may also be produced to reduce already existing autoimmune inflammation. Various treatments targeting IL-10 against SLE have also shown contradictory results. For example, IL-10 deficiency significantly enhances disease severity in MRL/lpr mice with increases in IFN-\(\gamma\) and IgG2a anti-dsDNA autoantibody production, which are suppressed by recombinant IL-10 treatment (67). In the current study, CD19 deficiency led to lower serum IL-10 levels in NZB/W mice throughout the disease course (Fig. 4D). In contrast, continuous anti–IL-10 mAb administration significantly delays disease development in NZB/W mice, which is attributed to increased TNF-\(\alpha\) production (68). These contradictory findings are most likely explained by the fact that multiple cell types are capable of producing IL-10, including B cells. Thereby, the positive and negative regulatory roles of IL-10 are likely to differ depending on the cell source of IL-10, as well as the timing of its production, duration, and levels of IL-10 expression. Thus, B10 cell IL-10 production is but one component of a complex regulatory network that balances protective and pathogenic immune responses.

In addition to B10 cells and Ig secretion, B cells regulate immune responses through multiple mechanisms that have been described recently (69). B cells contribute to Ag-presentation, cytokine production, the regulation of lymphoid organogenesis, effector T cell differentiation, and dendritic cell function. It is also noteworthy that B cells have other critical roles in lupus, presumably through their interaction with T cells. For example, B cell deficiency in MRL/lpr mice results in the complete absence of inflammatory T cell renal infiltration (70). B cell ablation in MRL/lpr mice using CD79a mAb decreases the relative abundance of CD4 memory T cells and also reduces T cell infiltration into the kidneys (71). In contrast, MRL/lpr mice engineered to have B cells expressing surface-bound but not secretory Ig develop nephritis, which is characterized by renal T cell infiltration (56). Thus, B cells play pathogenic roles via cytokine secretion or Ag presentation (72). Because lupus develops under the complex regulation of different B cell subsets and their functions, the selective targeting of B cell subsets might lead to promising therapies for this and other autoimmune disorders.

Although the adoptive transfer of CD1d\(^{hi}\)CD5\(^{+}\) B cells into CD19\(^{-/-}\) NZB/W mice significantly improved survival, this treatment did not cure the underlying disease (Fig. 5). Because CD19-positive transferred cells were detected in the spleens of CD19\(^{-/-}\) NZB/W mice 2 wk after injection, but not in 5 wk (data not shown), this may be partly explained by the eventual rejection of CD19-expressing WT B10 cells in CD19-deficient mice. However, this most likely reflects the complex etiology of the lupus-like diseases, and the involvement of multiple hematopoietic lineages in disease initiation and regulation. As an example, splenic T cell IL-10 mRNA levels were significantly reduced during the late stages of disease in CD19\(^{-/-}\) NZB/W mice (Fig. 6B). The spleen CD4\(^{+}\)Foxp3\(^{+}\) Treg cell subset was also significantly reduced in CD19\(^{-/-}\) NZB/W mice, while Treg cells expanded during disease progression in WT NZB/W mice (Fig. 6A). Consistent with this finding, the adoptive transfer of CD1d\(^{hi}\)CD5\(^{+}\) B cells from WT NZB/W mice significantly increased Treg cell numbers in CD19\(^{-/-}\) NZB/W mice (Fig. 6C). These results indicate that CD19 expression by B cells or the presence or absence of B10 cells also has a significant influence on Treg cell development and/or activation in NZB/W mice that remains to be explored. Thus, effective treatments or a cure for lupus-like disease is likely to require the modulation of not only B cell and B10 cell functions, but also T cell and Treg cell functions that significantly modulate disease.

Disclosures
T.F.T. is a paid consultant for MedImmune, Inc. and is a consultant and shareholder for Angelica Therapeutics, Inc.

References


