Development of self-reactive germinal center B cells and plasma cells in autoimmune FcγRIIB-deficient mice

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Abnormalities in expression levels of the IgG inhibitory Fc gamma receptor IIB (FcγRIIB) are associated with the development of immunoglobulin (Ig) G serum autoantibodies and systemic autoimmunity in mice and humans. We used Ig gene cloning from single isolated B cells to examine the checkpoints that regulate development of autoreactive germinal center (GC) B cells and plasma cells in FcγRIIB-deficient mice. We found that loss of FcγRIIB was associated with an increase in poly- and autoreactive IgG$^+$ GC B cells, including hallmark anti-nuclear antibody–expressing cells that possess characteristic Ig gene features and cells producing kidney-reactive autoantibodies. In the absence of FcγRIIB, autoreactive B cells actively participated in GC reactions and somatic mutations contributed to the generation of highly autoreactive IgG antibodies. In contrast, the frequency of autoreactive IgG$^+$ B cells was much lower in spleen and bone marrow plasma cells, suggesting the existence of an FcγRIIB-independent checkpoint for autoreactivity between the GC and the plasma cell compartment.

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Abbreviations used: ANA, antinuclear antibody; CDR, complementarity determining region; dsDNA, double-stranded DNA; FcγRIIB, IgG inhibitory Fc γ receptor IIB; GC, germinal center; GL, germline; IFA, indirect immunofluorescence assay; RF, reading frame.

The autoimmune disease systemic lupus erythematosus is characterized by high titers of serum IgG autoantibodies to nuclear antigens (Sherer et al., 2004). Anti–double-stranded DNA (dsDNA) and anti-nucleosome IgG antibodies are hallmark lupus autoantibodies in mice and humans, which correlate with clinical symptoms and contribute to renal pathology (Reveille, 2004). Ig gene analysis of monoclonal antinuclear antibodies (ANAs) from autoimmune mice and humans has shown that the majority of these antibodies carry somatic mutations and show signs of antigen-mediated selection, suggesting that they developed in response to antigenic stimulation (Shlomchik et al., 1987, 1990; van Es et al., 1991; Winkler et al., 1992; Wellmann et al., 2005; Mietzner et al., 2008). Because somatic mutations and affinity maturation are hallmark features of T cell–dependent germinal center (GC) reactions, it has been inferred that these autoantibodies develop in GCs. However, in all studies reported to date autoantibodies were obtained from hybridomas or EBV transformed stable cell lines and, therefore the precise origin of the cells that expressed the autoantibody and whether or not they arose in GCs in vivo is not known.

The IgG inhibitory Fc γ receptor IIB (FcγRIIB) plays an important role in maintaining self-tolerance (Tarasenko et al., 2007). Low levels of FcγRIIB, which negatively regulates activating FcγR-mediated signals in myeloid cells and antigen receptor-mediated signals in B cells, are associated with lupus in mice and humans (Jiang et al., 1999, 2000; Pritchard et al., 2000; Qin et al., 2000; Ravetch and Bolland, 2001; Rao et al., 2002; Rahman and Manser, 2005; Mackay et al., 2006; Rahman et al., 2007b; Su et al., 2007; Lee et al., 2009).

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Mice deficient for FcγRIIB spontaneously develop high serum IgG ANAs with age, which precedes the onset of nephritis in a strain-specific manner (Bolland and Ravetch, 2000). FcγRIIB is expressed on myeloid cells and B cells, but B cell–specific overexpression of FcγRIIB is sufficient to reduce IgG autoantibody levels, lupus-like disease, and mortality, thus demonstrating the B cell–intrinsic importance of FcγRIIB for the regulation of autoreactive B cells (McGaha et al., 2005; Brownlie et al., 2008). A role for FcγRIIB in maintaining peripheral self-tolerance at the plasma cell level was suggested by the finding that loss of FcγRIIB leads to expansion of IgG^+ spleen and bone marrow plasma cells and hypergammaglobulinemia (Fukuyama et al., 2005; Rahman et al., 2007b; Xiang et al., 2007). However, the role of FcγRIIB in regulating autoreactive GC B cells has only been explored in Ig gene transgenic mouse models (Paul et al., 2007; Rahman et al., 2007a). Thus, how loss of FcγRIIB expression influences the frequency at which autoreactive and ANA-expressing B cells participate in GC reactions and develop into plasma cells under physiological conditions is unknown.

To address this question and to determine the frequency of autoreactive GC B cells and plasma cells in mice with an unrestricted antibody repertoire, we analyzed the GC B cell and spleen and bone marrow plasma cell antibody repertoire in $FcγRIIB^{-/-}$ mice and healthy C57BL/6 control mice. Cloning and expression of 360 monoclonal antibodies from single cells revealed that $FcγRIIB^{-/-}$ GC B cells are enriched for somatically mutated self-reactive antibodies including high-affinity anti-dsDNA and kidney-specific autoantibodies. Such antibodies were also detected in the plasma cell compartment of $FcγRIIB^{-/-}$ mice but at much lower frequency than in GC B cells. Increased frequencies of GC B cells with positively charged IgH complementarity determining region (CDR) 3 were associated with high IgG serum anti-DNA autoantibody levels and disease progression, but anti-nuclear and anti-kidney reactive GC B cells were present at high frequency even in mice with low anti-DNA IgG serum levels. In wild-type mice, low-level self-reactive and polyreactive antibodies were expressed by spleen plasma cells, but high-affinity lupus-associated IgG autoantibodies were not detected. In summary, our data demonstrate a role for $FcγRIIB^-$ in the development and differentiation of autoreactive GC B cells and provide direct proof that dsDNA self-reactive B cells participate in GC reactions in diseased animals. In addition, we demonstrate that FcγRIIB-independent self-tolerance mechanisms dominate the regulation of self-reactive GC B cells before differentiation into plasma cells.

RESULTS

$FcγRIIB^{-/-}$ GC B cells are enriched for IgGs with charged IgH CDR3s

To compare the wild-type and $FcγRIIB^{-/-}$ B cell repertoires, we isolated single IgM^+ mature naive and IgG^+ GC B cells from spleen and plasma cells from spleen and bone marrow of six 4–8-mo-old autoimmune $FcγRIIB^{-/-}$ mice (1RII–3RII and 5RII–7RII) and of three controls (1B6–3B6; Fig. S1, A–C). All $FcγRIIB^{-/-}$ mice showed autoimmunity with high serum IgG anti-dsDNA and anti-nucleosome autoantibody titers, splenomegaly, increased frequencies of splenic GC B cells and plasma cells in spleen and bone marrow, and IgG immune complex deposition in the kidney (Fig. S1 and Table S1). $GL7^+Fas^+IgD^-$ GC B cells resembled normal GC B cells in that they were located exclusively within the follicle and frequently expressed IgG2b/c antibodies (Fig. S1 D). Out of 811 single B cells, we amplified 70 different VH region genes from 9 out of 16 different families and 67 different Vκ region genes belonging to 16 out of 19 different families demonstrating the unbiased amplification of the expressed C57BL/6 V gene repertoire by our nested PCR strategy (Fig. 1 and Tables S2–S4; Tiller et al., 2009). Major consistent differences in the VH and JH gene usage, Vκ and Jκ gene usage, DH gene usage, IgH CDR3 length, and IgG subclass distribution between wild-type and $FcγRIIB^{-/-}$ mice or between GC B cells and spleen and bone marrow plasma cells were not observed (Fig. 1, A–F; and Figs. S2 and S3). However, $FcγRIIB^{-/-}$ mice showed a significant increase in DH gene reading frame (RF) 1 usage associated with decreased RF2 usage as compared with wild-type mice ($P = 0.003$; Fig. 1 D), and IgG antibodies from GC B cells of $FcγRIIB^{-/-}$ mice showed, on average, significantly lower numbers of VH gene somatic mutations than wild-type GC B cells (Fig. 1 G). $FcγRIIB^{-/-}$ GC B cells were also significantly enriched for antibodies with highly positively charged IgH CDR3, a feature which is associated with antibody autoreactivity (Fig. 1 H; Barbas et al., 1995; Casali and Schettino, 1996). The high frequency of positively charged amino acids in IgH CDR3 of $FcγRIIB^{-/-}$ GC B cells was not associated with abnormalities in Ig DH gene hydrophobic RF usage (Fig. 1 D and Fig. S2 D).

In summary, the presence of serum IgG autoantibodies in $FcγRIIB^{-/-}$ mice was not associated with a general skewing of the Ig gene repertoire. However, GC B cells of $FcγRIIb^{-/-}$ mice showed lower numbers of somatic mutations, and IgG antibodies with highly positively charged IgH CDR3 were enriched in GC B cells but not in spleen or bone marrow plasma cells compared with wild-type controls.

IgG^+ $FcγRIIB^{-/-}$ GC B cells with positively charged IgH CDR3 accumulate over time and are associated with disease progression

Despite the early presence of IgG serum autoantibodies, only ~20% of $FcγRIIB^{-/-}$ mice develop T cell–dependent disease symptoms, including high anti-DNA serum IgG levels, splenomegaly, and nephritis, and die from kidney failure by the age of 9 mo (Fig. 2 A and not depicted). To determine if changes in the IgG^+ GC B cell repertoire are detectable before the onset of overt disease, we analyzed the Ig gene repertoire and IgH CDR3 of GC B cells from three $FcγRIIB^{-/-}$ mice with modest increase in spleen size and low or undetectable levels of serum IgG autoantibodies for the presence of positively charged amino acids (Fig. 2 and Tables S1 and S4). No differences in IgH or Igκ gene usage between nondiseased

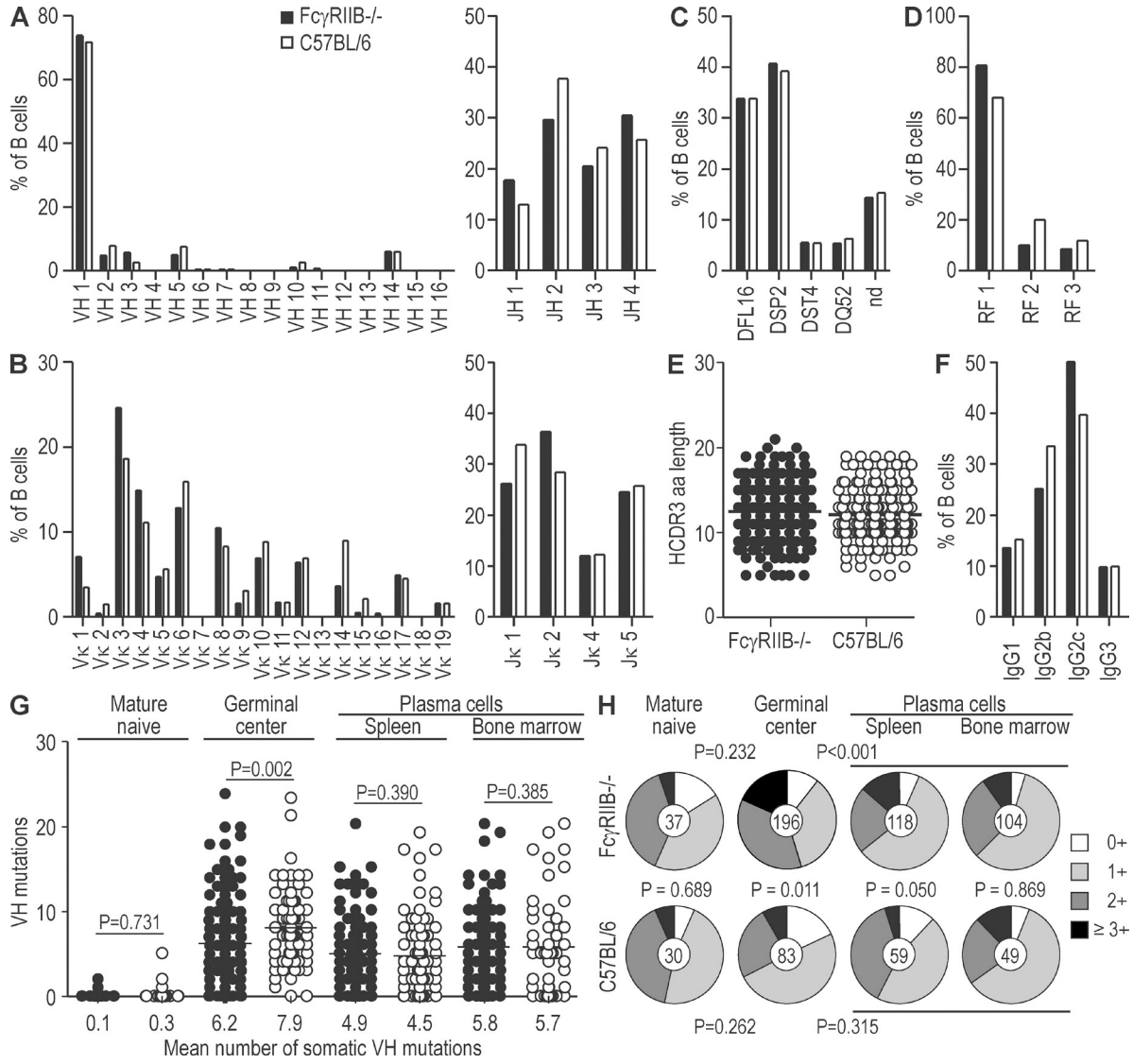

Figure 1. Ig gene analysis of mature naive B cell, GC B cell, and plasma cell antibodies from wild-type and FcγRIIB$^{-/-}$ mice. Data shown summarize the IgH and Igκ gene sequence analysis of 717 antibodies from single IgM$^+$ mature naive B cells and IgG$^+$ GC B cells and spleen and bone marrow plasma cells of six FcγRIIB$^{-/-}$ mice (black; Table S2, 1RII–3RII and 5RII–7RII) and three wild-type mice (white; Table S3, 1B6–3B6). (A) IgH V gene family and J gene usage. (B) Igκ V gene family and J gene usage. (C and D) Ig DH gene (C) and Ig DH gene RF (D) usage. (E) IgH CDR3 amino acid length. Horizontal lines indicate the mean IgH CDR3 amino acid length. (F) IgG1, IgG2c, IgG2b, and IgG3 isotype subclass distribution. (G) Number of somatic mutations in individual IgH V genes from FWR1 to FWR3, inclusively, of IgM$^+$ mature naive B cells, IgG$^+$ GC B cells, and IgG$^+$ spleen and bone marrow plasma cells of FcγRIIB$^{-/-}$ (filled circles) and wild-type (open circles) mice. Horizontal lines indicate the mean number of somatic mutations. (H) Frequency of IgH CDR3s with zero (white), one (light gray), two (dark gray), or three and greater than three (black) positive charges in IgM$^+$ mature naive B cells, IgG$^+$ GC B cells, and IgG$^+$ spleen and bone marrow plasma cells from FcγRIIB$^{-/-}$ and wild-type mice. The numbers of IgH sequences analyzed are indicated in the pie chart centers. P-values were calculated by Fisher's exact 2 × 5 test for DH gene and DH gene RF usage and IgG subclass distribution or Student's *t* test for IgH CDR3 amino acid length and somatic mutations but did not show significant differences between FcγRIIB$^{-/-}$ and wild-type mice (A–G). P-values for the analysis of IgH CDR3-positive charges in H were calculated by Fisher's exact 2 × 5 test and are indicated for the comparison of GC B cells to mature naive B cells and pooled spleen and bone marrow plasma cells from FcγRIIB$^{-/-}$ (top) and C57BL/6 mice (bottom), respectively, and for the comparison of mature naive B cells, GC B cells, spleen plasma cells, and bone marrow plasma cells between FcγRIIB$^{-/-}$ and C57BL/6 mice (center).

and diseased FcγRIIB$^{-/-}$ mice or nondiseased FcγRIIB$^{-/-}$ mice and wild-type mice were observed (Tables S2–S4 and not depicted). Despite high numbers of GC B cells, FcγRIIB$^{-/-}$ mice with low or negative anti-DNA serum IgG levels showed no significant increase in the frequency of GC B cells with positively charged IgH CDR3 ($P = 0.348$ as compared with B6 and $P = 0.044$ as compared with diseased FcγRIIB$^{-/-}$ mice). To further determine if increased frequencies of GC B cells with positively charged IgH CDR3 are a general feature of strong active GC responses, we analyzed IgG$^+$ GC B cells

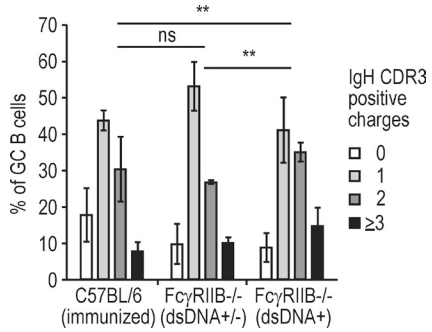

Figure 2. Increased frequency of GC B cells with positively charged IgH CDR3 are associated with lupus disease in FcγRIIB$^{-/-}$ mice. Bar graphs compare the frequency of IgH CDR3s with zero (white), one (light gray), two (dark gray), or greater than or equal to three (black) positive charges in 237 IgG$^+$ GC B cells from six FcγRIIB$^{-/-}$ mice (1RII–3RII and 5RII–7RII; Table S1) with overt lupus disease, as measured by high anti-dsDNA IgG serum levels (dsDNA$^+$) to IgG$^+$ GC B cells from three FcγRIIB$^{-/-}$ mice (4RII, 8RII, and 9RII) with low or negative anti-dsDNA serum IgGs (dsDNA$^{+/-}$; $n = 97$; Table S1) and to IgG$^+$ GC B cells from three wild-type mice at days 10, 14, and 21 after immunization with OVA in CFA ($n = 174$; Table S1). Error bars indicate standard deviation of means. GC B cells with positively charged IgH CDR3 were significantly enriched in FcγRIIB$^{-/-}$dsDNA$^+$ mice as compared with FcγRIIB$^{-/-}$ dsDNA$^{+/-}$ mice ($P = 0.008$) and to immunized wild-type mice ($P = 0.007$). GC B cells from FcγRIIB$^{-/-}$dsDNA$^{+/-}$ mice were not significantly different from immunized wild-type mice ($P = 0.109$). P-values were calculated by Fisher's exact 2 × 5 test.

from wild-type mice at different time points (days 10, 14, and 21) after immunization with OVA in CFA, which contains a complex mixture of mycobacterial antigens (Fig. 2). Immunization of wild-type mice did not increase the frequency of IgG$^+$ GC B cells with highly positively charged IgH CDR3s as compared with nonimmunized wild-type mice ($P = 0.109$). We conclude that increased frequencies of GC B cells with positively charged IgH CDR3 are associated with high levels of anti-DNA serum IgG and the development of overt disease symptoms in FcγRIIB$^{-/-}$ mice and are not a general feature of active inflammatory GC responses to diverse foreign antigens.

Limited diversity in antigen-experienced B cells from wild-type and FcγRIIB$^{-/-}$ mice

It has been suggested that serum IgG autoantibodies in systemic lupus erythematosus are the products of clonally expanded plasma cells, which originate from GC reactions in response to T cell–dependent stimulation (Shlomchik et al., 1990). Autoimmune FcγRIIB$^{-/-}$ mice showed splenomegaly and increased numbers of GC B cells and plasma cells in secondary lymphoid organs and bone marrow, respectively (Table S1). We therefore determined the degree of clonality by Ig gene sequence analysis. Single GC B cells and spleen and bone marrow plasma cells of individual C57BL/6 and FcγRIIB$^{-/-}$ mice frequently expressed Ig genes with identical IgH VDJ and Igκ VJ rearrangements but varying levels of somatic mutations, indicating that they were clonally related (Tables S2–S4). Clonal relatives were frequently distributed between the spleen and bone marrow plasma cell compartments in wild-type and FcγRIIB$^{-/-}$ mice, but little clonal overlap was observed between the GC and spleen or bone marrow plasma cell pool (Tables S2 and S3). Thus, the GC and plasma cell antibody repertoires are distinct and show limited clonal diversity irrespective of FcγRIIB expression.

Increased frequencies of polyreactive IgG$^+$ B cells in FcγRIIB$^{-/-}$ mice

Serum IgG autoantibodies and antibodies, which deposit in the kidney of lupus mice, are frequently polyreactive and show cross-reactivity with diverse self- and foreign antigens (Table S1; Pankewycz et al., 1987; Deshmukh et al., 2006). To measure the frequency of polyreactive IgG$^+$ plasma cells and to determine if polyreactive B cells participate in GC reactions, we cloned and expressed the IgH and matching Igκ chains from GC B cells and spleen and bone marrow plasma cells from FcγRIIB$^{-/-}$ and control mice. The recombinant monoclonal antibodies were assayed for polyreactivity by ELISA with three structurally diverse individual antigens: dsDNA, insulin, and LPS (Fig. 3 and Tables S2 and S3; Wardemann et al., 2003). IgG$^+$ GC B cell antibodies and bone marrow plasma cell antibodies from FcγRIIB$^{-/-}$ mice were more frequently polyreactive with all tested antigens than the wild-type control antibodies (Fig. 3 B; 35 and 14% in FcγRIIB$^{-/-}$ GC and bone marrow plasma cells, respectively, and 4 and 3% in wild-type GC and bone marrow plasma cells, respectively). In contrast, spleen plasma cells frequently expressed polyreactive antibodies in wild-type (18%) and FcγRIIB$^{-/-}$ (23%) mice, suggesting that the spleen is a reservoir of polyreactive plasma cells under normal circumstances and polyreactive spleen plasma cells are not strongly increased in the absence of FcγRIIB (Fig. 3 B). Sequence analysis showed that antibody polyreactivity was not associated with mutation levels or clonal expansion, and clonal relatives with different somatic mutation patterns frequently varied in their antibody polyreactivity (Tables S2 and S3). Thus, loss of FcγRIIB is associated with increased numbers of polyreactive IgG$^+$ GC B cells and bone marrow plasma cells, but splenic plasma cells frequently express polyreactive antibodies even in wild-type mice.

Nucleosome-reactive GC and plasma cell antibodies in FcγRIIB$^{-/-}$ mice

To measure the frequency of anti-nucleosome IgG$^+$ GC B cells and plasma cells, we analyzed the recombinant antibodies from FcγRIIB$^{-/-}$ and control mice for nucleosome reactivity by ELISA (Fig. 4). Antibodies with low-level nucleosome reactivity were found at similar frequency in plasma cells of FcγRIIB$^{-/-}$ and wild-type mice (Fig. 4; 29 and 31% for spleen plasma cells and 18 and 16% for bone marrow plasma cells of FcγRIIB$^{-/-}$ and wild-type mice, respectively). In contrast, 15% of FcγRIIB$^{-/-}$ GC B cells but only 4% of wild-type GC B cells showed low nucleosome reactivity (Fig. 4 A). To determine if the frequency of nucleosome-reactive GC

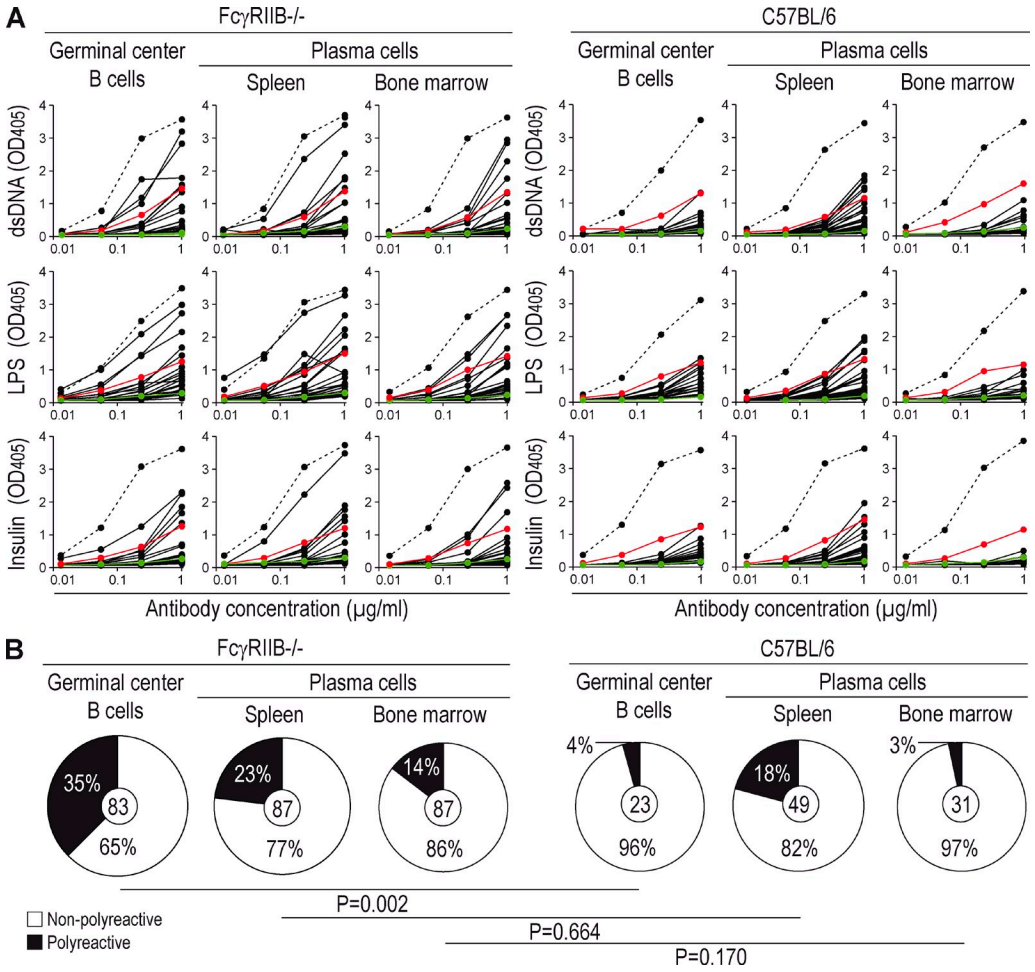

Figure 3. Polyreactivity of IgG⁺ antibodies from GC B cells and plasma cells from FcγRIIB$^{-/-}$ and wild-type mice. (A) Antibodies cloned (black lines) from IgG⁺ GC B cells and spleen and bone marrow plasma cells of FcγRIIB$^{-/-}$ (1RII–5RII) and wild-type (1B6–3B6) mice were tested for polyreactivity with dsDNA, insulin, and LPS by ELISA. Graphs show OD_{405} values at 1 μg/ml antibody concentration and three consecutive 1:4 dilutions of representative antibodies. Dashed lines represent the highly polyreactive positive control antibody ED38 (Wardemann et al., 2003). Red lines represent the low-polyreactive positive control antibody JB40 and green lines represent the nonpolyreactive negative control antibody mGO53 (Wardemann et al., 2003). (B) Pie charts summarize the frequency of nonpolyreactive antibodies (white) and polyreactive antibodies (black) in the respective compartments. All antibodies were tested in at least three independent experiments. The numbers of antibodies analyzed are indicated in the pie chart centers. P-values were calculated by Fisher's exact 2 × 2 test.

B cells in wild-type mice increased in active GC responses to foreign antigens, we cloned, expressed, and tested 80 antibodies from IgG⁺ GC B cells at days 10, 14, and 21 after immunization with OVA in CFA for nucleosome reactivity (Fig. 4 B). OVA/CFA immunization was not associated with increased frequencies of nucleosome-reactive GC B cells. Furthermore, IgG antibodies with high levels of anti-nucleosome reactivity (OD_{405} > 1.5× higher than the internal positive control) were only detected in FcγRIIB$^{-/-}$ GC and plasma cells and not in control cells (Fig. 4). Thus, nucleosome-reactive GC B cells are enriched only in the absence of FcγRIIB ($P = 0.001$ as compared with nonimmunized and immunized wild-type controls).

Anti-nucleosome reactivity has been associated with antibody polyreactivity (Mortensen et al., 2008). Indeed, 69% of nucleosome-reactive GC antibodies and 48 and 38% of nucleosome-reactive spleen and bone marrow plasma cells, respectively, were polyreactive in FcγRIIB$^{-/-}$ mice (Tables S2 and S4). In wild-type mice, 33% of nucleosome-reactive spleen plasma cells, but none of the bone marrow plasma cell antibodies, were polyreactive, thus confirming the result that polyreactivity is enriched in the spleen plasma cell compartment but not in bone marrow plasma cells under normal circumstances (Table S3).

In summary, low-affinity nucleosome-reactive IgG antibodies constitute part of the normal plasma cell compartment, but they were rare in GC B cells from wild-type mice independently of ongoing active immune responses to foreign antigens. Loss of FcγRIIB is associated with increased numbers of nucleosome-reactive GC B cells, including those producing highly reactive antibodies. These antibodies were also found among plasma cells in FcγRIIB$^{-/-}$ mice but were not detected in wild-type mice.

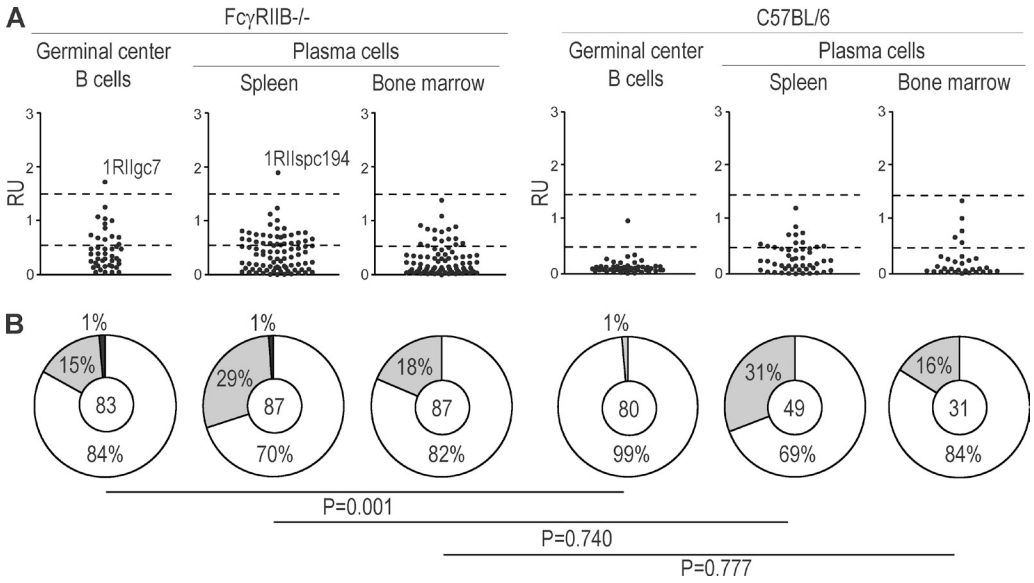

Figure 4. Nucleosome reactivity of IgG⁺ antibodies from GC B cells and plasma cells of FcγRIIB$^{-/-}$ and wild-type mice. Antibodies cloned from IgG⁺ GC B cells and spleen and bone marrow plasma cells of FcγRIIB$^{-/-}$ mice (1RII–5RII) and nonimmunized and OVA/CFA immunized wild-type (1B6–6B6) mice were tested for nucleosome reactivity by ELISA. (A) Data shown are normalized relative units (RU) of OD_{405} values at 3 µg/ml antibody concentrations for representative antibodies from FcγRIIB$^{-/-}$ and nonimmunized wild-type animals. Normalization was relative to the OD_{405} values of the internal positive control, which was set to 1. Dots represent individual antibodies. Dashed lines indicate 0.5 and 1.5× RU values of the positive control. The clone names of highly nucleosome reactive antibodies are indicated in the graphs. (B) Pie charts summarize the frequency of non-nucleosome-reactive (white; <0.5× RU value of positive control), low nucleosome-reactive (gray; 0.5–1.5× RU value of positive control), and high nucleosome-reactive antibodies (black; >1.5× RU value of positive control) in the respective compartments and mice. Data are representative of at least two independent experiments. The number of tested antibodies is indicated in the pie chart center. P-values were calculated by Fisher's exact 2 × 5 test.

FcγRIIB$^{-/-}$ GC B cells are enriched for ANAs

IgG ANAs are a hallmark of systemic lupus disease. These antibodies were found in the serum of the autoimmune FcγRIIB$^{-/-}$ mice analyzed in this study by indirect immunofluorescence assay (IFA) on the human larynx carcinoma cell line HEp-2 (Fig. S1 E). To determine the frequency of GC B cells and plasma cells expressing ANAs and to characterize their staining pattern, we tested all monoclonal antibodies by IFA with HEp-2 cells (Fig. 5, A–C). Various nuclear, cytoplasmic, and nuclear plus cytoplasmic HEp-2 cell antibody staining patterns were detected in wild-type and FcγRIIB$^{-/-}$ mice. However, the overall frequency of IgG ANAs was consistently higher in GC B cells of all FcγRIIB$^{-/-}$ mice (16%) as compared with GC B cells from immunized and nonimmunized wild-type controls (3%; P = 0.005; Fig. 5 B and not depicted). Furthermore, antibodies with a homogenous chromatin staining pattern, as judged by Hoechst costaining, were only found in FcγRIIB$^{-/-}$ mice (Fig. 5, A and C). Two such antibodies with high numbers of positively charged amino acids in IgH CDR3 (1RIIgc7 and 1RIIspc194; Fig. 5 D) were further tested for dsDNA reactivity by IFA with the flagellate *Crithidia luciliae* (Fig. 5 D). Antibody 1RIIgc7, but not 1RIIspc194, showed kinetoplast staining indicating reactivity with native dsDNA, and neither reacted with histones (Fig. 5 D and not depicted). Thus, antibody 1RIIgc7 and antibody 1RIIspc194 are IgG lupus autoantibodies with specificity for dsDNA and nucleosomes, respectively.

In summary, self-reactive antibodies constitute part of the normal GC B cell and plasma cell repertoire, but ANAs are rare and those that exist do not show chromatin reactivity. In contrast, highly autoreactive anti-dsDNA and anti-nucleosome IgG2b and IgG2c with positively charged IgH CDR3 and other chromatin and non-chromatin–reactive ANAs were cloned from GC B cells and plasma cells of autoimmune FcγRIIB$^{-/-}$ mice with serum antibodies of the same specificity. Thus, the overall frequency of ANAs was high in FcγRIIB$^{-/-}$ GC B cells but low in the plasma cell compartments.

FcγRIIB$^{-/-}$ GC B cells frequently express kidney-reactive autoantibodies

The presence of serum autoantibodies in FcγRIIB$^{-/-}$ mice is associated with immune complex deposition in the kidney and precedes the development of nephritis (Ehlers et al., 2006). By IFA with mouse kidney and control stomach sections, we found kidney-specific IgG autoantibodies to be expressed consistently by GC B cells of FcγRIIB$^{-/-}$ mice (12%) and at lower frequency by spleen (2%) and bone marrow plasma cells (3%; Fig. 6). The antibodies recognized different kidney structures including the glomeruli. Sequence analysis showed that some of the kidney-specific antibodies in one of the FcγRIIB$^{-/-}$ animals belonged to an expanded clone comprising six IgG2c spleen and bone marrow plasma cell antibodies with varying levels of somatic mutations (Fig. S4 and Table S2). Kidney-reactive GC and plasma cell antibodies

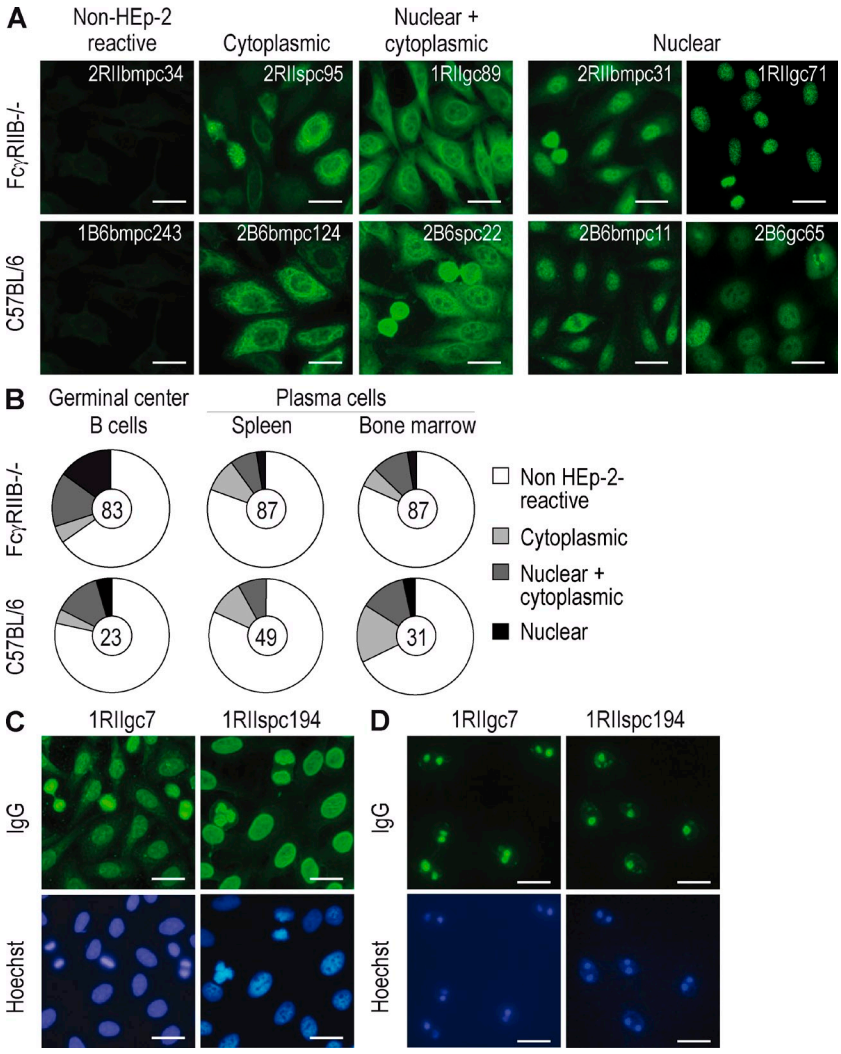

Figure 5. ANAs are enriched in GC B cells of FcγRIIB$^{-/-}$ mice. (A) Antibodies cloned from IgG$^+$ GC B cells and spleen and bone marrow plasma cells of FcγRIIB$^{-/-}$ (1RII–5RII) and wild-type (1B6–3B6) mice were tested for self-reactivity with HEp-2 cells by IFA. Examples of typical nonreactive antibodies, anti-cytoplasmic, anti-nuclear + cytoplasmic, and anti-nuclear HEp-2 cell staining patterns at 40× magnification are shown. Staining patterns of reactive antibodies were confirmed in at least two independent experiments. Bars, 25 µm. (B) Pie charts summarize the frequency of non–HEp-2 self-reactive (white) GC B cells and spleen and bone marrow plasma cell antibodies of wild-type and FcγRIIB$^{-/-}$ mice and of antibodies with nuclear (black), cytoplasmic (light gray), and nuclear + cytoplasmic (dark gray) HEp-2 cell staining patterns. The numbers of antibodies analyzed are indicated in the pie chart centers. (C) Strong homogenous nuclear HEp-2 cell staining patterns of IgG$^+$ FcγRIIB$^{-/-}$ GC B cell antibody 1RIIgc7 and spleen plasma cell antibody 1RIIspc194. Nuclei are visualized by Hoechst staining. Bars, 25 µm. (D) *Crithidia luciliae* IFA staining pattern at 100× magnification of the same antibodies as in C. Nuclei and kinetoplasts are shown by Hoechst staining. Bars, 10 µm. Data shown are representative of at least two independent experiments.

were rare in healthy mice. Immunization of wild-type mice did not increase the frequency of kidney-specific GC B cells ($P = 0.002$ vs. FcγRIIB$^{-/-}$; unpublished data). In contrast, stomach-specific autoantibodies and antibodies reactive with autoantigens in both tissues were found at similar frequency in GC B cells and plasma cells of FcγRIIB$^{-/-}$ and wild-type mice (Fig. 6 B and Tables S2 and S3). We conclude that kidney-specific autoantibodies are rare under normal circumstances but are enriched in the GC B cell and plasma cell compartment in autoimmune FcγRIIB$^{-/-}$ mice.

Somatic mutations in autoantibodies from FcγRIIB$^{-/-}$ mice

T cell help is required for the development of IgG serum autoantibodies and disease symptoms in FcγRIIB$^{-/-}$ mice (Fig. 7 A). To determine the role of somatic mutations in autoreactivity, we cloned and expressed the unmutated IgH and IgL V(D)J germline (GL) versions of six FcγRIIB$^{-/-}$-derived GC B cell and plasma cell autoantibodies (Fig. 7, B–E; and Table S5). Three of the antibodies were specific for unknown kidney (1RIIgc58 and 1RIIbmpc19) and stomach (2RIIspc95) autoantigens and two antibodies recognized dsDNA (1RIIgc7) and nucleosomes (1RIIspc194), respectively. The sixth antibody (lnpc203) was an IgG rheumatoid factor antibody from a FcγRIIB$^{-/-}$ lymph node plasma cell with high specificity to IgG2c as measured by ELISA and surface plasmon resonance (Fig. 7 C and not depicted). IgG rheumatoid factor antibodies were also present in the serum of two FcγRIIB$^{-/-}$ mice analyzed in this study. Five out of the six autoantibodies tested showed partial or complete loss of specificity and/or reactivity in the absence of somatic mutations (Fig. 7, B–E). For example, kidney reactivity of GL 1RIIgc58 was no longer limited to the glomeruli and, in contrast to their mutated counterparts, GL 2RIIspc95 and GL 1RIIbmpc19 lacked reactivity to defined stomach and kidney structures (Fig. 7 B). Loss of reactivity was also observed for the GL form of the anti-nucleosome antibody 1RIIspc194, and antibody lnpc203 lacked IgG2c rheumatoid factor reactivity in the absence of somatic mutations (Fig. 7, C and D). Only antibody 1RIIgc7 showed no measurable changes in ELISA reactivity to dsDNA in the absence of somatic mutations (Fig. 7 E). However, dsDNA reactivity was dependent on two non-GL Ig gene encoded arginine residues at the V-D and D-J junction in IgH CDR3 (Table S5). dsDNA reactivity of GL 1RIIgc7 was abrogated if the two arginines, which may be the product of somatic mutations, were replaced by serine residues (Fig. 7 E; GL 1RIIgc7S). In summary, we conclude that the GC B cells and plasma cells expressing highly reactive T cell–dependent IgG autoantibodies in FcγRIIB$^{-/-}$ mice can develop from non-self–reactive or low affinity self-reactive precursors by somatic mutation in GCs.

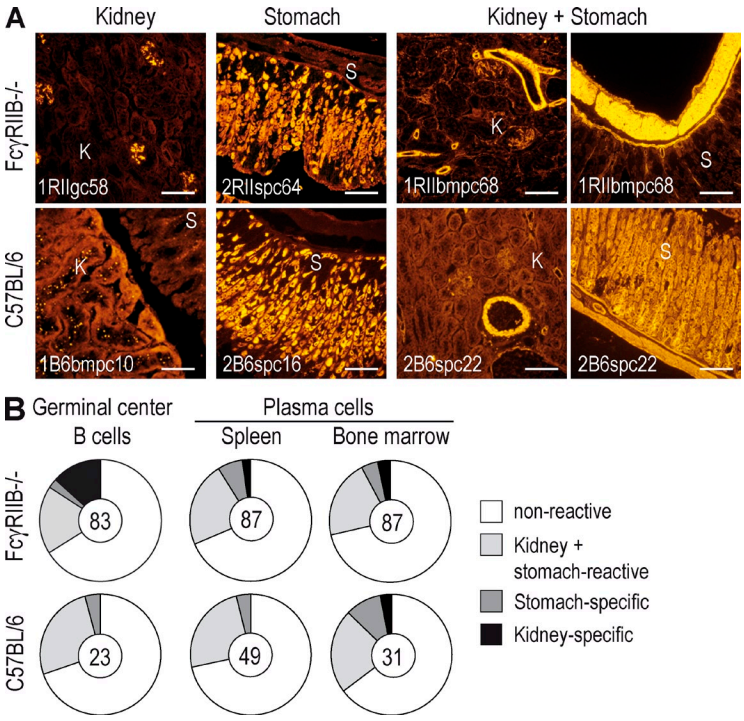

Figure 6. Kidney-reactive autoantibodies are enriched in GC B cells of FcγRIIB$^{-/-}$ mice. (A) Antibodies cloned from IgG$^+$ GC B cells and spleen and bone marrow plasma cells of FcγRIIB$^{-/-}$ (1RII–5RII) and wild-type (1B6–3B6) mice were tested for self-reactivity with mouse kidney (K) and stomach (S) tissue sections by IFA. Examples of typical staining patterns at 20× magnification from antibodies with specific kidney or stomach reactivity or with kidney plus stomach reactivity are shown. Data shown are representative of at least two independent experiments. Bars, 100 µm. (B) Pie charts summarize the frequency of IgG$^+$ GC B cells and spleen and bone marrow plasma cell antibodies from FcγRIIB$^{-/-}$ and wild-type mice lacking tissue reactivity (white) or showing kidney + stomach (light gray), stomach (dark gray), and kidney (black) tissue staining patterns. The numbers of antibodies analyzed are indicated in the pie chart centers.

DISCUSSION

Previous studies analyzed Ig gene-targeted mice carrying autoreactive antigen receptors to determine how FcγRIIB suppresses the activation of autoreactive B cells (Paul et al., 2007; Rahman et al., 2007a). However, presumably as a result of differences in the level of self-tolerance in naive B cells in the respective models, the results failed to demonstrate a clear role for FcγRIIB in regulating autoreactive GC B cells as opposed to the regulation of extrafollicular antibody-forming cells. Although diseased FcγRIIB-deficient mice with an unrestricted antibody repertoire showed no general defect in Ig gene recombination or early steps in B cell selection in our single cell analysis, GC B cells were significantly enriched for ANAs with positively charged IgH CDR3 and polyreactive and kidney-reactive autoantibodies. Thus, FcγRIIB is essential to suppress the emergence of autoreactive GC B cells.

GC B cells with positively charged IgH CDR3 were associated with high anti-DNA autoantibody levels and were not significantly increased in the absence of overt disease symptoms as measured by high serum anti-DNA IgG levels and splenomegaly, suggesting that such B cells accumulate over time and are associated with the onset of lupus symptoms. However, non-chromatin–reactive ANAs lacking positively charged IgH CDR3s and kidney- and polyreactive GC antibodies were also observed at high frequency in the absence of high serum anti-DNA IgG levels, suggesting that anti-DNA–reactive GC B cells accumulate over time. Indeed, progressive loss of self-tolerance with age, as measured by loss of follicular exclusion, increased plasma cell frequencies, and accumulation of IgG serum autoantibodies, has been observed in FcγRIIB$^{-/-}$ mice carrying a 3H9 anti-DNA IgH chain knockin allele (Paul et al., 2007).

Previous studies have also reported IgH CDR3 abnormalities in anti-dsDNA antibodies from lupus-prone mice, including D-D fusions and hydrophobic DH RF usage (Radic et al., 1989). However, atypical V-D-J rearrangements are not a general feature of ANAs, and FcγRIIB-deficient mice on a C57BL/6 background showed low hydrophobic RF usage and lacked D-D fusions. Thus, they may not be prone for the generation or selection of self-reactive B cells with atypical V-D-J rearrangements (Shefner et al., 1991; Ash-Lerner et al., 1997; Mueller et al., 1997; Klonowski et al., 1999).

A role for somatic mutations in the generation of autoantibodies, including polyreactive and anti-dsDNA antibodies, has been described previously in mice and humans (Diamond and Scharff, 1984; Shlomchik et al., 1987, 1990; van Es et al., 1991; Wellmann et al., 2005; Tiller et al., 2007; Mietzner et al., 2008). However, as a result of the use of stable cell lines, the origin of the cells expressing the autoantibodies was not known and studies in humans were limited to the memory B cell compartment. Thus, the question of whether the autoreactive B cells originated from GC or extrafollicular responses in vivo has not been answered. Our antibody cloning strategy now demonstrates that IgG autoantibodies, including ANAs and kidney-specific autoantibodies, are expressed by GC B cells in FcγRIIB$^{-/-}$ mice and that somatic mutations contribute to the generation of high-affinity IgG autoantibodies, which can develop from non-self–reactive and self-reactive precursors. Indeed, replacement of non-GL–encoded arginine residues abrogated dsDNA reactivity of antibody 1RIIgc7. As a result of terminal deoxynucleotidyl transferase–induced nontemplate nucleotides in CDR3, reversion experiments of Ig gene segment somatic mutations can never provide definite answers on the reactivity of the naive precursor B cell. Thus, our experiments cannot preclude that abnormal selection of self-reactive precursors contributes to the increased frequency of poly- and self-reactive GC B cells in FcγRIIB$^{-/-}$ mice. However, somatic mutations play

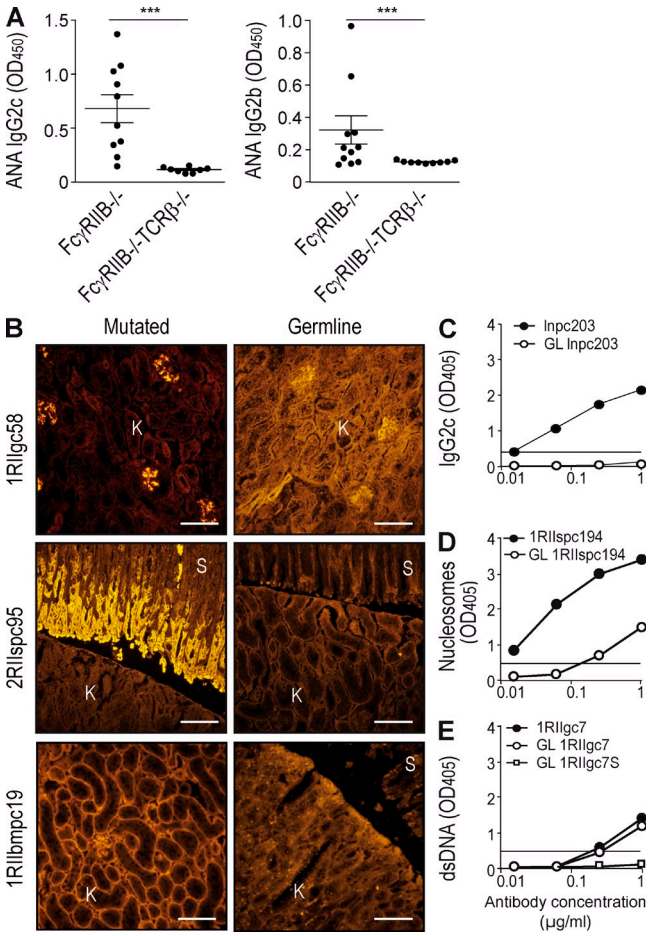

Figure 7. Somatic mutations generate specific high-affinity T cell–dependent autoantibodies in FcγRIIB$^{-/-}$ mice. (A) ANA-ELISA–reactive IgG2b and IgG2c autoantibodies in diluted serum (1:100) from FcγRIIB$^{-/-}$ and FcγRIIB$^{-/-}$TCR-β$^{-/-}$ mice. Horizontal lines indicate mean OD_{405} values. Error bars indicate standard deviation. (B) Indirect immunofluorescence staining patterns of mutated kidney (K) and stomach (S) tissue-reactive antibodies and of their unmutated GL forms cloned from FcγRIIB$^{-/-}$ mice are shown (20× magnification). Bars, 100 µm. (C) Anti-IgG2c ELISA for the mutated IgG2c-specific rheumatoid factor antibody Inpc203 (closed circles) and its unmutated GL form (open circles). (D) Anti-nucleosome ELISA for the mutated (filled circles) and GL (open circles) form of the nucleosome-specific antibody 1RIIspc194. (E) Anti-dsDNA ELISA of the mutated (closed circles) and GL (open circles) form of the anti-dsDNA 1RIIgc7. dsDNA reactivity of unmutated 1RIIgc7 is abrogated if two IgH CDR3 arginine residues are replaced by serines (GL 1RII-gc7S, squares). Horizontal lines in C–E show cutoff OD_{405} values for reactive (top) and nonreactive (bottom) antibodies. Graphs are representative of at least two independent experiments.

a major role in the development of IgG autoantibodies in terminal deoxynucleotidyl transferase–deficient lupus mice (Guo et al., 2010). Lack of negative signaling by FcγRIIB may specifically favor the accumulation of T cell–dependent chromatin-reactive GC B cells and, ultimately, the development of lupus disease as a result of the strong BCR cross-linking capacity of abundant DNA- and RNA-containing self-antigens, which promote activation of autoreactive B cells via toll-like receptors (Leadbetter et al., 2002; Lau et al., 2005; Avalos et al., 2010).

Surprisingly, the frequency of polyreactive and autoreactive B cells was lower in the plasma cell compartments than in GCs of FcγRIIB$^{-/-}$ mice, suggesting that autoreactive GC B cells are still partly regulated based on their antibody reactivity independently of FcγRIIB and before differentiation into plasma cells. Slow differentiation rates of GC B cells into plasma cells may prevent the frequent detection of clonally expanded B cells in the GC and in the plasma cell compartment at the same time (Takahashi et al., 1998). However, little overlap of clonally related cells between the GC B cell and plasma cell repertoires may also reflect the fact that high antibody affinity is a prerequisite for the differentiation of GC B cells into plasma cells (Shih et al., 2002; Phan et al., 2006; Tarlinton et al., 2008). Therefore, in analogy to humoral immune responses to foreign antigens, only GC B cells expressing high-affinity autoantibodies may develop into plasma cells and contribute to serum autoantibody production, which may be a rare event given the high frequencies of polyreactive and self-reactive GC B cells in the absence of FcγRIIB. Competition for antigen presented in the form of immune complexes on follicular dendritic cells has been considered a bottleneck for selection of antigen-specific GC B cells. FcγRIIB is expressed on follicular dendritic cells, but the B cell–intrinsic importance of FcγRIIB for the development of autoimmunity has been demonstrated by bone marrow transfer and cell-specific overexpression experiments (Bolland and Ravetch, 2000; McGaha et al., 2005; Brownlie et al., 2008). Furthermore, recent evidence suggests that selection of GC B cells is limited predominantly by competition for access to T follicular helper cells, which play an important role in preventing the differentiation of autoreactive GC B cells into effector B cells (Haberman and Shlomchik, 2003; Allen et al., 2007).

Spleen and bone marrow harbor niches for long-term plasma cell survival, and clonally related cells were frequently found in both organs (Manz et al., 1997; Slifka et al., 1998). However, spleen plasma cells had, on average, lower mutation numbers and were enriched for polyreactivity, suggesting that the spleen harbors a pool of plasma cells, which are excluded from the bone marrow under physiological circumstances. Marginal zone B cell and B1 cell–derived plasma cells expressing natural polyreactive and low level self-reactive antigen receptors may contribute to the relative increase in polyreactive spleen plasma cells not observed in bone marrow (Martin and Kearney, 2000). However, the development of IgG autoantibodies and lupus disease in FcγRIIB$^{-/-}$ mice strictly depends on T cell help, suggesting that T cell–independent MZ and B1 cell–derived plasma cells do not contribute to pathogenicity.

In summary, our analysis allowed, for the first time, the dissection of the GC and plasma cell antibody response under physiological circumstances on a cellular level. The data demonstrate an important role for FcγRIIB in the regulation of autoreactive IgG^{+} GC B cells, which develop from non-self–reactive or low-level self-reactive precursors by somatic

mutations. FcγRIIB has been shown to control plasma cell homeostasis (Xiang et al., 2007). However, the development of high-affinity autoreactive IgG^+ plasma cells in $Fc\gamma RIIB^{-/-}$ mice is a relatively rare event given the high numbers of autoreactive IgG^+ GC B cells, and we did not observe any signs for the preferential clonal expansion of chromatin-reactive B cells. The findings suggest the existence of an FcγRIIB-independent checkpoint for autoreactivity before development of GC B cells into spleen or bone marrow plasma cells, which has been predicted previously by observations made in Ig gene transgenic mice (Erikson et al., 1991; Culton et al., 2006). Accumulation of T cell–dependent high-affinity autoreactive IgG^+ GC B cells over time and limited differentiation of such cells into plasma cells may explain the late onset of autoantibody development and lupus disease in the absence of FcγRIIB.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from Charles River Laboratories. FcγRIIB-deficient C57BL/6 mice (B6.129S4-*Fcgr2b*^{tm1Ttk}) were supplied by J. Ravetch (The Rockefeller University, New York; NY; Takai et al., 1996; Bolland and Ravetch, 2000). TCR-β–deficient mice (B6.129P2-*Tcrb*$^{tm1Mom/J}$; The Jackson Laboratory) were bred to FcγRIIB-deficient C57BL/6 mice (B6.129S4-*Fcgr2b*^{tm1Ttk}) to obtain $Fc\gamma RIIB^{-/-}$TCR-$\beta^{-/-}$ mice. All mice were maintained under specific pathogen-free conditions. Animal experiments were approved by the Landesamt für Gesundheit und Soziales, Berlin.

Flow cytometry. For FACS analyses, the following anti–mouse antibodies were used according to standard protocols: anti–CD138-PE (clone N418), anti–Fas-PE (clone Jo2), anti–GL7-FITC (clone GL7), anti–IgD-biotin (clone 11.26c), anti–B220–peridinin chlorophyll protein complex (clone RA3-6B2), anti–CD21-FITC (clone 7G6), anti–CD23-biotin (clone B3/B4), anti–IgM–cyanine 5 (clone M41), and streptavidin-PE (all obtained from BD or in house-preparations).

Single B cell sorting and Ig gene cloning. Single cell sorting, RT-PCR, and Ig gene cloning were performed as previously described (Tiller et al., 2009). In brief, single $CD21^{dull}CD23^+IgM^+B220^+$ mature naive B cells, $FAS^+GL7^+IgD^-$ GC splenocytes, and $CD138^{high}$ plasma cells from spleen and bone marrow of the indicated mice were isolated into 96-well PCR plates using a FACSVantage cell sorter with DiVa option (BD). GC B cells from immunized C57BL/6 mice ($n = 3$) were isolated from pooled draining lymph nodes at days 10, 14, and 21, respectively, after subcutaneous immunization with a mixture of 100 μg OVA in 50 μl PBS (EMD) and 50 μl CFA (Sigma-Aldrich). Cells were lysed and cDNA was prepared as described. IgH chain and Igκ light chain gene transcripts were amplified by seminested PCR for IgM (naive mature B cells) or IgG (GC B cells and plasma cells) heavy chain and by nested PCR for Igκ, respectively (Tiller et al., 2009). PCR products were sequenced.

Ig gene sequence analysis. Ig gene nucleotide sequences (Tables S2–S4, accession numbers FR688150–FR689535) are available from the European Molecular Biology Laboratory Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/). Ig gene sequence analysis was performed by IgBlast (http://www.ncbi.nlm.nih.gov/igblast/) to identify GL V(D)J genes with highest homology. IgH CDR3 was defined as the sequence between the conserved VH gene encoded cysteine at Kabat position 92 (International ImMunoGeneTics 104) and the tryptophan at Kabat position 103 (International ImMunoGeneTics 118) encoded by the JH gene (Ivanov et al., 2005). Ig DH gene RF was annotated as defined by Ichihara et al. (1989). Numbers of positively charged amino acids in IgH CDR3 summarize the number of arginines, lysines, and histidines. Somatic mutations were counted in V genes from CDR1 to framework region 3, inclusively, as defined by the Ig Blast. Isotype subclasses were determined by comparison to the annotated sequences on the International ImMunoGeneTics homepage (http://imgt.cines.fr) or to the genomic C57BL/6 reference sequence of the National Center for Biotechnology Information m37 assembly (accessed via http://www.ensembl.org).

Antibody production, ELISA, and IFA. Ig gene cloning and antibody expression was performed as previously described (Tiller et al., 2009). In brief, mouse variable region genes were cloned into expression vectors encoding the human constant Igγ1 and Igκ regions, respectively, to generate chimeric recombinant antibodies (Tiller et al., 2009). Recombinant monoclonal antibodies were expressed in human embryonic kidney HEK293T cells and supernatants of known antibody concentrations were tested for polyreactivity with dsDNA, insulin, and LPS as previously described (Wardemann et al., 2003; Tiller et al., 2008). Commercially available nucleosome, dsDNA, and histone diagnostic ELISAs (all from Orgentec) were performed under blocking conditions with 2% BSA. Nucleosome ELISAs were internally controlled using commercially available anti-nucleosome IgG-positive human control serum (Orgentec). Protein G purified antibodies were tested for self-reactivity with fixed HEp-2 cells, *Crithidia luciliae*, and fixed mouse stomach and kidney sections (all from Orgentec) by IFA at 100 μg/ml under moist conditions at room temperature. Polyclonal goat anti–human IgG–cyanine 3 (Jackson ImmunoResearch Laboratories) was used at a 1:500 dilution to detect bound recombinant antibodies. Mouse sera were tested at 1:100 dilutions for reactivity with HEp-2 cells by IFA and by ANA Detect ELISA (both Orgentec). Bound serum IgG antibodies were detected using individual or mixed FITC-labeled polyclonal goat anti–mouse IgG1, IgG2b, IgG2c, and IgG3 antibodies (all from Bethyl Laboratories, Inc.). 1 μg/ml Hoechst 33343 (Invitrogen) was used to counterstain cell nuclei. Serum IgG reactivity with dsDNA, nucleosomes, and IgG antibodies (rheumatoid factor reactivity) was tested by ELISA at 1:100 mouse serum dilutions (all from Orgentec). Bound serum IgG antibodies were detected using horseradish peroxidase–labeled polyclonal goat anti–mouse IgG2b and IgG2c antibodies (all from Bethyl Laboratories, Inc.). Slides were mounted with Fluoromount G (SouthernBiotech) and examined on a fluorescence microscope (Axioplan 2; Carl Zeiss, Inc.). Control stainings with PBS and control sera were performed as suggested by the manufacturer and were included in all experiments. All images were acquired at equal exposure times.

Ig gene reversion of somatic mutations. Somatically mutated Ig genes were reverted into their unmutated GL counterparts using an overlap PCR strategy as previously described (Tiller et al., 2008). In brief, GL V gene transcripts were amplified from previously cloned unmutated V genes amplified from naive B cells. Mutated CDR3-J sequences were reverted independently by PCR using forward primers with a minimum of 10 nt complementary to the GL V gene PCR product in combination with J gene–specific reverse primers. PCRs were performed at 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s for 30 cycles. Equal ratios of the reverted V and CDR3-J gene PCR products were fused under the same conditions in a third 20-cycle overlap PCR with V gene and J gene-specific primers containing restriction sites, which allow direct expression vector cloning. The successful reversion of somatic mutations was confirmed by sequence analysis of the cloned products.

Statistics. P-values for Ig gene repertoire analyses, analysis of positive charges in IgH CDR3, and antibody reactivity were calculated by 2×2 or 2×5 Fisher's Exact test or χ^2 test. P-values for IgH CDR3 aa length were calculated by Student's *t* test. P-values for V gene somatic mutation numbers were calculated by Mann-Whitney Wilcoxon test.

Online supplemental material. Fig. S1 shows representative FACS plots of mature naive B cells, GC B cells, and spleen and bone marrow plasma cells of $Fc\gamma RIIB^{-/-}$ and wild-type mice, $Fc\gamma RIIB^{-/-}$ spleen IFA for GL7/IgM/CD4, MOMA-1/IgG2b/c, and GL7/IgG2b/c, and representative nuclear HEp-2 cell IFA staining patterns of IgG serum antibodies from $Fc\gamma RIIB^{-/-}$ and wild-type mice. Figs. S2 and S3 provide detailed information on the

IgH and Igκ gene repertoire, respectively, in the individual B cell subpopulations (mature naive B cells, GC B cells, spleen plasma cells, and bone marrow plasma cells) in FcγRIIB$^{-/-}$ and wild-type C57BL/6 mice. Fig. S4 shows clonal relationships for kidney-reactive spleen and bone marrow plasma cells from one FcγRIIB$^{-/-}$ mouse. Table S1 provides information on the serology and other features from the respective FcγRIIB$^{-/-}$ and wild-type mice analyzed here. Tables S2 and S3 provide Ig gene repertoire and antibody reactivity information of all antibodies from FcγRIIB$^{-/-}$ with high anti-DNA serum IgG autoantibodies and wild-type mice. Table S4 provides Ig gene repertoire and antibody reactivity information of GC B cells from FcγRIIB$^{-/-}$ with low or negative anti-DNA serum IgG autoantibodies. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100171/DC1.

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