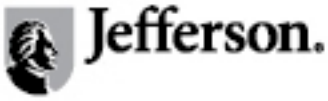

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Expression of the autoimmune Fcgr2b NZW allele fails to be upregulated in germinal center B cells and is associated with increased IgG production

S.M. Ziaur Rahman
Thomas Jefferson University, zrahman@mail.jci.tju.edu

H. Niu
University of Florida

D. Perry
University of Florida

Timothy L. Manser
Thomas Jefferson University

L. Morel
University of Florida

Expression of the autoimmune Fcgr2b NZW allele fails to be upregulated in germinal center B cells and is associated with increased IgG production

ZSM Rahman[1], H Niu[2], D Perry[2], E Wakeland[3], T Manser[1] and L Morel[2]

[1]Department of Microbiology and Immunology, The Kimmel Cancer Center, Jefferson Medical College, Philadelphia, PA, USA
[2]Department of Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, FL, USA
[3]Department of Immunology, University of Texas Southwestern Medical Center, Dallas, TX, USA

Corresponding author:
Dr. L. Morel
Department of Pathology, Immunology and Laboratory Medicine
University of Florida
1600 Archer Road
Gainesville, FL 32610-0275
Email: morel@ufl.edu

Abstract

The inhibitory receptor FcγRIIb regulates B-cell functions. Genetic studies have associated Fcgr2b polymorphisms and lupus susceptibility in both humans and murine models, in which B cells express reduced FcγRIIb levels. Furthermore, FcγRIIb absence results in lupus on the appropriate genetic background, and lentiviral-mediated FcγRIIb overexpression prevents disease in the NZM2410 lupus mouse. The NZM2410/NZW allele Fcgr2b is, however, located in-between Sle1a and Sle1b, two potent susceptibility loci, making it difficult to evaluate $Fcr2b^{NZW}$ independent contribution. By using two congenic strains that each carries only Sle1a (B6.Sle1a(15–353)), or $Fcr2b^{NZW}$ in the absence of Sle1a or Sle1b (B6.Sle1(111–148)), we show that the $Fcr2b^{NZW}$ allele does not upregulate its expression on germinal center B cells and plasma cells, as does the C57BL/6 allele on B6.Sle1a(15–353) B cells. Furthermore, in the absence of the flanking Sle1a and Sle1b, $Fcr2b^{NZW}$ does not produce an autoimmune phenotype, but is associated with an increased number of class-switched plasma cells. These results show that while a lower level of FcγRIIb does not by itself induce the development of autoreactive B cells, it has the potential to amplify the contribution of autoreactive B cells induced by other lupus-susceptibility loci by enhancing the production of class-switched plasma cells.

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Introduction

Both forward and reverse genetic approaches in lupus patients and in murine models have shown a high level of complexity in the mechanisms by which genetic variation contributes to lupus susceptibility.[1] A number of linkage and association studies have associated the Fcγ receptors (FcgRs) with lupus susceptibility in both humans and mice. Among the FcγRs, FcγRIIb plays a unique role in inducing inhibitory signals in B cells upon immune complex binding.[2] In humans, a -343 G/C promoter polymorphism and a Ile232Thr trans-membrane polymorphism, both associated with loss of function, have been associated with lupus.[3] The strength of the association between each of these polymorphisms and disease varied among populations, most likely due to interactions with other susceptibility alleles segregating differently among these populations. Functionally, two recent studies have reported that lupus patients present a defective upregulation of FcγRIIb expression in selected late B-cell developmental stages, namely, memory B cells and plasma cells (PC).[4,5] In the mouse, FcγRIIb is required for the maintenance of systemic tolerance on a C57BL/6 (B6) background,[6] and a B-cell receptor l transgenic model has suggested that it constitutes a checkpoint at the post-germinal center (GC)/plasma cell stage.[7] Several lupus-prone strains show reduced FcγRIIb levels attributed to a promoter region polymorphism for NZB, BXSB and MRL.[8,9] However, using B6.Sle1/NZW and NZW mice, we have recently shown that failed upregulation of FcγRIIb on GC B cells is not associated with the promoter region polymorphism, but rather with additional polymorphisms in the putative regulatory regions 3 and 4 in the third intron shared by both NZB and NZW alleles.[10] A direct demonstration of the involvement of FcγRIIb in lupus pathogenesis was dramatically demonstrated in both the NZM2410 and BXSB models, in which full disease abrogation was achieved by partial restoration of FcγRIIb expression on hematopoeitic cells.[11]

The B6.Sle1 congenic interval corresponds to the 37 cM long telomeric end of chromosome 1 derived from the NZM2410/NZW genome.[12] The location within this interval of the Fcgr2b gene makes this the primary observed on B6.Sle1 GC B cells.[10] However, it is not clear whether the polymorphisms in the FcγRIIb NZW allele alone are responsible for the perturbed expression of FcγRIIb on GC B cells in B6.Sle1 mice or whether other susceptibility genes located within the Sle1 interval might act epistatically in producing this altered expression. In addition, Fcgr2b is located between Sle1a and Sle1b, and we have reported that a congenic strain B6.Sle1(111–148) that contains Fcgr2b but lacks both Sle1a and Sle1b does not produce autoantibodies.[13] The B6.Sle1(111–148) strain contains in addition to $Fcgr2b^{NZW}$ a number of genes of NZW origin, including two FcR homolog genes Fcrla and FcRlb. It however allows the evaluation of $Fcgr2b^{NZW}$ independently from Sle1a and Sle1b. In the present study, we report that $Fcgr2b^{NZW}$ fails to upregulate

on GC B cells independently from Sle1a and Sle1b, and that it is associated with a greater number of class-switched PCs in the spleen and bone marrow (BM).

Results

Perturbed upregulation of FcγRIIb expression on GC B cells in B6.Sle1 mice

As previously described,[10] we have established an operative definition of GC B-cell phenotype for flow cytometry using peptide nucleic acid (PNA), in combination with anti-B220 and anti-IgD. Based on B220 and PNA staining, we first separated $B220^{hi}$ mature B cells consisting of both PNA þ and PNA^{neg} (rectangular gate, Figure 1a, left panel) populations from total lymphocytes obtained from sheep red blood cells (SRBC)-immunized C57BL/6 (B6) mice on day 9 of the immune response. We then defined $B220^{hi}$ PNA^{neg} IgD þ and $B220^{hi}$ PNA þ IgD^{neg} cells as non-GC and GC B cells, respectively (rectangular gates, Figure 1a, right panel).

Using these definitions, we first measured the levels of FcγRIIb on splenic B-cell subpopulations, by performing four-color flow cytometry analysis from B6 and B6.Sle1 mice on day 9 of the anti-SRBC response. Fcgr2b-deficient ($B6.RIIB^{-/-}$) mice were used as negative controls. The mAb 2.4G2 that recognizes the extracellular domains of both FcγRII and III was used since no mAb specific for the NZM2410/NZW/Sle1 FcγRIIb allele (Ly17.1 allotype) is available. As shown in Figure 1b, compared with $B220^{hi}$ PNA^{neg} IgD^{+} non-GC B cells (red line in histograms), the levels of 2.4G2 staining were elevated on $B220^{hi}$ PNAþ IgD^{neg} GC B cells (blue line in histograms) in B6 mice (Figure 1b, lower left panel) while background levels of staining on both populations of B cells were observed in $B6.RIIB^{-/-}$ mice (Figure 1b, right most panel). As previously described,[10] and illustrated in Figure 1b, in contrast to splenic GC B cells in B6 mice (lower left panel), we found little or no elevation of 2.4G2 staining levels on B6.Sle1 splenic GC B cells (blue line in histograms for $B220^{hi}$ PNAþ IgD^{neg} cells, lower middle panel) as compared with non-GC B cells (red line in histograms, lower middle panel).

We also investigated whether the altered expression of FcγRIIb we observed by flow cytometry on B6.Sle1 GC B cells was apparent on GC B cells and follicular dendritic cells (FDCs) were defined histologically. Parallel spleen sections obtained on day 9 post-SRBC immunization of B6, B6.Sle1 and $B6.RIIB^{-/-}$ mice were stained with 2.4G2 and anti-IgD (Figure 1c, left panels) and FDC-M1 and GL7 (Figure 1c, right panels) to evaluate the expression of FcγRIIb on GC B cells and FDCs, respectively. Although 2.4G2 staining in B6 and B6.Sle1 GC FDC-rich regions was comparable, this staining was undetectable in B6.Sle1 GC areas deficient in FDCs. The background level of staining in both FDC-rich and FDC-poor regions of GCs in $B6.RIIB^{-/-}$ mice indicated that FcγRIII is not expressed at levels detectable with 2.4G2 on these cells. Together, these results confirmed our recently reported data showing

a defect in the upregulation of FcγRIIb expression on GC B cells in autoimmune-prone B6.Sle1 mice.[10]

The Fcgr2b allele in B6.Sle1(111–148) is of NZW origin

We have already excluded Fcgr2b from the Sle1a and Sle1b intervals,[13] but both these loci could interact with the $Fcgr2b^{NZW}$ allele and contribute to the failed FcγRIIb upregulation in Sle1 GC B cells. To test this hypothesis, we genotyped the two overlapping strains represented in Figure 2a: B6.Sle1a(15–353), previously associated with autoreactive T cells[14] and B6.Sle1(111–148), previously described as not producing autoantibodies.[13] A third intron deletion (Figure 2b) specific to the NZW allele[10] determined that the Sle1a(15–353) interval contained the $Fcgr2b^{B6}$ allele, mapping the telomeric recombination breakpoint between D1MIT270 (included) and Fcgr2b (excluded). In contrast, the Sle1(111–148) interval contained the $Fcgr2b^{NZW}$ allele, placing the telomeric recombination breakpoint between the Fcgr2b third intron (included) and D1MIT146 (excluded). We have not determined yet the exact location of the recombination breakpoint, and whether it is telomeric to the Fcgr2b promoter (Figure 2a, inset). The two strains also differed by their centromeric region centered on the D1MIT15 marker, where Sle1a was mapped.[13]

The NZW origin of Fcgr2b in the B6.Sle1(111–148) strain was confirmed at the protein level. The mAb K9.361, which is specific for the FcγRIIb Ly17.2 allotype, was used to measure the levels of FcγRIIb on B-cell subpopulations. Four-color flow cytometry analysis of splenocytes obtained on day 9 of the anti-SRBC response revealed background levels of K9.361 staining on B6.Sle1(111–148) B-cell subpopulations as compared to the strong staining of B6 control and B6.Sle1a(15–353) GC (blue line in histograms) and non-GC (red line in histograms) B cells (Figure 3a). In addition, the levels of K9.361 staining on both B6 and B6.Sle1a(15–353) GC B cells were at least fivefold higher compared with their non-GC counterparts. We also performed histological analysis (Figure 3b) of spleen sections obtained on day 9 of the anti-SRBC response by staining with anti-IgD (red) and K9.361 (green). While elevated levels of K9.361 staining were seen on IgD^{neg} B cells in B6 and B6.Sle1a(15–353) GCs, this staining was undetectable in B6.Sle1(111–148) GCs, further supporting the conclusion that the Fcgr2b allele in B6.Sle1(111–148) mice is not of B6 but of NZW/NZM2410 origin.

Failed upregulation of FcγRIIb on B6.Sle1(111–148) GC B cells

Using the protocol described above, we evaluated the levels of FcγRIIb on GC and non-GC B-cell populations in splenocytes obtained on day 9 of the anti-SRBC responses of B6, B6.Sle1a(15–353) and B6.Sle1(111–148) mice (Figure 4a). In contrast to B6 GC B cells (blue line in histograms, lower left panel), little or no elevation of 2.4G2 staining levels was observed on B6.Sle1(111–148) GC B cells (blue line in histograms, lower right panel). Interestingly, however, we found levels of 2.4G2 staining on B6.Sle1a(15–353) GC B cells at least eightfold higher than that on

non-GC B cells (Figure 4a, lower middle panel), analogous to that observed on B6 splenic GCs.

To corroborate these findings, we next performed histological analysis as described above in Figure 1c. As shown in Figure 4b, two sets of parallel spleen sections from each genotype were stained. Although 2.4G2 staining in the FDC-rich regions in GCs of all three strains was comparable, elevated levels of 2.4G2 staining was observed in the FDC-poor regions of B6 and B6.Sle1(15–353) GCs as compared to substantially lower or undetectable levels of staining in these regions in B6.Sle1(111–148) GCs (shown in arrows, right most column), analogous to that observed in B6.Sle1 GCs (Figure 1c).

Staining of B6.RIIB$^{-/-}$ mice indicated that the mAb 2.4G2 predominantly detects FcγRIIb expression on GC B cells. Nonetheless, since this mAb recognizes the extracellular domains of both FcγRIIb and FcγRIII, a fraction of the 2.4G2 staining differences observed in B6.Sle1 or B6.Sle1(111–148) GCs could have resulted from altered expression of FcγRIII. Therefore, we sorted B220 þ PNA þ IgD^{neg} GC B cells obtained on day 9 of the SRBC response and measured Fcgr2b mRNA levels via real-time RT-PCR. Fcgr2b mRNA levels in B6.Sle1(111–148) GC B cells were threefold lower than those in B6 control and B6.Sle1(15–353) GC B cells (Figure 4c). In total, these data indicated a defect in the upregulation of FcγRIIb expression on B6.Sle1(111–148) GC B cells similar to that observed in the B6.Sle1 strain.

The Fcgr2b^{NZW} allele is associated with an increased IgG production

We screened a new cohort of 10-to 12-month-old B6.Sle1(111–148) mice and confirmed as previously reported that these mice do not produce anti-chromatin or anti-dsDNA antibodies (data not shown). To further determine the functional consequences of the expression of the Fcgr2b^{NZW} allele, we compared antibody levels in SRBC-immunized mice. There was no significant difference between the three strains in levels of either SRBC-specific IgM or total IgM (Figures 5a and d). In contrast, B6.Sle1(111–148) mice produced significantly higher levels of SRBC-specific IgG and total IgG (Figures 5b and e). This resulted in a significantly skewed IgG/IgM ratio for both the response to the T-dependent antigen and the total circulating immunoglobulin (Figures 5c and f).

We have recently demonstrated that Fcgr2b deficiency regulated the production of autoreactive primary anti-production body-forming cells (AFCs) by BCR transgenic B cells independent of GC events.[15] We, therefore, compared the effect of the Fcgr2b NZW and B6 alleles on AFC formation 6 and 14 days after SRBC immunization. At 6 days in the spleen and 14 days in the BM, FcγRIIb expression on AFCs mirrored that on GC B cells with a significantly reduced level associated with the NZW allele (Figure 6a). Surface IgM expression on both CD138+ B220+ plasmablasts and CD138+ B220$^{-/lo}$ PC was significantly lower in B6.Sle1(111–148) than in either B6 or B6.Sle1a(15–353) mice (data not shown). Consistent with the surface expression, the percentage of intra- cellular IgM+ was also significantly lower in both splenic and

BM PCs at 6 and 14 days post immunization (p.i.) (Figure 6b), while the percentage of intracellular IgG+ PCs was significantly higher in B6.Sle1(111–148) spleens at 6 days p.i. (Figure 6c). Finally, the percentage of total CD138+ B220$^{-/lo}$ PCs was significantly higher in B6.Sle1(111–148) spleens at 6 and 14 days p.i. and BM 6 days p.i. (data not shown). These results were confirmed by the presence of a larger number of IgG+ AFCs in the spleen (although it did not reach statistical significance) and the BM (Po0.03) at 6 days p.i. in B6.Sle1(111–148) mice (Figure 6d). The same type of analysis was performed on mice following secondary immunization, and we did not observe any specific difference in B6.Sle1(111–148) mice (data not shown). Together, these data suggest that Fcgr2b^{NZW} regulates the primary AFC pathway by increasing their number and by accelerating class switching.

Discussion

We have used NZM2410/NZW-derived congenic strains to analyze the phenotypes associated with the Fcgr2b^{NZW} allele independently from the flanking lupus susceptibility loci Sle1a and Sle1b. We have used a combination of genomic, transcriptional and histological methods to show that the 2–3 Mb congenic interval of B6.Sle1(111–148) contains the NZW allele of Fcgr2b, while the strain B6.Sle1a(15–353) that we have used previously to analyze Sle1a phenotypes contains the B6 allele of Fcgr2b.

As we have previously described for the NZW and B6.Sle1 strains,[10] B6.Sle1(111–148) B cells fail to upregulate FcγRIIb at the GC stage, indicating that this phenotype does not require the expression of the Sle1a and Sle1b loci. The B6.Sle1(111–148) interval contains 15 other transcripts of known genes (www.ensembl.org/Mus_musculus/ based on the NCBI m36 mouse assembly). At least 12 of these genes are shared with B6.Sle1a(15–353) and can, therefore, be excluded from impacting Fcrg2b expression. There are, however, two FcR homolog genes Fcrla (Freb2)[16] and Fcrlb (FcRY or Freb1)[17] that are contained in the B6.Sle1(111–148) interval, and potentially not in the B6.Sle1a(15–353) interval since they are situated in the region of recombination between the D1MIT270 and D1MIT147 markers. These two FcR family members are differentially expressed on B cells, including GC B cells, but their function is yet unknown.[18,19] Interestingly, FCRL3,a member of this family in humans, is associated with RA and other autoimmune conditions, including lupus.[20] We are currently investigating whether one or both of these genes present allelic differences between NZW and B6. If it were the case, it would be possible that these genes could indirectly regulate Fcrg2b on GC B cells. The most likely explanation, however, is that the failure to upregulate Fcgr2b expression on GC B cells is regulated in cis by the Fcgr2b NZW allele.

In spite of the low expression of FcγRIIb on GC B cells, the number and size of GCs were equivalent between B6.Sle1(111–148) and B6 mice. We have found, however, that B6.Sle1(111–148) mice produced significantly more anti-SRBC IgG and total IgG, which was correlated with a greater number of primary AFCs that downregulated surface and intracellular IgM in favor of IgG. We have confirmed

recent results showing FcγRIIb expression on PC,[21] but contrary to the other autoimmune strains NZB and MRL in which FcγRIIb is virtually absent on the PC surface, the NZW allele is associated with only a modest reduction of expression on PCs. Future experiments will have to be performed to assess whether this modest reduction is sufficient to decrease PC turnover through decreased apoptosis in Fcgr2b^{NZW}-carrying mice, as it has been shown for Fcgr2b-deficient mice.[21] Interestingly, the kinetics of FcγRIIb expression on PC as well as the absence of Fcgr2b^{NZW}-specific phenotype in secondary immune responses corroborates our previous findings[15] that this receptor regulates allele does not result by itself in the production of primary T-dependent immune responses.

Overall, our results indicate that Fcgr2b^{NZW} functions an Fcgr2b hypomorph in regulating AFC numbers and class switching independent of GC reactions. This concurs with previous findings that showed that an Fcgr2b deficiency allows the accumulation of autoreactive PC.[7,10] Interestingly, however, the Fcgr2b NZW allele does not result by itself in the production of autoreactive B cells, but acts as a modifier of the fate of autoreactive B cells induced by the expression of other as loci. It is not clear to what extent primary AFC foci contribute to systemic autoimmunity. However, at least in the MRL/lpr model, autoreactive B cells expand and hypermutate outside of GCs,[22] which suggests that primary AFCs constitute a key regulatory pathway in the maintenance of B-cell tolerance. We are in the process of testing whether a similar GC 'bypass' also occurs in the NZM2410 strain, and what role, if any, Fcgr2b plays in this context.

We have previously documented the highly interactive nature of the Sle1 loci,[23] and we have specifically shown that the interval including Sle1a, Sle1b and the intervening region resulted in autoimmune phenotypes that were much stronger than the simple additive effects of Sle1a plus Sle1b. We speculate that Fcgr2b^{NZW} contributes to these epistatic effects. In addition, we have previously shown that both NZM2410 and NZW mice present an accumulation of PC in the spleen.[24] Although we were not able to map the entire phenotype to any single Sle locus, it is also temping to speculate that reduced FcγRIIb expression may contribute to this phenotype, as suggested by our data in Figure 6. Interestingly, close examination of our results in Figures 5 and 6 suggests that Sle1a also impacts the immune response to SRBC immunization, including the number of class-switched PCs. Detailed studies of the interaction between Fcgr2b^{NZW} and the other Sle loci in the context of plasma cell development will be necessary to fully understand how the restoration of normal FcγRIIb expression on NZM2410 B cells was sufficient to abrogate disease.[11]

Materials and methods

Mice and immunization

The B6.NZM2410-Sle1 (B6.Sle1), B6.NZM2410-Sle1a(15– 353) (B6.Sle1a(15–353))

and B6.NZM2410-Sle1(111–148) (B6.Sle1(111–148)) mice have already been described.[12,13] These mice along with B6 controls were bred at the University of Florida Animal Care Center. B6;129S4Fcgr2b^{tm1Rav}/J (B6.RIIB$^{-/-}$) were kindly supplied by Dr Jeffrey Ravetch (The Rockefeller University, New York, NY, USA). All mice were maintained in a pathogen-free barrier facility and all procedures were approved by the JU or UF IACUCs. Cohorts of at least three mice per strain were immunized intra-peritoneally with 200 µl of SRBC diluted 10% v/v in sterile 0.9% saline solution. Spleens, BM and terminal sera were collected after either 6, 9, or 14 days, as indicated in the text. All mice were used between 8 and 20 weeks of age, except for the autoantibody screens, and cohorts of both males and females were used without significant differences.

Congenic strain genotyping

All available MIT microsatellite markers located within 170 and 173 Mb on chromosome 1 were screened for polymorphism between B6 and NZW and then used to assess the haplotype distribution across the B6.Sle1a(15– 353) and B6.Sle1(111–148) intervals. The NZW or B6 allelic origin of the Fcgr2b gene was determined with PCR primers designed to detect the 4921–4944 deletion in the third intron (region 4 described by Rahman and Manser[10]). The sequences were for the sense primer: 5′-TGCCCCCTCCTCTTATCC and 5′-TGCATGTATGTG CATGTGT for the antisense primer.

Antibodies and other reagents

Antibodies and other reagents used for flow cytometry and immunohistology included the following: PE, fluorescein isothiocyanate (FITC) and PE-Texas red-anti-B220 (clone RA3–6B2); PE-anti-FcγRII/FcγRIII (clone 2.4G2); PE-anti-IgD (clones 11–26); APC-anti-IgM (clone Igh6); biotin-anti-CD138 (clones 281–282), Streptavidin (SA)-CyChrome; rat IgG Ab to mouse FDCs (FDC-M1, BD Pharmingen, San Diego, CA, USA); biotin-anti-IgD (clones 11–26, Southern Biotechnology Associates, Birmingham, AL, USA); FITC-goat anti-mouse IgG (Southern Biotechnology); SA-PE; avidin-Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA); FITC-peanut lectin (agglutinin, PNA, Vector Laboratories, Burlingame, CA, USA); biotin-$(Fab')_2$ mouse anti-rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA). The K9.361 mAb was biotinylated in house from the hybridoma kindly provided by Dr U Hammerling (Sloan-Kettering Memorial Hospital, New York, NY, USA). Intracellular staining was performed with BD Pharmingen reagents as recommended by the manufacturer.

Immunohistology

Spleen cryostat sections (5–6 mm) were prepared as described.[25] Immunohistology was performed using the antibodies listed above and the stained sections were analyzed using a fluorescence microscopy (Leitz Diaplan, Wetzlar, Germany) and images were captured as described.[26]

Flow cytometry

Four-color flow cytometric analysis was performed on cell suspensions prepared from spleens of naive and immunized mice stained with multiple combinations of antibodies listed above. Biotinylated Abs were detected with SA-CyChrome. Stained cells were analyzed using a Coulter Epics XL/MCL or a Facscalibur (BD Biosciences) analyzer. Data were analyzed using the FlowJo software (Treestar, San Carlos, CA, USA).

GC B-cell sorting, RNA extraction and real-time RT–PCR

$B220^{high}$ IgD^{neg} PNA^{+} GC B cells were sorted using a MoFlo fluorescent activated high-speed sorter (Dako Cytomation, Glostrup, Denmark). RNA purification, reverse transcription of RNA, real-time RT-PCR and generating raw RQ (relative quantification) values for FcγRIIb gene expression in GC B cells were performed as described.[10]

Antibody measurements

All antibodies were detected by enzyme-linked immunosorbent assay (ELISA) in Immunolon II plates (Dynex, Chantilly, VA, USA). For anti-SRBC antibodies, the plates were coated with SRBC diluted at 1:1000 in PBS for 1 h at room temperature, then fixed with 0.5% gluteraldehyde. After blocking (3% BSA, 0.1% gelatin, 3 mM EDTA), sera were incubated in duplicate at 1:200 dilution for 2 h, and bound IgG antibodies were detected with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Chemicon International, Temecula, CA, USA) at a 1:1000 dilution. Total IgM and IgG were detected by sandwich ELISA with sera diluted 1:200 000 as previously described.[27] Anti-chromatin IgG antibodies were detected in 10-to 12-month-old mice with plates coated with total histone and dsDNA as previously described.[28]

Enzyme-linked immunospot (ELISPOT) assay

AFCs were enumerated by ELISPOT as previously described.[24] Serially diluted RBC-depleted spleen and BM cells were added to multiscreen filter plates (Millipore, MA, USA) coated with 5 μg/ml goat anti-mouse IgG for 6 h at 37 1C. Bound cells were detected with HRP-conjugated-anti-IgG (Southern Biotechnology), and developed by 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich, St Louis, MO, USA). AFCs were then counted and measured using a Bioreader 4000 Pro-x (BioSys, Germany).

Statistical analysis

Statistical significance was evaluated with the GraphPad Prism 4 software package and the specific tests were reported in the text (either Bonferroni's multiple comparison or variance F tests).

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Figures & Captions

Figure 1

FcγRIIB expression on germinal center (GC) B cells and follicular dendritic cells (FDCs) in C57BL/6 and b6.Sle1 mice.

(a) Splenocytes from sheep red blood cells (SRBC)-immunized B6 mice on day 9 of the immune response were stained with peptide nucleic acid (PNA), in combination with anti-B220 and anti-IgD and analyzed by flow cytometry. In the left panel, $B220^{hi}$ and PNA^{+} /PNA^{neg} mature cells are shown in the rectangular gate. In the right panel, $B220^{hi}$ PNA^{+} /IgD^{neg} GC and $B220^{high}$ PNA^{neg} IgDþ non-GC B cells are shown in two rectangular gates.

(b) Levels of FcγRIIb measured by staining with 2.4G2 (bottom row) on $B220^{hi}$ PNA^{+} /IgD^{neg} GC (blue histograms) and $B220^{hi}$ PNA^{neg} IgDþ non-GC (red histograms) B cells are shown for SRBC-immunized B6, B6.Sle1 and $B6.RIIB^{-/-}$ mice on day 9 of the primary GC response.

(c) (c) Adjacent spleen sections obtained from these mice were stained with 2.4G2 (red) and anti-IgD (green), shown in the left column and FDC-M1 (red) and GL7 (green), shown in the right column. These data represent those obtained from three to five mice of each strain.

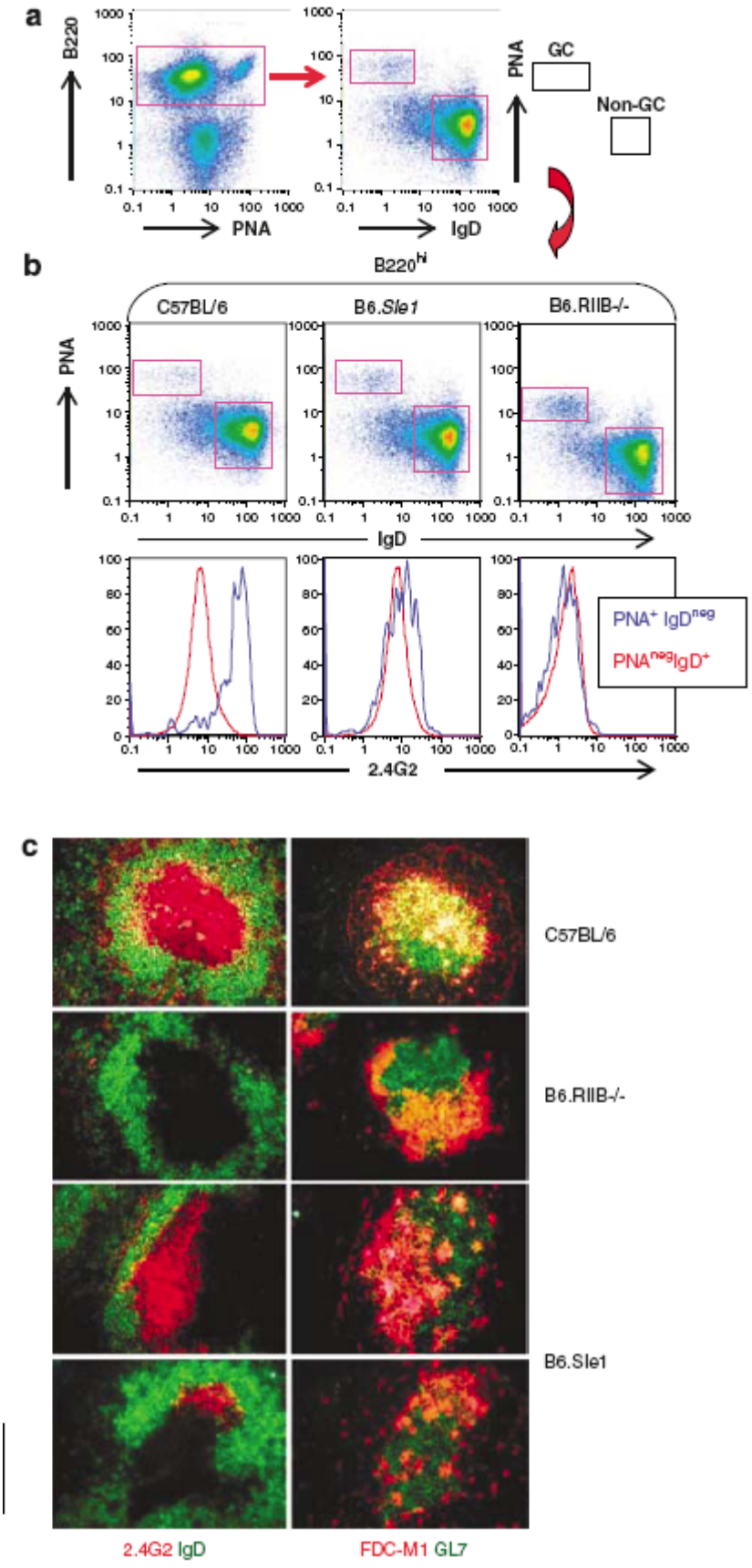

a
B220
PNA
IgD
GC
Non-GC
B220hi
b
C57BL/6
B6.Sle1
B6.RIIB-/-
PNA
IgD
2.4G2
PNA+ IgDneg
PNAneg IgD+
c
C57BL/6
B6.RIIB-/-
B6.Sle1
2.4G2 IgD
FDC-M1 GL7

Figure 2

The B6.Sle1a(15–353) and B6.Sle1(111–148) congenic strains differ by the B6 or NZW origin of Fcgr2b.

(a) Map of the two congenic intervals with, from top to bottom, the distance in Mb, the position of key polymorphic microsatellite markers used to define the interval termini, the position of the Fcgr2b gene and the two intervals. The gray bars represent the areas of known NZW (NZM2410) derivation, and the white bars represent the areas of recombination between the NZW and B6 genomes. The boxed inset on the right shows an enlargement of the telomeric termini between markers D1MIT147 and D1MIT146 relative to the Fcg2rb transcript.

(b) PCR amplification of the region 4 deletion identifying the NZW allele in B6.Sle1(111–148) as compared to B6.Sle1a(15–353), which has the B6 alleles.

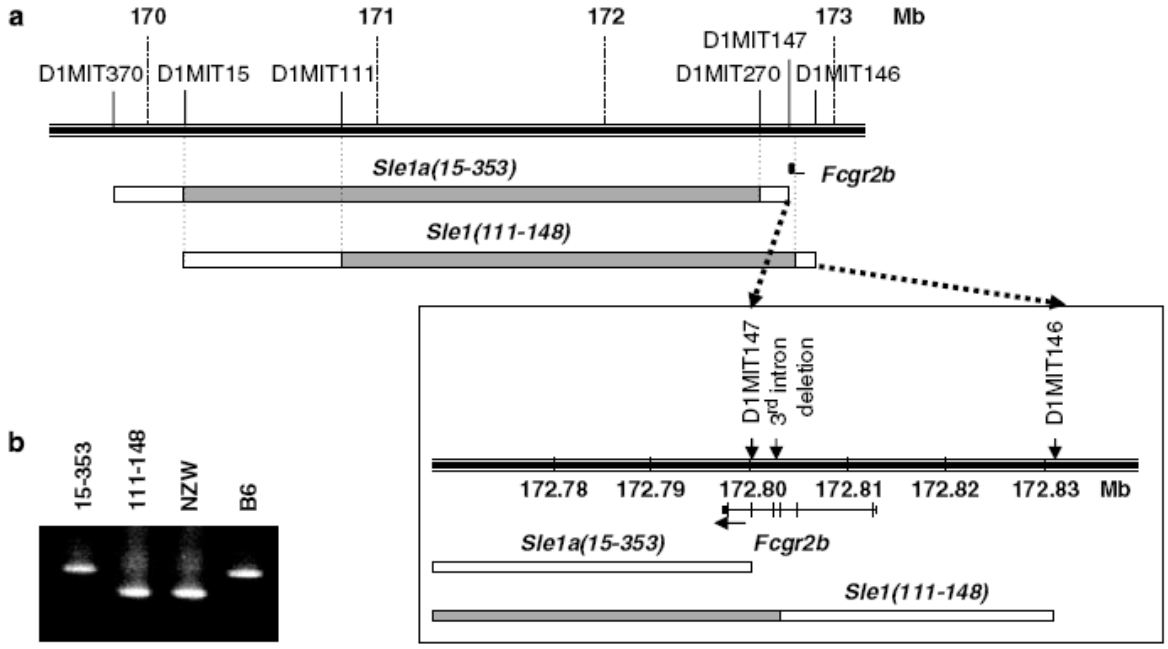
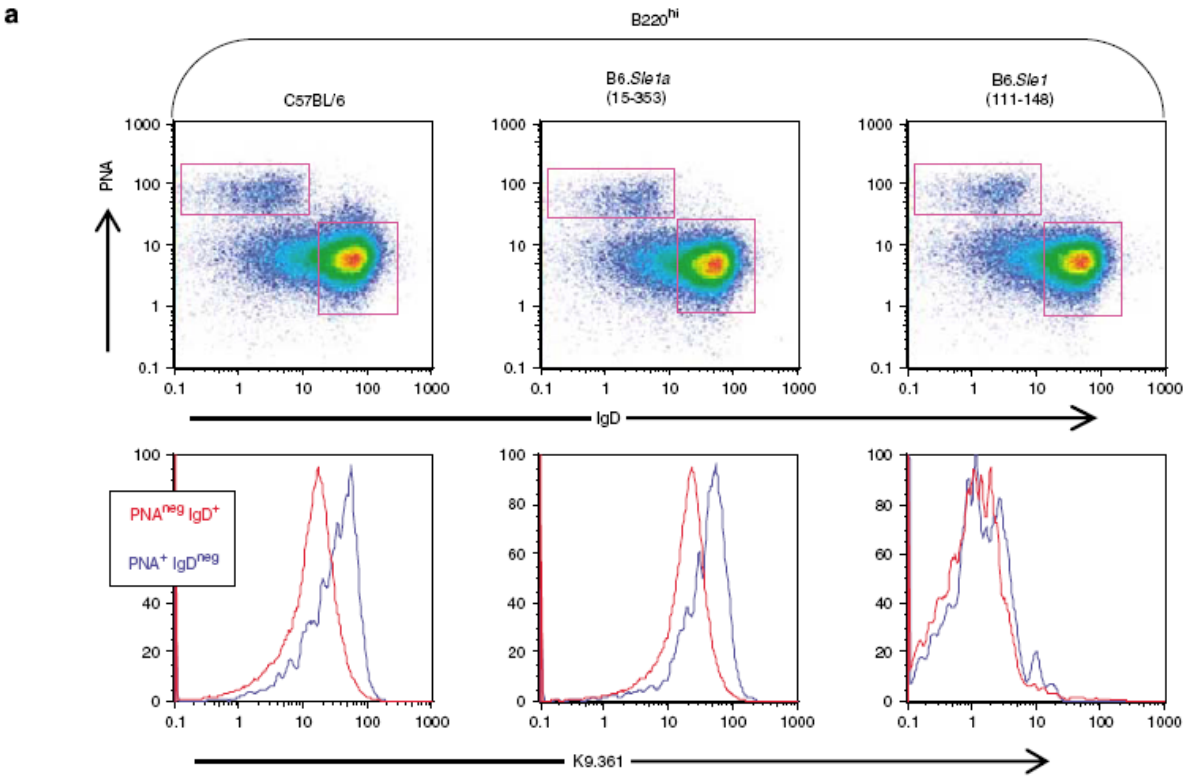


Figure 3

B6.Sle1(111–148) mice contain a Fcgr2b allele of NZW origin.

(a) Splenocytes from sheep red blood cells (SRBC)-immunized B6, B6.Sle1a(15–353) and B6.Sle1(111–148) mice obtained on day 9 of the immune response were stained with peptide nucleic acid (PNA), in combination with anti-B220 and anti-IgD. Levels of FcγRIIb measured by the mAb K9.361 that specifically stains the Fcgr2b allele of B6 origin (Ly17.2 allotype) on $B220^{hi}$ PNAþ /IgD^{neg} germinal center (GC) (blue histograms) and $B220^{hi}$ PNA^{neg} IgDþ non-GC (red histograms) B cells are shown.

(b) Spleen sections obtained on day 9 of the anti-SRBC response of B6, B6.Sle1a(15–353) and B6.Sle1(111–148) mice were stained with anti-IgD (red) and K9.361 (green). These data are representative of at least three mice of each genotype.



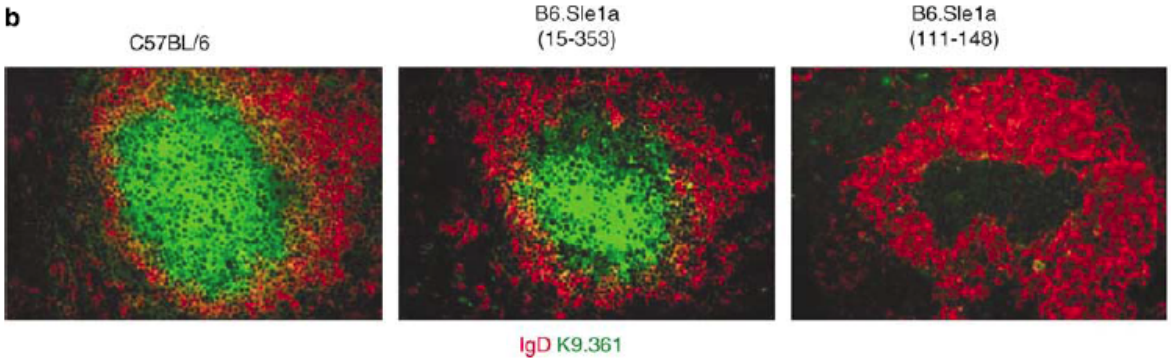
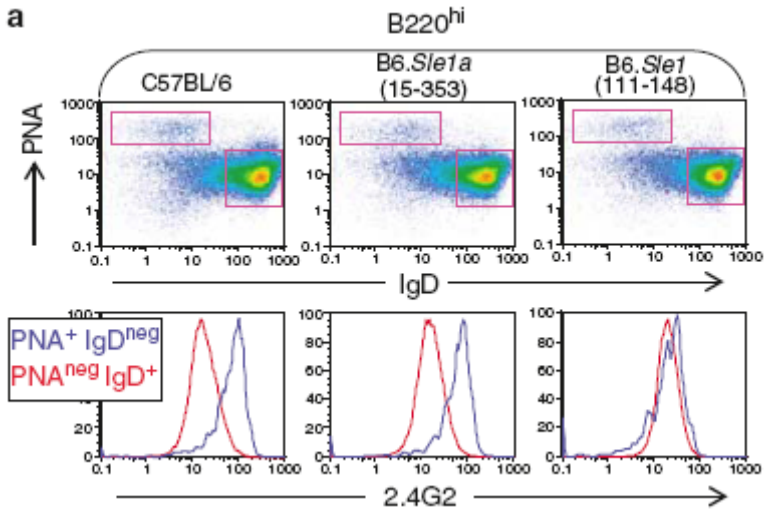


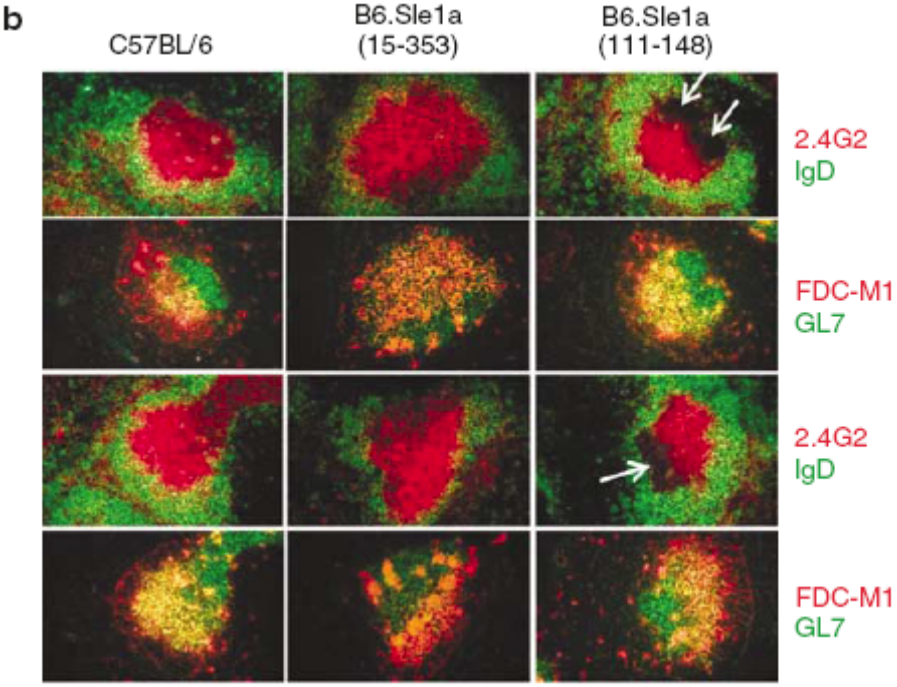
Figure 4

Impaired FcγRIIb expression on B cells and follicular dendritic cells (FDCs) in B6.Sle1(111–148), but not in B6.Sle1a(15–353) mice.

(a) Splenocytes obtained on day 9 of the anti-sheep red blood cells (SRBC) response of B6, B6.Sle1a(15–353) and B6.Sle1(111–148) mice were stained and analyzed, and data are illustrated as detailed in Figures 1a and b.

(b) Two sets of adjacent spleen sections obtained on day 9 of the anti-SRBC response of B6, B6.Sle1a(15–353) and B6.Sle1(111–148) mice were stained and illustrated as detailed in the legend to Figure 1c. Substantially lower or undetectable levels of 2.4G2 staining, in IgD^{-} GL7þ and FDC poor regions in B6.Sle1(111–148) GCs, are shown by arrows, right most column. These data are representative from five to seven mice of each genotype. (c) RNA was extracted from FACS-purified $B220^{hi}$ PNA^{+} /IgD^{neg} germinal center (GC) B cells obtained on day 9 of the SRBC response of B6, B6.Sle1a(15–353) and B6.Sle1(111– 148) mice. Raw RQ values of Fcgr2b expression in B6 (open bar), B6.Sle1a(15–353) (green bar) and B6.Sle1(111–148) (red bar) GC B cells were obtained via real-time RT-PCR by setting the RQ value for B6 GC B cells as 1. Real-time RT-PCR amplification was performed in quadruplet, from which the means and maximum relative quantification (RQ as calculated by the ABI software) shown as error bars were generated. These data are representative of pooled samples of two to three mice of each genotype obtained from three experiments.


a
B220hi
C57BL/6
B6.Sle1a (15-353)
B6.Sle1 (111-148)
PNA
IgD
PNA+ IgDneg
PNAneg IgD+
2.4G2


b
C57BL/6
B6.Sle1a (15-353)
B6.Sle1a (111-148)
2.4G2
IgD
FDC-M1
GL7
2.4G2
IgD
FDC-M1
GL7

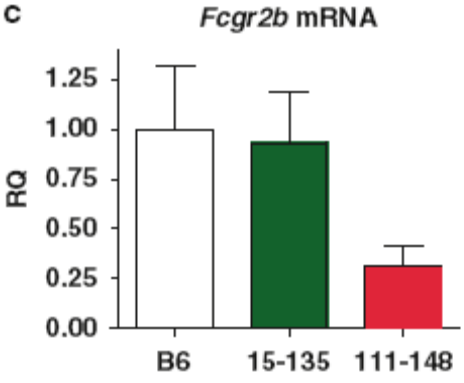

c
Fcgr2b mRNA
RQ
1.25
1.00
0.75
0.50
0.25
0.00
B6
15-135
111-148

Figure 5

IgG production is increased in B6.Sle1a(111–148) mice.

Anti-sheep red blood cell (SRBC) IgM (a) and IgG (b) antibodies, as well as total IgM (d) and IgG (e) were measured on day 9 after primary immunization in the three congenic strains. The graphs on the right show the anti-SBRC (c) and total (f) IgG/IgM ratios. Each symbol represents a mouse. **P < 0.01; ***P < 0.001 from Bonferroni's multiple comparison tests.

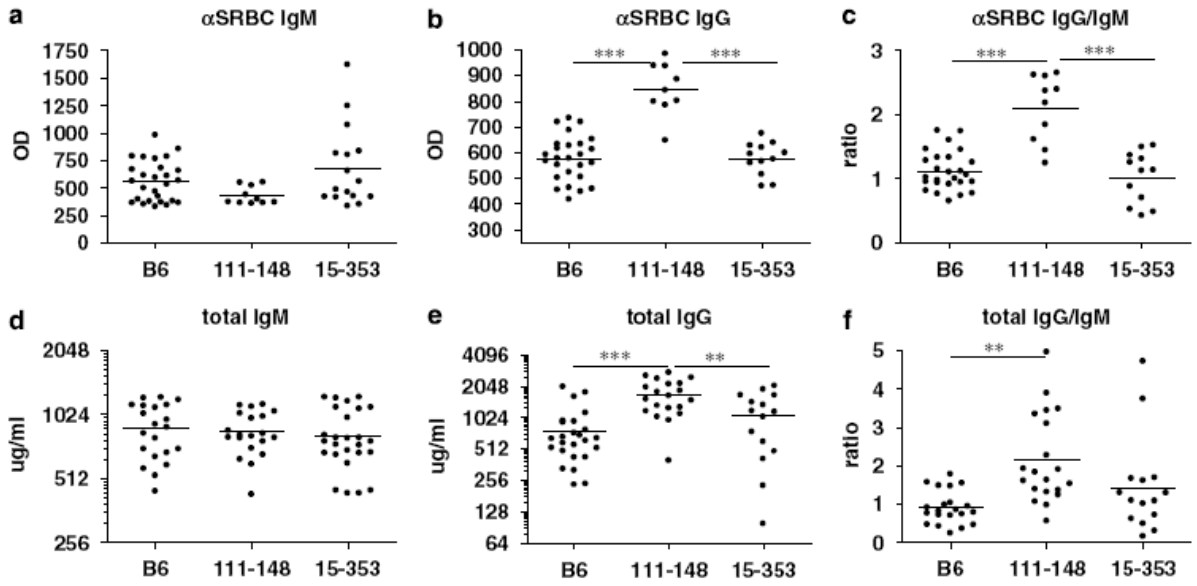


Figure 6

B6.Sle1(111–148) antibody-forming cells (AFCs) produce less IgM and more IgG than congenic mice carrying the Fcgr2b B6 allele.

Splenic (graphs on the left) and bone marrow (BM) (graphs on the right) $CD138^{+}$ $B220^{-/lo}$ plasma cells (PC) were evaluated in 6 and 14 days after sheep red blood cells (SRBC) immunization.

(a) Surface FcγRIIb expression.

(b) Intracellular IgM expression.

(c) Intracellular IgG expression.

(d) Number of IgG þ AFC measured by ELISPOT. Mean and s.d. of six mice (6 days p.i.) and three mice (14 days p.i.) per strain. *P < 0.05; **P < 0.01 from Bonferroni's multiple comparison tests. Representative flow cytometry histograms gated on $CD138^{+}$ $B220^{-/lo}$ PC in the spleen 6 days p.i. are shown on the right, with filled gray

histograms representing C57BL/6, and solid and broken lines representing B6.Sle1(111–148) and B6.Sle1a(15–353), respectively.