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B cells expressing the transcription factor T-bet drive lupus-like autoimmunity

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B cells contribute to multiple aspects of autoimmune disorders and may play a role in triggering disease. Thus, targeting B cells may be a promising strategy for treating autoimmune disorders. Better understanding of the B cell subsets that are responsible for the development of autoimmunity will be critical for developing efficient therapies. Here we have reported that B cells expressing the transcription factor T-bet promote the rapid appearance of autoantibodies and germinal centers in spontaneous murine models of systemic lupus erythematosus (SLE). Conditional deletion of T-bet from B cells impaired the formation of germinal centers and mitigated the development of kidney damage and rapid mortality in SLE mice. B cell–specific deletion of T-bet was also associated with lower activation of both B cells and T cells. Taken together, our results suggest that targeting T-bet–expressing B cells may be a potential target for therapy for autoimmune diseases.

Introduction

Autoimmunity is the third most common type of disease in the United States. Although such diseases can be treated, there is no cure for autoimmunity; therefore, it is extremely important to study the mechanisms that trigger these diseases. Innate and adaptive immunity are involved in the development and progression of autoimmune diseases. B cells are known to be involved in different aspects of autoimmune diseases and may contribute in a number of ways including the secretion of autoantibodies, processing and presentation of autoantigen to T cells, and production of inflammatory cytokines. Therefore, B cells are promising targets for treatment of autoimmune diseases (1–3).

Indeed, this idea has been put into practice and B cell depletion therapy has been tested for multiple autoimmune diseases. The results of B cell depletion in systemic lupus erythematosus (SLE) are still controversial (4). However, such therapies have been effective in some patients with rheumatoid arthritis (RA) and multiple sclerosis (MS) (5, 6). It is not yet known why B cell depletion is effective for some but not all diseases and for some but not all patients with a particular malady. One possibility is that the depletion therapies might not affect all B cell subsets equally well and different diseases, or different patients, might have involvements of different B cell subsets. Nevertheless, B cells are attractive targets for the treatment of many different autoimmune disorders and more targeted approaches focusing on pathogenic autoreactive B cells (as opposed to depletion of all B cells) may be tremendously beneficial.

A novel subset of B cells named age-associated B cells (ABCs) has recently been identified by others and ourselves (7–10). Unlike other B cells, ABCs express high levels of CD11c and the transcription factor T-bet. T-bet was subsequently demonstrated to be necessary and sufficient for the appearance of this subset (7, 11), and triggering of the B cell antigen receptor (BCR), IFN-γ receptor (IFN-γR), and TLR7 on B cells induces high levels of T-bet expression (12).

Our previous data demonstrated that T-bet$^+$ ABCs appear in autoimmune patients and in autoimmune-prone mice (7, 13). These cells produce high amounts of autoantibodies upon stimulation in vitro, suggesting that they are major precursors of autoantibody-secreting cells (7). Moreover, our recent findings indicate that ABCs are very potent antigen-presenting cells and therefore might participate in autoimmune responses by presenting self-antigen to autoreactive T cells (14). In agreement with our findings, a recent study by Becker et al. demonstrated elevated levels of T-bet expression in B cells obtained from peripheral blood mononuclear cells of SLE patients when compared with healthy donors, suggesting that T-bet expression in B cells may be critical for the development of lupus in humans (15). Others have reported that T-bet–expressing B cells are associated with Crohn's disease activity (16), and an increased expression of T-bet in B cells was found in a patient with MS and celiac disease (17), altogether suggesting an important role for T-bet–expressing B cells in human autoimmunity (18).

Therefore, we hypothesized that ablation of ABCs will prevent or delay the development of lupus-like autoimmunity. We tested this hypothesis by conditionally deleting T-bet from B cells in the SLE1,2,3 mouse model of SLE. Our data demonstrate that this deletion leads to reduced kidney pathology, prolonged survival, and delayed appearance of autoantibodies in these SLE mice. Moreover, our data suggest that T-bet expression in B cells is required for the rapid formation of spontaneous germinal centers (GCs) that develop without purposeful immunization or infection during such autoimmune responses (19, 20). The results indicate a critical role for T-bet expression in B cells for the generation of efficient autoimmune responses and the development of lupus-

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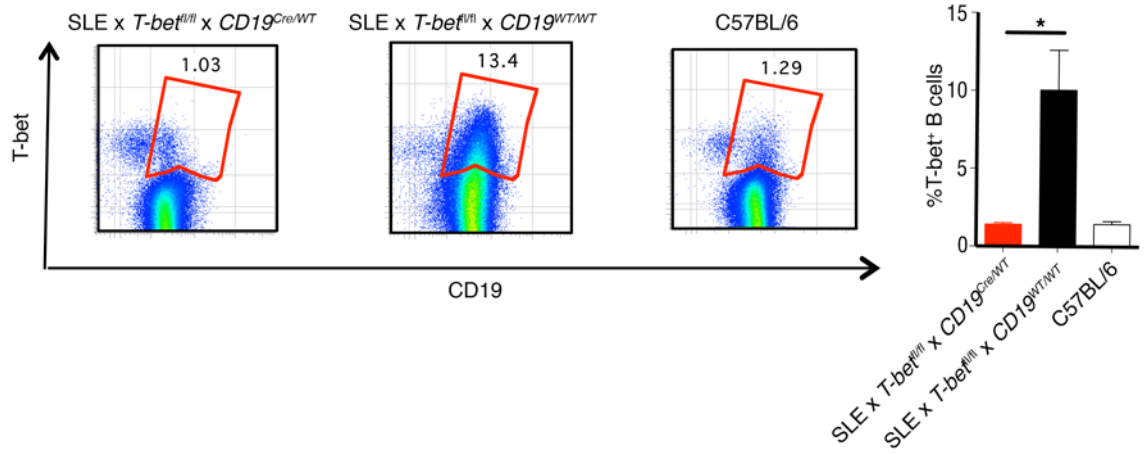


Figure 1. Generation of SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice. Representative FACS plots and quantification of the frequency of T-bet$^+$ B cells in spleens of 4-month-old C57BL/6, SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$, and SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ littermate controls. Numbers represent the percentage of cells in the gates. B cells were gated as live, B220$^+$CD19$^+$CD4$^-$CD8$^-$. n = 4 mice per group. Data are presented as mean ± SEM and are representative of more than 5 independent experiments. *P < 0.05 by unpaired 2-tailed Student's t test.

like autoimmunity, and suggest that specific targeting of T-bet$^+$ B cells might be a useful therapy for some autoimmune diseases.

Results

Generation of autoimmune-prone mice with a B cell–specific deletion of T-bet. B6.SLE1,2,3 mice (referred to hereafter as SLE mice) were used as a model of spontaneous lupus-like autoimmunity. These mice express intervals of chromosomes 1, 4, and 7 derived from NZM2410 animals on the C57BL/6 background. These genetic intervals have been shown to drive lupus-like disease since SLE, unlike B6, animals contain activated lymphocytes, autoantibodies, and develop glomerulonephritis, with a female bias (21–23). We confirmed that, like other mouse models of SLE, SLE mice accumulate T-bet$^+$ ABCs (Figure 1 and ref. 7).

To study the effect of T-bet expression in B cells in autoimmunity, we intercrossed *CD19*$^{Cre/WT}$ and *T-bet*$^{fl/fl}$ with SLE mice, generating SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice. This strain contains all of the intervals (from chromosomes 1, 4, and 7) necessary for predisposition to autoimmunity and lacks T-bet expression in B cells. The successful transfer of all intervals specific for SLE mice was checked by PCR (see Methods). B cell–specific *T-bet* deletion in the SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice was confirmed by both PCR and intracellular staining (Figure 1). We confirmed that the *T-bet* deletion was B cell specific and that T cells continued to express normal levels of T-bet (data not shown).

Thus, we have successfully generated autoimmune-prone mice with a B cell–specific *T-bet* deletion.

In these animals Cre is driven by the *CD19* promoter with concomitant deletion of *CD19*; thus, B cells in such mice are heterozygous for expression of CD19. To check that this did not affect disease onset, we confirmed that autoimmunity occurred similarly in SLE × *CD19*$^{WT/WT}$ and SLE × *CD19*$^{Cre/WT}$ animals (data not shown), so SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ littermate controls were used for all experiments.

In the absence of T-bet in B cells, SLE mice demonstrate improved kidney function and better survival rates. First we asked whether T-bet expression in B cells was required for the development of kidney pathology, a definitive measure of organ pathology in lupus. First, we measured proteinuria levels in SLE mice with or without T-bet expression in B cells. SLE mice in our facility develop proteinuria by 7 months of age; therefore, we tested 7-month-old mice for the presence of proteinuria. The data indicate that the proteinuria score was significantly reduced in the absence of T-bet–expressing B cells (Figure 2A). The mice were subsequently tested again until they were 12 months old. Only one additional SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mouse (out of 9) developed proteinuria during this time frame, indicating that development of proteinuria was prevented rather than delayed in SLE mice with B cell–specific *T-bet* deletion (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI91250DS1).

To further evaluate kidney disease in the mice, the kidney glomeruli of SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ and SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ littermate controls were evaluated by a blinded expert observer. The kidneys were obtained at the time of sacrifice when the mice were 12 months old, or earlier if the mouse exhibited high proteinuria and/or lost 10% of body weight. The results, shown in Figure 2, B–E, indicate that approximately 80% of glomeruli demonstrated hypercellularity and endocapillary proliferation in SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ littermate controls. In contrast, less than 30% of the glomeruli were affected in the SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice.

To confirm that the kidney pathology was associated with immune complex deposition, immunofluorescence histology was performed. Kidney sections were stained with anti-IgG and anti-C3 antibodies to detect immune complex deposition. Our results indicate that, in the absence of T-bet expression in B cells, SLE mice contained less immune complex formation in the kidneys since the size and/or intensity of IgG/C3 stained glomeruli were significantly reduced (Figure 2, F–H).

Together, these data indicate that T-bet expression in B cells plays a critical role for the development of kidney pathology during lupus-like autoimmunity.

Improved survival of SLE mice with B cell–specific T-bet deletion. Since kidney failure is the major cause of death of SLE mice, we asked whether the improved kidney function in SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$

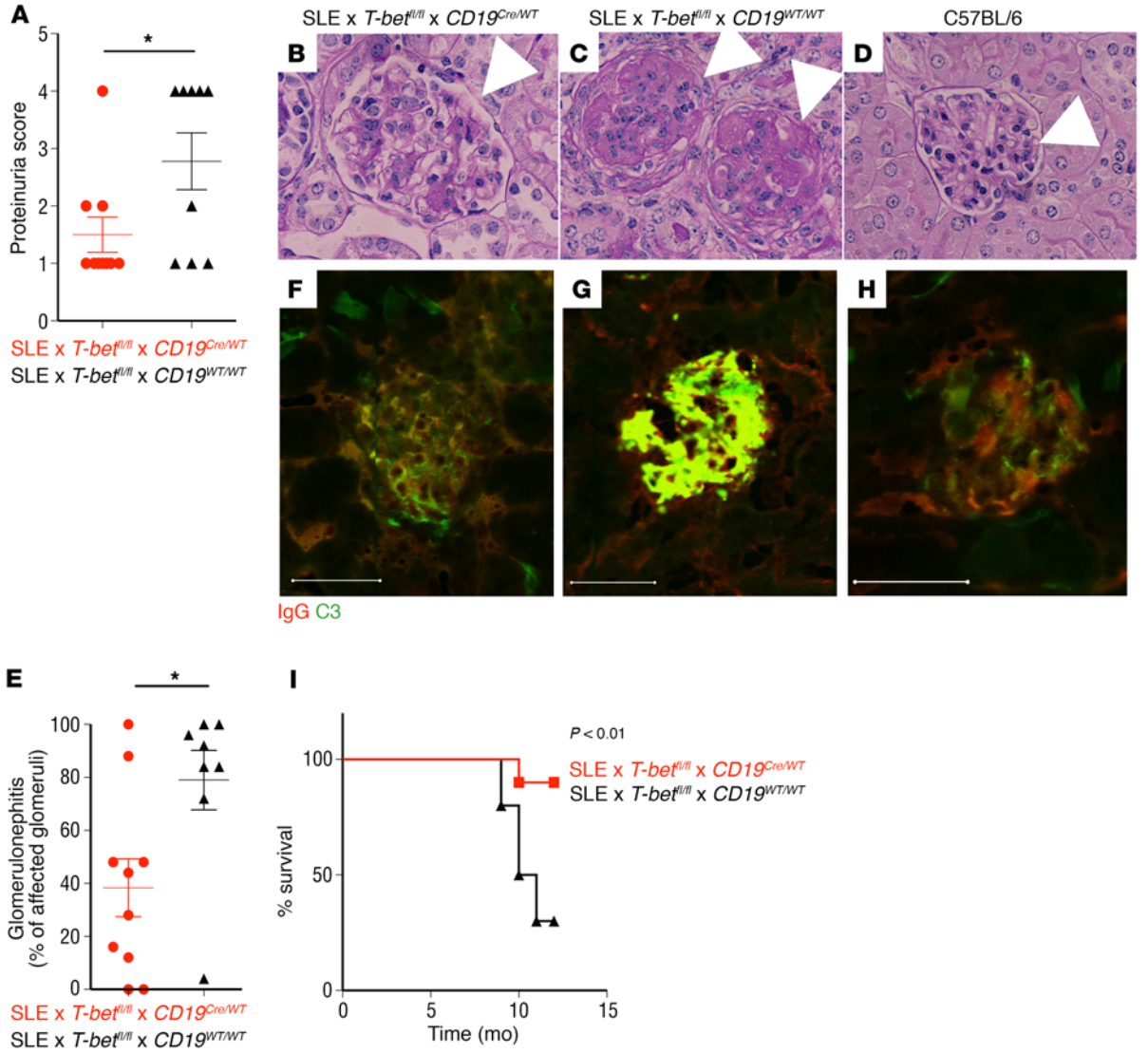


Figure 2. B cell–specific T-bet expression is responsible for the development of kidney pathology and rapid mortality in lupus-like autoimmunity. (**A**) Proteinuria scores of 7-month-old mice (1 = trace, 2 = 30 mg/dl, 3 = 100 mg/dl, 4 ≥ 500 mg/dl). (**B**–**D**) Kidney sections from 12-month-old mice were stained with periodic acid–Schiff from SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ (**B**), and SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ (**C**), and C57BL/6 (**D**) mice. A representative glomerulus is shown in each section. Arrowheads indicate glomeruli. Hypercellularity and endocapillary proliferation were seen in most glomeruli from the SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ mice. Original magnification, ×400. (**E**) Twenty-five glomeruli for each mouse were evaluated in a blinded analysis in kidney sections. The percentage of affected glomeruli is shown for each individual mouse. (**F**–**H**) Five kidneys of 12-month-old mice per genotype were analyzed by immunofluorescence histology (IFH). Representative IFH staining of kidney glomeruli stained with anti-IgG (red) and anti-C3 (green) is shown for: SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ (**F**), SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ (**G**), and C57BL/6 (**H**) mice. Scale bars: 50 μm. (**I**) Survival of SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$, and SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ littermate control mice was followed over the same 12 months (n = 10 mice per group). Comparison of the survival curves and statistical analysis were performed using Prism software. *$P < 0.05$. Significance determined by Student's t test (**A** and **E**) or Gehan-Berslow-Wilcoxon test (**I**).

animals also affected their mortality rates. As demonstrated in Figure 2I, the survival of SLE mice was dramatically improved in the absence of T-bet expression in B cells when compared with littermate controls. Only 25% of T-bet–sufficient SLE mice survived until 12 months of age when, in contrast, 90% of SLE mice with B cell–specific *T-bet* deletion survived until the same age (Figure 2I). This finding demonstrates that T-bet expression in B cells plays a critical role in the development and the consequences of lupus-like autoimmunity.

Overall our data demonstrate that T-bet expression in B cells is essential for the development of kidney pathology and rapid mortality in SLE mice.

B cell–specific T-bet deletion leads to reduced titers of serum IgG2a in SLE mice. Next we asked which changes in the immune system lead to the improved kidney function and reduced mortality in SLE mice that lack B cell–intrinsic T-bet. Multiple groups have previously reported that T-bet expression in B cells is critical for switching B cell isotype expression to IgG2a/c (described herein as IgG2a) (24–26). Therefore, we measured the presence of different IgG subclasses in the sera of SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice compared with SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ littermate controls. Figure 3A demonstrates that the serum concentrations of total IgG were comparable between the experimental and control animals; how-

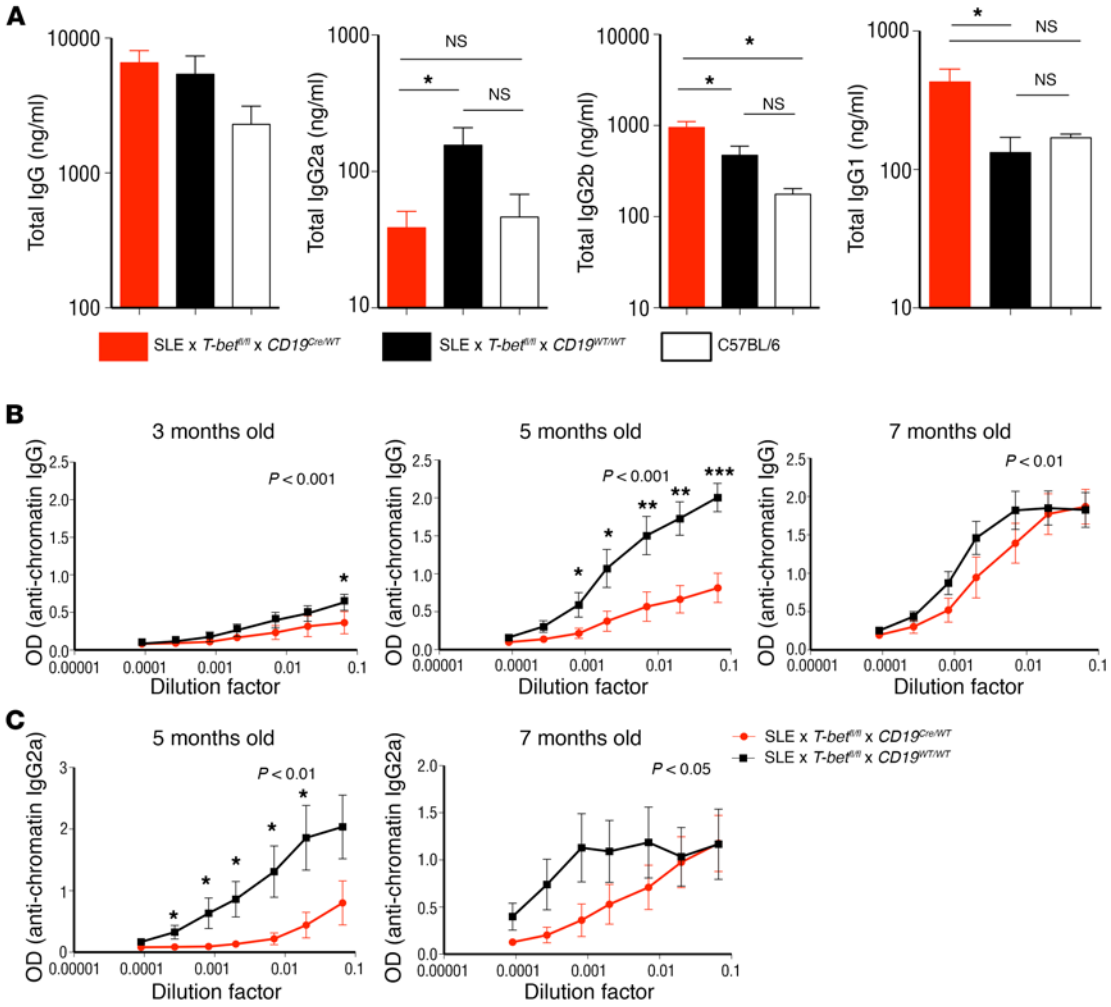


Figure 3. B cell–specific *T-bet* deletion delays the appearance of autoantibodies in SLE mice. (**A**) Concentrations of total or different subclasses of IgG in the serum of 4-month-old C57BL/6 or SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ and SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ littermate controls (n = 5). Data are presented as mean ± SEM. *P < 0.05 by 1-way ANOVA followed by Newman-Keuls analysis. (**B** and **C**) Titers of anti-chromatin total IgG (**B**) or IgG2a (**C**) in the serum of SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ (red) and SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ (black) littermate controls at different ages as indicated (n = 10–12 mice per group). P values for **B** and **C** were calculated using 2-way ANOVA. **P < 0.01, ***P < 0.001. NS, not significant.

ever, the isotype distribution was altered in the absence of T-bet expression in B cells. In particular, as predicted by previous reports (25–27), in the absence of T-bet$^+$ B cells there was a significant reduction in IgG2a levels, such that it decreased to levels seen in normal C57BL/6 animals. This was accompanied by increased levels of IgG2b and IgG1 in SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice compared with their Cre-negative littermate controls.

B cell–specific T-bet deletion leads to reduction of autoantibodies in SLE mice. The presence of high titers of autoantibodies is essential for the development of kidney failure during the development of lupus-like autoimmunity. Therefore, next we evaluated how T-bet expression in B cells affected the development of autoantibodies. We monitored the appearance, over time, of serum anti-chromatin IgG levels in the SLE mice in the absence or presence of T-bet expression in B cells. The appearance of anti-chromatin total IgG autoantibodies (Figure 3B) and of anti-chromatin IgG2a in particular (Figure 3C) was significantly delayed in the absence of T-bet expression in B cells, when compared with littermate controls. The difference was most marked when the mice were 5 months old. However, later in life, the difference between the SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ and SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ control mice lessened, although the titers in T-bet–deficient animals were still significantly lower than those in mice containing T-bet$^+$ B cells. This suggests that another transcription factor (or a combination of transcription factors) can compensate for the lack of T-bet expression in B cells, leading to the activation of autoreactive B cells and their production of autoantibodies. One of the obvious candidates for such a transcription factor is EOMES, which has been reported to compensate for lack of T-bet expression in T cells (28). However, we were unable to detect EOMES expression in T-bet–deficient B cells (data not shown), suggesting the involvement of some other yet unknown transcription factor.

B cell–specific T-bet deletion affects both B and T cell compartments during autoimmune responses. To find out why the absence of T-bet expression in B cells led to the delayed appearance of autoantibodies and reduced kidney damage, we compared the B cell compartments of SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ and SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ littermate control mice. Our data indicate that the absence of T-bet expression in B cells correlated with a reduction in frequencies and

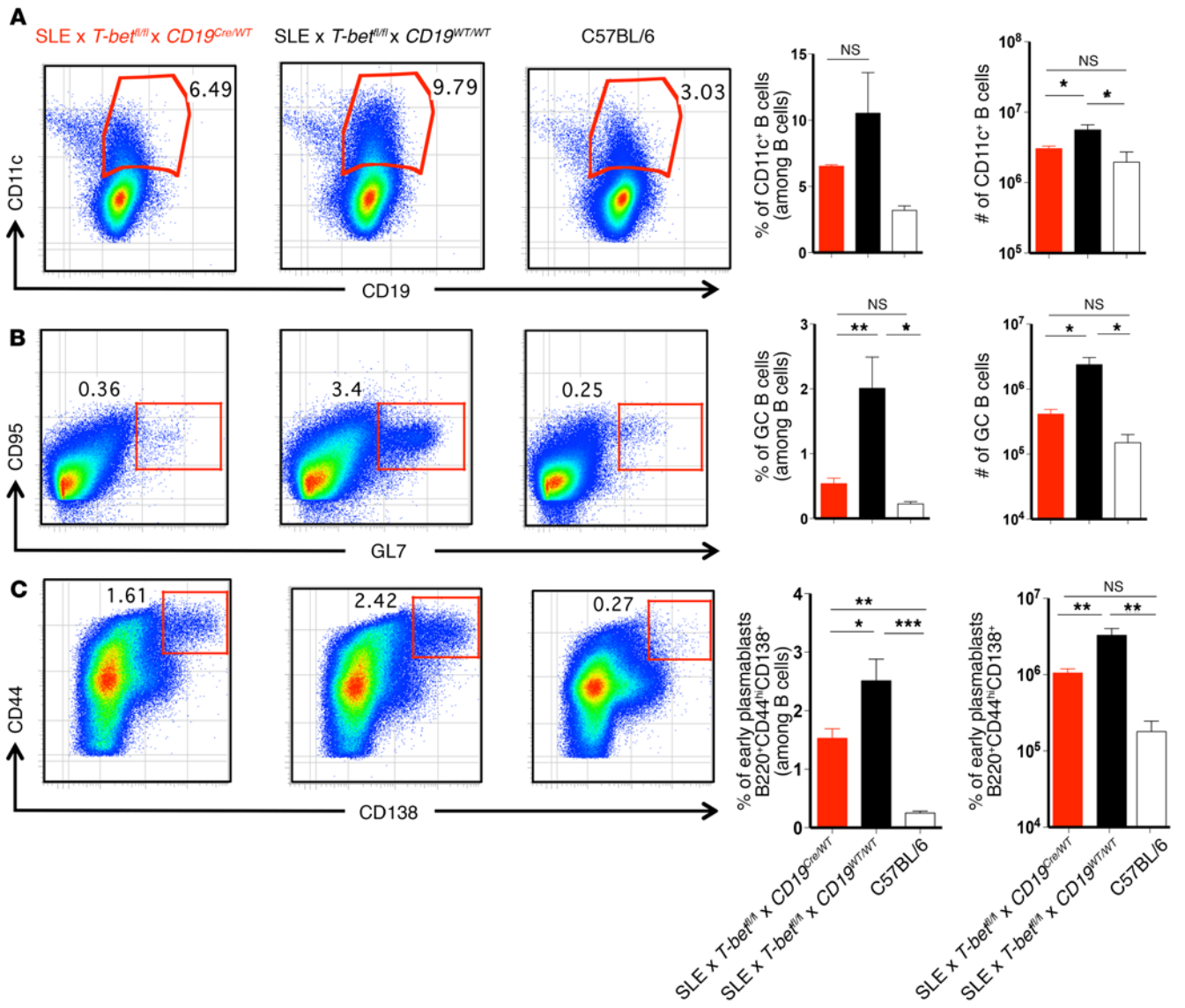


Figure 4. Reduced appearance of CD11c⁺ ABCs, GC B cells, and early plasmablasts in SLE mice with B cell–specific *T-bet* deletion. Representative FACS plots and quantification of the frequency of (**A**) CD11c⁺ B cells, (**B**) GC B cells, and (**C**) early plasmablasts in the spleens of 4-month-old C57BL/6 (white bars), SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ (red bars), and SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ (black bars) mice. B cells were gated as live, B220⁺CD19⁺CD4⁻CD8⁻. Bar graphs represent mean ± SEM (n = 4 mice per group, representative of 3 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001 by 1-way ANOVA followed by Newman-Keuls analysis. NS, not significant.

numbers of CD11c⁺ B cells (Figure 4A). Surprisingly, we detected significantly lower frequencies and numbers of GC B cells in mice with a B cell–specific deletion of *T-bet* (Figure 4B).

Our data also demonstrate significant reduction in frequencies and numbers of early plasmablasts in SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice compared with littermate controls (Figure 4C).

Together these data indicate that, in the absence of their T-bet expression, B cells fail to facilitate the formation of GCs during spontaneous autoimmune responses and do not differentiate into plasmablasts, a failure that probably results in decreased autoantibody production.

Antigen (Ag) presentation by B cells plays an important role in spontaneous T cell activation during autoimmune responses (29). Moreover, it has been previously demonstrated that T-bet⁺ ABCs are potent Ag-presenting cells (14); therefore, the absence of T-bet expression in B cells might affect T cell activation in autoimmunity. To test this hypothesis, we evaluated the T cell compartments in SLE mice in the presence or absence of T-bet expression in B cells. Our data indicate that there was a significant reduction in the percentage and numbers of activated/memory CD4⁺ T cells but not of CD8⁺ T cells in the absence of T-bet–expressing B cells (Figure 5, A and B). On the other hand, the percentages and numbers of IFN-γ–producing CD4⁺ T cells were not affected by the absence of T-bet–expressing B cells, whereas the percentages (but not absolute numbers) of IFN-γ⁺ CD8⁺ cells did drop significantly (Figure 5, C and D).

GC B cells, preplasmablasts, and activated T cells do appear in aged SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice (>7 months old) (Supplemental Figure 2), which is in line with our observation of the delayed appearance of autoantibodies in these mice (Figure 3), suggest-

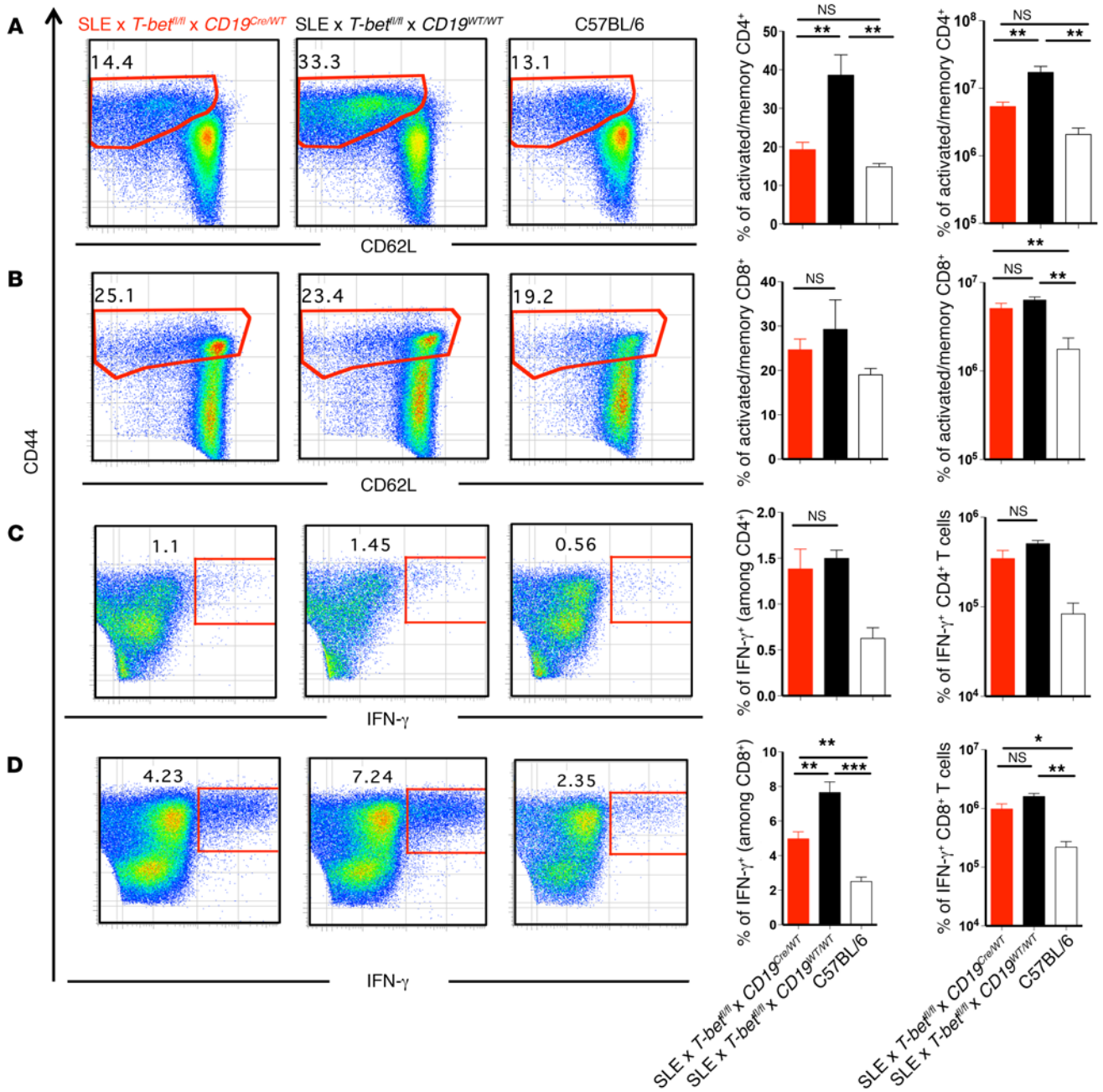


Figure 5. Reduced T cell activation in SLE mice with B cell–specific *T-bet* deletion. Representative FACS plots and quantification of the frequency of (**A**) activated/memory CD4⁺ T cells, (**B**) activated/memory CD8⁺ T cells, (**C**) IFN-γ⁺ CD4⁺ T cells, and (**D**) IFN-γ⁺ CD8⁺ T cells in the spleens of 4-month-old C57BL/6 (white bars), SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ (red bars), and SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ (black bars) mice. T cells were gated as live, B220⁻, CD19⁻, CD4⁺ (or CD4⁻), CD8⁺ (or CD8⁻). Bar graphs represent mean ± SEM (n = 4 mice per group, representative of 3 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001 by 1-way ANOVA followed by Newman-Keuls analysis. NS, not significant.

ing that the autoimmune response is substantially delayed in SLE mice in the absence of T-bet–expressing B cells.

Overall, these data indicate that T-bet expression in B cells during spontaneous autoimmune responses is critical for B cell activation and generation of GC B cells and early plasmablasts (CD44⁺CD138⁺). Moreover, T-bet expression in B cells is also required for the efficient T cell activation during an autoimmune response, perhaps via its effect on B cell Ag-presenting abilities. These changes in B and T cell activation ultimately lead to the reduced titers of autoantibodies and reduction in immune complex formation and deposition in kidneys, which in turn improves the survival of SLE mice.

B cell–intrinsic T-bet expression is critical for the development of spontaneous GCs during autoimmunity. The low frequency of GC B cells in SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice (Figure 4B) suggests that the animals might have impaired formation of spontaneous GCs. To test this, we confirmed by histological analysis of spleens that SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice exhibited a defect in the formation of spontaneous GCs when compared with littermate controls (Figure 6, A–C).

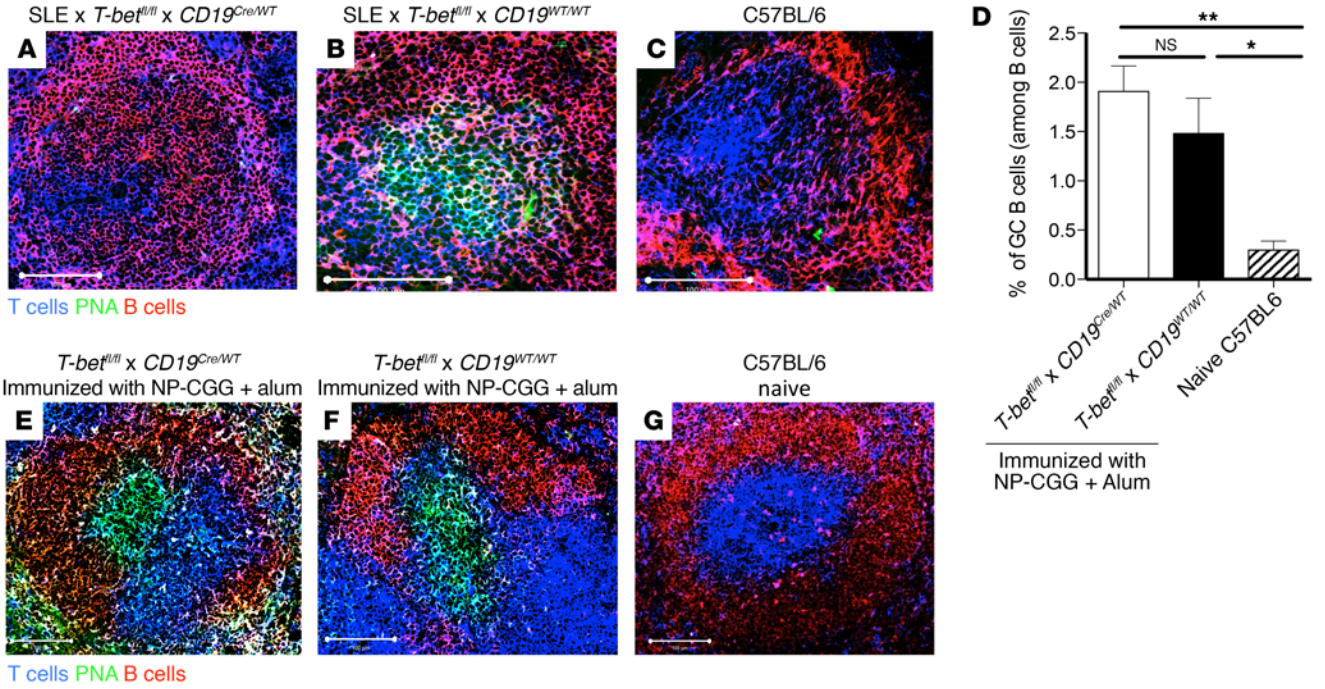


Figure 6. T-bet expression in B cells is required for the formation of spontaneous GCs, but is dispensable for the formation GCs following deliberate immunization. (**A**–**C**) Representative immunofluorescent staining of 5 spleen sections obtained from (**A**) SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$, (**B**) SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$, or (**C**) C57BL/6 mice. (**D**) Percentage of GC B cells identified by FACS as B220$^+$CD19$^+$CD4$^-$CD8$^-$GL7$^+$CD95$^+$ and (**E**–**G**) representative immunofluorescent staining of spleens sections obtained from (**E**) *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$, (**F**) *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ mice immunized with NP-CGG and alum (day 11) (n = 4 mice per group), or (**G**) naive C57BL/6 mice. n = 4 mice per group, representative of 3 independent experiments. Scale bars: 100 μm. $^*P < 0.05$, $^{**}P < 0.01$ by 1-way ANOVA followed by Newman-Keuls analysis. NS, not significant; PNA, peanut agglutinin.

A requirement for T-bet expression in B cells in the formation of GCs was unexpected. Therefore, we wondered whether this requirement was common to all circumstances leading to the creation of GCs, or applied only to their appearance in spontaneous autoimmunity. To answer this question we immunized *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ and *T-bet*$^{fl/fl}$ × *CD19*$^{WT/WT}$ mice with a conventional protocol involving nitrophenylated chicken γ globulin (NP-CGG) with alum, and analyzed for the presence of GCs in spleens 10 days after immunization. As demonstrated in Figure 6, the absence of T-bet expression in B cells did not affect the appearance of GC B cells (Figure 6D) or GCs (Figure 6, E–G) in response to deliberate immunization with Ag plus an adjuvant.

Together, these data indicate that T-bet expression in B cells is required for the formation of spontaneous GCs during autoimmune responses, but is dispensable for the formation of GCs in response to deliberate immunization, at least with an alum-adjuvanted Ag. These findings are in line with recent reports indicating that IFN-γR expression in B cells is required for the formation of spontaneous GCs during autoimmunity (30, 31) and indicate differential requirements for spontaneous and deliberate GC formation. This finding should be further examined in the future.

T-bet expression in B cells is required for the appearance of autoantibodies in Mer$^{-/-}$ *and B6.Nba2 mice*. Our data concern the role of T-bet$^+$ B cells in the SLE model of lupus-like disease. However, other mouse models for this malady exist, so we asked whether the effects we observed were confined to SLE mice or were also apparent in other mouse models of SLE. To approach this question we used 2 other mouse strains that are known to develop lupus-like autoantibodies: B6 mice lacking MerTK (*Mer*$^{-/-}$ mice), which have a defect in the clearance of apoptotic cells and generate autoantibodies with age (32), and B6.Nba2 animals (referred to hereafter as Nba2 mice), which express the autoimmune-predisposing chromosome 1 locus of NZB animals (33). Both *Mer*$^{-/-}$ and Nba2 mice produce autoantibodies, but do not develop kidney pathology (32, 34). We intercrossed these mice with *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice, generating *Mer*$^{-/-}$ × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ or Nba2 × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice with B cell–specific *T-bet* deletions.

As demonstrated in Figure 7A, *Mer*$^{-/-}$ animals with a B cell–specific *T-bet* deletion had significantly decreased titers of anti-chromatin antibodies in comparison with their *Mer*$^{-/-}$ littermate controls that contained T-bet$^+$ B cells. B cell–specific *T-bet* deletion in *Mer*$^{-/-}$ mice also resulted in a reduced frequency of GC B cells (Figure 7B). The appearance of spontaneous GCs in such animals was also significantly reduced (data not shown). Similar effects on autoantibody production were detected in Nba2 × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice (Figure 7C).

Since, as mentioned above, *Mer*$^{-/-}$ and Nba2 mice do not develop kidney pathology, we could not assess the effects of B cell–intrinsic *T-bet* deletion on the development of these clinical features using these models of SLE. Nevertheless, these data show that our findings about the need for T-bet$^+$ B cells for the appearance of autoimmune symptoms in SLE lupus-like disease is extendable to 2 other models of spontaneous autoantibody production. In combination with the recent discoveries of similar B cells in human patients (16, 17), discussed below, our data suggest that ABCs may be targets of interest in treatment of such diseases.

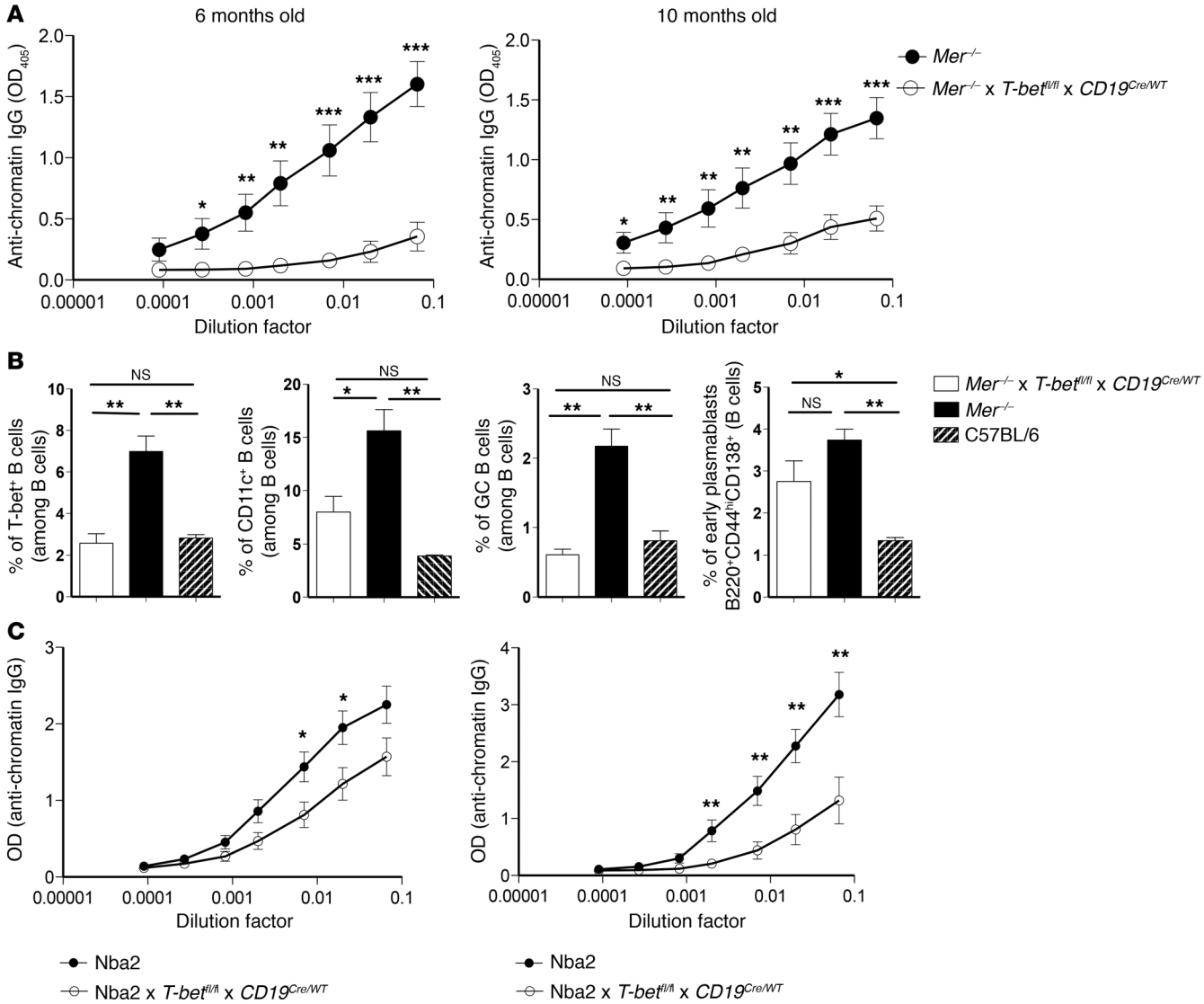


Figure 7. *Mer*$^{-/-}$ and Nba2 mice with B cell–specific *T-bet* deletion exhibit reduced titers of autoantibodies, and reduced frequency of CD11c$^+$ and GC B cells. (**A**) Titers of anti-chromatin IgG in the serum of *Mer*$^{-/-}$ and *Mer*$^{-/-}$ × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice at different ages (as indicated) assessed by ELISA (n = 10 mice per group). (**B**) Quantification of frequency of T-bet$^+$CD11c$^+$ B cells, GC B cells, and early plasmablasts in 4-month-old *Mer*$^{-/-}$ × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ versus *Mer*$^{-/-}$ mice. Bar graphs represent mean ± SEM (n = 4 mice per group, representative of 3 independent experiments). (**C**) Titers of anti-chromatin IgG and IgG2a in the serum of Nba2 and Nba2 × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice assessed by ELISA (n = 10 mice per group). *P < 0.05, **P < 0.01, ***P < 0.001 by 1-way ANOVA followed by Newman-Keuls analysis. NS, not significant.

Discussion

In this report we demonstrate that T-bet expression in B cells is critical for the rapid initiation and progression of autoimmune responses, end-organ damage, and early mortality during lupus-like autoimmunity. Even though autoantibody titers in mice lacking T-bet in B cells increase somewhat in older animals (>7 months old), these titers are still significantly lower than those in T-bet–sufficient controls. Perhaps this consistent difference in autoantibody titer, particularly with respect to IgG2a titers, is why the animals with T-bet–deficient B cells do not suffer accumulation of immune complexes in kidneys and end-organ damage and are much less likely to die an early death. Alternatively, or in addition, the T-bet–deficient B cells might produce autoantibodies of lower affinity, which might be consequently less pathogenic. These possibilities will be approached in future studies.

The observation that the titers of autoantibodies of all IgG subclasses were reduced in the SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice was unexpected. We expected to find a reduction in autoreactive IgG2a titers, since T-bet has been reported to be required for IgG2a isotype switching (24, 26, 35, 36) and B cell–specific T-bet expression has been demonstrated by us (11) and others (10) to be critical for the production of anti-viral IgG2a but not other IgG subclasses. However, we thought that the lack of IgG2a-producing cells might be compensated by increased production of autoantibodies of other types. Surprisingly, though, autoantibody titers in toto were reduced, suggesting reduced activation of all B cells, regardless of their antibody isotype, in the absence of B cell–intrinsic T-bet expression. A similar observation applies to the fact that spontaneous GCs were also reduced in mice lacking T-bet in B cells. These findings are in line

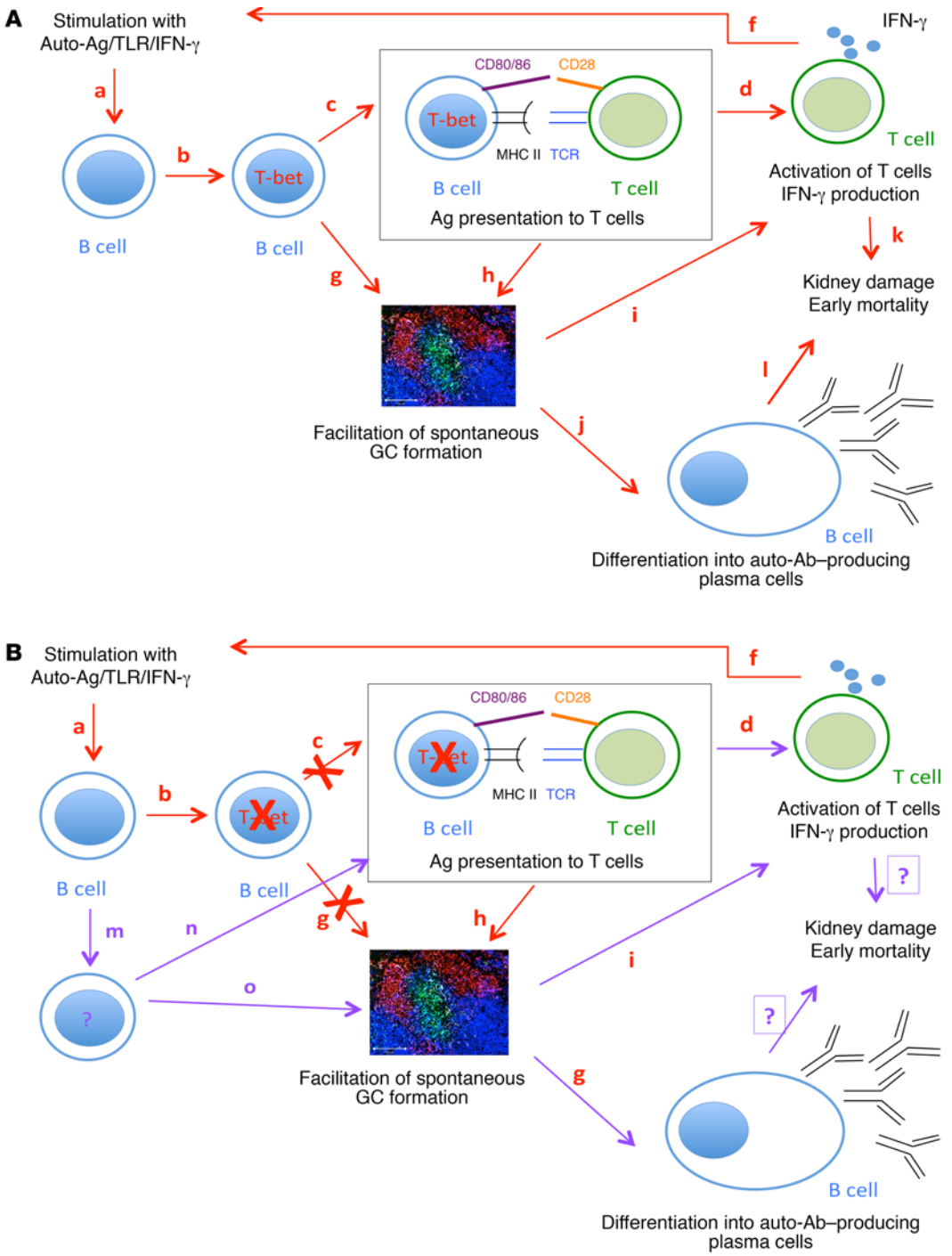


Figure 8. Model. (**A**) Development of a lupus-like autoimmune response in the presence of B cell–intrinsic T-bet. Synergistic stimulation of B cells with auto-antigen (auto-Ag), TLR ligand, and IFN-γ leads to the B cell–intrinsic T-bet expression (arrows a and b). T-bet$^+$ B cells present auto-Ag to T cells (c), leading to subsequent T cell activation, IFN-γ production (d), and GC formation (h). Simultaneously, the GC reaction leads to further T cell activation (i) and differentiation of the B cells into autoantibody-producing (auto-Ab–producing) plasma cells (j). Finally, activated autoreactive T cells and autoantibody-producing plasma cells facilitate kidney damage (k and l). (**B**) The effect of B cell–intrinsic *T-bet* deletion on the development of the autoimmune response. Alternative pathway of B cell activation takes place when T-bet cannot be expressed by B cells (m), and also leads to the GC development and T cell activation (n and o); however, this pathway is delayed, less efficient, and generates less end-organ damage. Scale bars: 100 μm.

with the recent report by Barnett et al., in which they concluded that T-bet expression in B cells regulates a broad antiviral gene expression program, not only anti-viral antibody production (10).

How could T-bet$^+$ B cells affect GC formation and the responses of other autoreactive B cells? We have previously shown that ABCs, the T-bet–expressing B cells, are excellent Ag-presenting cells (14), so the consequences of the absence of such B cells may be related to lowered stimulation of auto-Ag–specific T cells. Our model (Figure 8) indicates that upon stimulation via BCR/TLR/IFN-γR, B cells upregulate T-bet expression (Figure

8, arrows a and b, and ref. 11) and also a number of surface proteins (MHCII, CD80, and CD86) that improve their ability to present Ag to T cells. Moreover, we have also shown that ABCs are located at the T cell/B cell border in spleens — a location that will increase the likelihood of contact between Ag-specific T cells and Ag-presenting ABCs (14) — and that ABCs are precursors for autoantibody production (7), indicating that their BCRs are autoreactive. All these features indicate that ABCs will be ideal presenters of auto-Ags to T cells. Their autoreactive BCRs will concentrate auto-Ags inside the B cells, increasing the number of MHCII/auto-Ag peptides on their surfaces and their high levels of MHCII, CD80, and CD86 will improve their presenting activities (Figure 8, arrow c). This increased Ag presentation will favor the formation of spontaneous GCs (Figure 8, arrows g and h), an idea that is supported by the lack of spontaneous GCs in SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice. T cell activation leads to the enhanced production of IFN-γ, which in turn may feed back, providing IFN-γ for the induction of T-bet expression in B cells (Figure 8, arrow f). Formation of spontaneous GCs leads to the generation of plasma cells producing high-affinity autoantibodies (Figure 8, arrow j) and T cell activation (Figure 8, arrow i). Finally, the presence of activated T cells and autoantibody-producing plasma cells leads to the formation of immune complexes and their deposition in the kidneys, facilitating end-organ damage and early mortality (Figure 8, arrows k and l). Therefore, ablation of B cell–intrinsic T-bet reduces Ag presentation by B cells, diminishing T cell activation, formation of spontaneous GCs, and enabling of B cell differentiation into autoantibody-producing plasma cells. All of these events eventually lead to the prevention of kidney damage and prolonged survival (Figure 8, arrows k and l).

Our data indicate, however, that autoimmune responses are not completely shut down in the absence of B cell–intrinsic T-bet. For instance, we found that autoantibodies, spontaneous GCs, and T cell activation occur in SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice that are more than 7 months old, albeit later than in T-bet–sufficient animals. These data suggest the presence of the alternative, less efficient, T-bet–independent pathway (Figure 8B, arrows m, n, and o). It is not clear whether this alternative pathway also leads to the development of end-organ damage and death of the animals later in life. So far our data indicate that the appearance of autoantibodies is delayed by 2 months in the absence of B cell–intrinsic T-bet. However, their development of proteinuria must be delayed by much more than 2 months since the vast majority of SLE littermates with B cell–intrinsic *T-bet* deletion do not develop proteinuria even by the time they are 12 months old (only 10% of these mice contain > 500 mg/dl of protein urine when they are 12 months old). In addition, 80% of T-bet–sufficient SLE mice die by the time they are 12 months old, whereas 80% of their littermates that lack B cell–intrinsic T-bet survive beyond that age. Moreover, we were able to keep an additional, smaller group of SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice until they were 18 months old without apparent morbidity. Altogether, these data suggest that the delayed response to auto-Ags that occurs in mice lacking T-bet$^+$ B cells is less pathogenic and less damaging for SLE animals.

Ongoing and future studies will be directed to identify which transcription factor(s) is(are) responsible for the activation of autoreactive B cells in the absence of T-bet. We have established that the T-bet–related transcription factor, EOMES, is not expressed by T-bet–deficient B cells in SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ mice, so it must not act as the replacement for T-bet. It has recently been reported that Bcl-6 expression in B cells is required for the formation of spontaneous GCs and the production of autoreactive antibodies in autoimmune mice (31). Therefore, BCL-6 might be involved in the T-bet–independent development of autoimmune responses. Preliminary RNA sequencing analyses reveal several factors that might play a role, including PLZF, a transcription factor that is also expressed in activated T cells and NKT cells (37, 38). These possibilities will be explored in the future. In addition, we will investigate how and why this alternative pathway does not lead to the development of end-organ damage.

Surprisingly and contrastingly, deliberate immunization with conventional Ag plus adjuvant did not require B cell–specific T-bet expression. Others have previously reported different requirements for GC formation in spontaneous autoimmunity versus deliberate immunization, with IFN-γR needed for the former but not the latter event (30, 31). These observations, together with another observation that TLR7 expression in B cells is needed for GC appearance in spontaneous autoimmunity (39), are in line with our findings with respect to T-bet$^+$ ABCs, since ABC formation depends on IFN-γ and TLR7 signaling in B cells (11). However, the results of some others differ from those reported here in one respect, since some, but not all other studies found that T-bet expression in B cells was not required for spontaneous GC formation (30, 31).

Together these data suggest that the initiation of immune responses and the formation of GCs in response to auto-Ag is different compared with the immune response to foreign Ag. Why should this be true? One possibility is that the deliberate addition of adjuvant in immunizations involving foreign Ag automatically generates excellent Ag-presenting cells (dendritic cells) (40). Contrastingly, no such exogenous adjuvant is present during the generation of autoimmune responses in mice. Consequently, some other cell type, the T-bet$^+$ B cells, may be needed to fulfill the need for a good Ag-presenting cell. An idea like this provides one additional explanation for the relative rarity of autoimmune diseases. Only under circumstances that can generate, without external stimulation, ABCs (via BCR, TLR7, and IFN-γR engagement), thus providing an alternative to conventional Ag-presenting cells, can autoimmunity be induced and/or exacerbated.

However, the crucial question is whether these data could be applicable to autoimmune diseases in humans. In order to answer this question, first a T-bet–dependent pathway of B cell activation in autoimmunity has to be demonstrated in humans. Fortunately, several groups, including us, have reported the presence of T-bet–expressing B cells in autoimmune patients. Thus, we have reported a high frequency of T-bet–expressing B cells in RA and scleroderma patients (7). We demonstrated that T-bet$^+$ B cells in humans could be identified as CD21$^-$CD11c$^+$, a phenotype that is similar to that which we observe in mice. B cells with a similar phenotype (CD21$^-$CD19^{hi}CD11c$^+$) have also been reported to be enriched in the blood of common variable immunodeficiency patients with autoimmune cytopenia (41, 42), SLE (43), RA (42), and Sjøgren's syndrome (44). Supporting these original findings, several groups have recently reported the appearance of T-bet$^+$ B cells in autoimmune patients

suffering from SLE (15), MS (17, 45), and Crohn's disease (16). Together these data strongly suggest that T-bet–dependent activation of autoreactive B cells takes place during the development and/or progression of human autoimmunity. Moreover, several studies have indicated that human $CD21^{-}CD19^{hi}$ B cells (the phenotype associated with T-bet expression) are enriched in autoreactivity, supporting the idea that T-bet–expressing B cells are key mediators of human autoimmunity (9, 44). In addition, gene expression profiles of human $CD21^{lo}CD19^{hi}$ B cells show that such cells are similar to those in mice (both subsets are $CD21^{lo}CD23^{lo}CD19^{hi}CD86^{hi}CD11c^{hi}FAS^{hi}$T-$bet^{hi}$; see refs. 7, 9, 18). Overall, we can conclude that human T-bet^{+} B cells very closely recapitulate their mouse counterparts, suggesting their overall similar function in the development of autoimmunity. Therefore, T-bet^{+} B cells appear to be very promising targets for treatment of human autoimmunity.

Development of a depleting agent that will specifically target T-bet^{+} B cells is a crucial step in order to apply this research to human disease. This is an extremely challenging task since so far no unique surface markers have been identified on T-bet^{+} B cells.

Methods

Mice. SLE, C57BL/6, and *CD19*^{Cre} mice were purchased from The Jackson Laboratory and bred at the National Jewish Health animal facility. *T-bet*$^{fl/fl}$ mice were provided by Laurie Glimcher (Dana-Farber Cancer Institute, Boston, Massachusetts, USA) and were intercrossed with *CD19*^{Cre} and SLE animals to generate SLE × *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ animals in which T-bet is specifically absent in B cells. SLE × *T-bet*$^{fl/fl}$ littermates were used as controls for all experiments. Blood collection and proteinuria assessment were performed on all experimental animals monthly. Mice that exhibited high levels of autoantibodies, high proteinuria (above 500 mg/dl), and/or weight loss were sacrificed. Nba2 and *Mer*$^{-/-}$ mice were also crossed to *T-bet*$^{fl/fl}$ × *CD19*$^{Cre/WT}$ and the appearance of autoantibodies was assessed monthly. Female mice were used for all experiments. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the National Jewish Health Animal Care and Use Committee.

Genotyping for SLE NZM2410–derived genomic intervals. Genotyping of all mice containing SLE NZM2410–derived genomic intervals was performed using oligos for D1Mit15, D1Mit17, D1Mit47, D4Mit9, D4Mit12, D4Mit17, D7Mit70, D7Mit145, D7Mit158, and *Ckmm* markers as described previously (46).

Flow cytometric analysis. Cells were stained with antibodies against mouse CD4 (clone GK1.5), CD8 (clone 53-6.7), B220 (clone RA3-6B2), CD11c (clone N418), CD19 (clone 1D3) CD44 (clone IM7), CD62L (clone MEL-14), GL7 (clone GL-7), CD138 (clone 281-2), and CD95 (clone 15A7) purchased from eBioscience, BD Pharmingen, or BioLegend. For intracellular T-bet staining, cells were surface stained, washed in PBS, and stained using Fixable Viability Dye (eBioscience), fixed and permeabilized with the FOXP3 staining buffer set, and stained with anti–human/mouse T-bet antibodies (eBioscience, clone 4B10). For intracellular IFN-γ staining, cells were cultured in the presence of PMA/ionomycin at 5×10^6 cells/ml in 24-well plates for 18 hours, after which Golgi Plug (eBioscience) was added for another 5 hours. Cells were washed, surface stained, fixed with permeabilization/fixation buffer (eBioscience), and stained with in-house-made rat anti–mouse IFN-γ antibodies, produced by the hybridoma MP6-XMG1.2 (previously described in ref. 47). Cells were analyzed by flow cytometry on a CyAn (Beckman Coulter) instrument and data were analyzed using FlowJo software (Tree Star).

ELISA. Plates were coated with goat anti–mouse total IgG antibodies (Jackson ImmunoResearch, catalog 115-005-003) (for total IgG), NP (for NP-specific IgG), or with in-house-made calf chromatin (for anti-chromatin IgG). Calf chromatin was made using Calf thymi. In brief, thymi were washed in sucrose/Tris/PMSF (STP) buffer, and homogenized using a Dounce homogenizer. The homogenate was then strained, washed with STP buffer, and then nuclei were resuspended in Tris/KCl/$MgCl_2$ (TKM) buffer. The mixture was underlaid with 60% sucrose/TKM, and centrifuged at 170 *g* for 10 minutes at 4°C with no brake. Nuclei at the interface were collected and washed in STP. The nuclei were incubated on ice for 1 hour. Afterwards, the nuclei were centrifuged at 3,600 *g* for 10 minutes and resuspended in 1 mM EDTA, causing lysis of the nuclei, following by centrifugation at 11,000 *g* for 10 minutes at 4°C. The chromatin-containing pellet was washed 5 times in 0.1 mM EDTA. The final pellet was resuspended in 1 mM EDTA, aliquoted, and stored at –20°C. Serum IgG levels (total or isotype specific) were detected using alkaline phosphatase–conjugated anti-IgG (SouthernBiotech, catalog 1030-04), anti-IgG1, anti-IgG2c, anti-IgG2b, and anti-IgG3 (Jackson ImmunoResearch).

Proteinuria. The levels of protein in mouse urea (proteinuria) were detected using Chemstrip 2 GP (Roche). Proteinuria was scored as: 1 = trace, 2 = up to 30 mg/dl, 3 = 30–100 mg/dl, and 4 = 500 mg/dl or higher.

Kidney histology. Kidneys were harvested from mice as indicated and were stored in formalin until paraffin wax preparation, sectioning, and subsequent staining with periodic acid–Schiff. To assess histologic injury of the kidneys, 25 glomeruli from each kidney were examined by a blinded observer by using a BX51 microscope (Olympus). Glomeruli were assessed for mesangial hypercellularity, endocapillary proliferation, and crescent formation. The percentage of glomeruli in each kidney that demonstrated these findings was calculated, and the mean percentages for each group were compared. The pathology scores were performed by a board-certified nephrologist with extensive experience with mouse models of lupus nephritis.

Immunofluorescence microscopy of kidney immune complexes. Kidneys were examined by immunofluorescence microscopy for the presence of immune complexes by staining for IgG and C3. The kidneys were harvested as indicated, cut in halves, frozen in OCT, and stored at –80°C until use. Kidney sections (7 μm) were fixed with acetone for 1 minute and then rehydrated with PBS. The sections were blocked for 1 hour in PBS with 2% BSA and 0.05% Tween at room temperature and then incubated with antibodies. Polyclonal goat anti-C3 FITC-conjugated (MP Biomedicals, catalog 55500) and donkey anti–mouse IgG Cy5-congugated antibodies (Jackson ImmunoResearch, catalog 715-175-150) were used at 1:100 dilution. After that the sections were washed, and then mounted. The images were acquired under ×20 and ×10 objectives with a Zeiss LSM 700 confocal microscope using Zen software. Images were analyzed using Slidebook software (3i). To quantify the area and relative fluorescence intensity of glomeruli, regions of interest were drawn around glomeruli. The area and mean fluorescence intensity of the region of interest were calculated using Slidebook software.

Immunofluorescence microscopy of spleen GCs. Spleens were harvested from mice as indicated, frozen in OCT, and kept at –80°C until

used. Sections were rehydrated, blocked for 1 hour in PBS with 2% BSA and 0.05% Tween, and stained with FITC-labeled anti–peanut agglutinin antibodies (catalog FL-1071, 1:200), anti-CD4-allophycocyanin (clone GK1.5, 1:100), and anti-IgD-Alexa550 (clone 1-3.5, 1:100) (all Vector Laboratories) for 1 hour at RT. Images were acquired using ×20 objectives with Zeiss LSM 700 confocal microscope using Zen software and analyzed using Slidebook software. For GC frequency, total GC numbers were quantified in 5 separate ×10-field areas per spleen section in a blinded manner.

NP-CGG immunization. Mice were immunized i.p. with 100 g/mouse of NP-CGG (Biosearch Technologies) with alum and sacrificed on day 11. Spleens and serum were harvested. Spleens were cut in halves and used for flow cytometry and immunofluorescence histology.

Statistics. Data were analyzed with Prism 5 (GraphPad Software) using unpaired *t* test, 1-way ANOVA, or 2-way ANOVA (as indicated) followed by Newman-Keuls analysis. Graphs show the mean ± SEM of the results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, with *P* values less than 0.05 considered significant.

Study approval. All animal work was approved by the National Jewish Health Animal Care and Use Committee.

Author contributions

KR, AVR, JWK, and PM designed research studies. KR, AVR, JMM, and JMT conducted experiments. KR, AVR, JMM, and JMT acquired data. KR, AVR, and PM analyzed data. KR and PM wrote the manuscript.

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