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The Inhibiting Fc Receptor for IgG, FcγRIIB, Is a Modifier of Autoimmune Susceptibility

Peter Boross,*1,2 Victoria L. Arandhara,*1,3 Javier Martin-Ramirez,*1,4 Marie-Laure Santiago-Raber,† Francesco Carlucci,‡ Roelof Flierman,§ Jos van der Kaa,* Cor Breukel,* Jill W. C. Claassens,* Marcel Camps,¶ Erik Lubberts,‖ Daniela Salvatori,†,‡ Maria Pia Rastaldi,† Ferry Ossendorp,¶ Mohamed R. Daha,§ H. Terence Cook,‡ Shozo Izui,† Marina Botto,§ and J. Sjef Verbeek*

FcγRIIB-deficient mice generated in 129 background (FcγRIIB129 −/−) if back-crossed into C57BL/6 background exhibit a hyperactive phenotype and develop lethal lupus. Both in mice and humans, the Fcγr2b gene is located within a genomic interval on chromosome 1 associated with lupus susceptibility. In mice, the 129-derived haplotype of this interval, named Sle1, causes loss of self-tolerance in the context of the B6 genome, hampering the analysis of the specific contribution of FcγRIIB deficiency to the development of lupus in FcγRIIB129 −/− mice. Moreover, in humans genetic linkage studies revealed contradictory results regarding the association of “loss of function” mutations in the Fcγr2b gene and susceptibility to systemic lupus erythematosus. In this study, we demonstrate that FcγRIIB −/− mice generated by gene targeting in B6-derived ES cells (FcγRIIBb6 −/−), lacking the 129-derived flanking Sle1 region, exhibit a hyperactive phenotype but fail to develop lupus indicating that in FcγRIIB129 −/− mice, not FcγRIIB deficiency but epistatic interactions between the C57BL/6 genome and the 129-derived Fcγr2b flanking region cause loss of tolerance. The contribution to the development of autoimmune disease by the resulting autoreactive B cells is amplified by the absence of FcγRIIB, culminating in lethal lupus. In the presence of the Taa lupus-susceptibility locus, FcγRIIBb6 −/− mice do develop lethal lupus, confirming that FcγRIIB deficiency only amplifies spontaneous autoimmunity determined by other loci. The Journal of Immunology, 2011, 187: 1304–1313.

The hyperactive phenotype of FcγRIIB KO mice confirms the important role of this inhibiting receptor for IgG in the negative regulation of activating Fc receptors on myeloid effector cells and in a negative feedback mechanism in B cells, controlling Ab production. However, the lupus phenotype of FcγRIIB −/− mice generated by gene targeting in 129-derived ES cells (FcγRIIB129 −/−) and back-crossed into C57BL/6 (B6) background (1) is surprising because genetic studies revealed that lupus susceptibility is a multigenic phenotype (2).

Genetic and functional studies have revealed contradictory data, making the role of FcγRIIB in autoimmunity controversial. The Fcγr2b gene is located in a locus within the distal region of mouse chromosome (Chr) 1, which is orthologous with an SLE-associated locus on human Chr 1. All lupus-prone mouse strains share the lupus-associated Sle1 haplotype of that locus, but B6 mice do not. In B6 congenic mice, the Sle1 locus causes loss of tolerance leading to anti-nuclear Ab (ANA) production, mild splenomegaly, but no fatal glomerulonephritis. The following observations in autoimmune-prone mice and systemic lupus erythematosis (SLE) patients suggest that the association with lupus of the Sle1 locus and its human ortholog can be attributed, at least partially, to polymorphisms in the Fcγr2b gene. Autoimmune-prone strains share a promoter haplotype and/or polymorphisms in the third intron of the Fcγr2b gene, which are associated with

T
reduced FcγRIIB expression in germinal center B cells and plasma cells (3, 4). In humans, the frequency of homozygosity for a single amino acid polymorphism (232T) in FcγRIIB, resulting in “loss of function” (5), is at least doubled in individuals with SLE compared with that in healthy controls in three Asian and one Caucasian cohort (reviewed in Ref. 6).

Several other observations, however, argue against a role of FcγRIIB in lupus. In humans, the association between the 232T polymorphism and SLE could not be confirmed with an African–American or Caucasian–American cohort (7). A recent genomewide association study in women of European ancestry with SLE identified susceptibility variants in 15 different genes but not in the Fcyr2b gene (8). Moreover, a strong SLE association in Caucasians of a “gain of function” mutation in FcγRIIB, resulting in increased promoter activity, has been reported (9). In mice, within the Sle1 locus four subloci, Sle1a–d, have been identified. In congenic B6 mice the presence of Sleb is sufficient for the loss of tolerance resulting in ANA production. Surprisingly, genetic fine mapping placed the Fcyr2b gene not in, but just between, the Slela and Sleb subloci (10). Moreover, a B6 strain congenic for the Fcyrb allele derived from the NZW autoimmune-prone strain did not develop autoimmunity (11).

Importantly, the lupus-associated Sle1b haplotype is also present in the non-autoimmune strain 129. In agreement with this, a B6 strain congenic for the 129-derived distal part of Chr 1, encompassing the Sleib locus, named Sle6, develops an autoimmune phenotype (12). As a consequence, it may be the presence of the Sle6 locus that determines the autoimmune phenotype of FcγRIIB129+/−/ mice. To analyze the biological function of FcγRIIB independently from the confounding effect of the Sle6 locus, we generated an FcγRIIB KO strain (FcγRIIB−/−) by gene targeting in B6-derived ES cells. FcγRIIBb6−/− mice displayed a hyperergic phenotype but did not develop spontaneous autoimmunity. However, when the FcγRIIBb6−/− strain was crossed with the autoimmune-prone Yaa B6 strain, FcγRIIBb6−/− Yaa offspring did develop fatal lupus. These observations indicate that FcγRIIB deficiency does not cause autoimmunity but amplifies spontaneous autoimmunity determined by autoimmune susceptibility loci, such as Sle6 or Yaa.

Materials and Methods

Generation of the conditional C57BL/6 FcγRIIB KO mouse model

For the generation of FcγRIIB−/− mice on C57BL/6 background (FcγRIIB−/−), a targeting vector was constructed based on a 12-kb fragment derived from the BAC clone RPCI23-87B18 of the RPCI 23 Female (C57BL/6) mouse BAC genomic library (BACPAC Resources Center, Children’s Hospital Oakland Research Institute, Oakland, CA). By using conventional cloning techniques, a neomycin selection cassette, flanked by loxP sites, was inserted in intron 3, and a third loxP site was inserted in the intron (Supplemental Fig. 1A). Gene targeting was performed in C57BL/6-derived ES cells. FcγRIIBb6−/− mice displayed a hyperergic phenotype but did not develop spontaneous autoimmunity. However, when the FcγRIIBb6−/− strain was crossed with the autoimmune-prone Yaa B6 strain, FcγRIIBb6−/− Yaa offspring did develop fatal lupus. These observations indicate that FcγRIIB deficiency does not cause autoimmunity but amplifies spontaneous autoimmunity determined by autoimmune susceptibility loci, such as Sle6 or Yaa.

The phenotype of FcγRIIBb6−/−/− mouse was analyzed in a series of in vitro and in vivo assays and compared with the phenotype of the FcγRIIBb6−/− mouse (generated with 129-derived ES cells and subsequently back-crossed eight generations on C57BL/6 background). FcγRIIBb6−/− and C57BL/6d mice, which responded similarly in all assays, were used as controls. FcγRIIBb6−/− and FcγRIIBb6−/− mice developed normally and showed normal breeding characteristics. No gross abnormalities were observed in these mice. The expression of FcγRIIB on splenic lymphocytes and the resulting F2 with C57BL/6 congenic strain, in which no FcγRIIB expression was observed in FcγRIIBb6−/− mice (Supplemental Fig. 1E). Similar results of FcγRIIB expression were obtained with B cells and myeloid cells isolated from peripheral blood, bone marrow, and peritoneal cavity (data not shown).

Mice

FcγRIIB129−/− mice, generated on 129 background, were a gift of Dr. T. Takai (Department of Experimental Immunology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) (13) and back-crossed in our facility into C57BL/6d (Charles River, Maastricht, The Netherlands) background for eight generations. FcγRIIB−/− mice back-crossed into C57BL/6d background for 13 generations have been described previously (14). The C57BL6.Yaa congenic strain was generated as described (15). C57BL6.Yaa FcγRIIBb6−/− Yaa mice were generated by intersecting F1 progeny from FcγRIIBb6−/− females and C57BL6/Yaa males.

The ElaCre deleter strain (n = 12 on C57BL/6d background), was a kind gift of Dr. Heiner Westphal (National Institute of Child Health and Human Development, Bethesda, MD). C57BL/6d control mice were purchased from Charles River. Mice were housed and all experiments were performed at the specified pathogen free animal facilities of the Leiden University Medical Center except for the aging experiment with cohorts of male mice of the C57BL6/Yaa congenic strain and the FcγRIIBb6−/− Yaa strain, which was performed at the animal facilities of the University of Geneva. All experiments were approved by the local ethical committee and performed in accordance with national guidelines and regulations. Six- to twelve-week-old mice were used for the experiments unless stated otherwise. Mice were routinely checked for their genotype by PCR and cytfluorimetry.

Simple sequence length polymorphism and single nucleotide polymorphism analysis

Genotyping of the mice was carried out using simple sequence length polymorphism (SSLP) markers (listed in Supplemental Table I), standard PCR, and either 4% Metaphor agarose (Cambrex/Bioscience Rockland, Rockland, ME) or 16% polyacrylamide gels stained with ethidium bromide. The positions and sequences were determined from the Ensembl (http://www.ensembl.org/index.html) and Mouse Genome Informatics (https://www.informatics.jax.org) databases. Single nucleotide polymorphisms (SNPs) in the FcγRIIB flanking regions reported to be different between the 129 and C57BL/6 mouse strains (Supplemental Table I) were amplified by PCR. The PCR products were purified and their nucleotide sequence determined.

Immunization with TNP–KLH

At day 0, mice were immunized i.p. with 100 μg 2,4,6, trinitrophenyl hapten conjugated keyhole limpet hemocyanin (TNP–KLH) (Biosearch Technologies) in alum (Merck) and boosted on day 49 with 100 μg TNP–KLH without alum. Serial blood samples were collected via the retro-orbital plexus on days 21, 28, and 56 after the primary immunization. The Ab response was determined by ELISA. Plates were coated with TNP–KLH (20 μg/ml) in 0.1 M carbonate buffer, pH 9.5, overnight at 4°C. After washing with PBS/0.1% BSA, 0.05% Tween 20, plates were blocked with PBS/1% BSA for 1 h at room temperature in a humid chamber, and samples, diluted in PBS (1:16,000), were incubated overnight at 4°C. Plates were washed five times with PBS/0.05% Tween 20 and incubated with rabbit anti-mouse IgG HRP (1:2000) or rabbit anti-mouse IgG HRP (1:1000) in PBS/1% BSA/0.01% Tween 20 for 2 h at room temperature. Plates were developed using ABTS (Sigma), and their OD was read at 405 nm. Samples were compared with a standard curve prepared from the pooled sera.

In vitro phagocytosis assay

Bone marrow-derived macrophages were cultured from mice in 15% L-cell conditioned medium (L929) for 8 d, pretreated overnight with 20 ng/ml recombinant IL-4 to increase FcγRIIB expression, and subsequently incubated at 37°C for 1 h with TNP-conjugated sheep RBCs (SRBCs) opsonized with anti-TNP IgG Abs. The ingested SRBCs were counted using a light microscope.
Passive cutaneous anaphylaxis
Mice were injected intradermally in the ear with 20 μl of varying concentrations (1–3 μg) IgG1 anti-TNP mAb and 2 h later given an i.v. injection of 500 μg human serum albumin (HSA)–TNP in 100 μl PBS with 1% Evans blue. After 30 min, extravasation was visualized by blue staining of the ear as described previously (14).

Monitoring the development of spontaneous autoimmune disease
Mice were monitored up to the age of 12 mo, and mortality was recorded. Moribund animals were killed before the 12 mo deadline and were counted as deceased. Blood and urine was collected periodically, and serological analyses were performed by ELISA to determine the autoantibody levels. After sacrifice, the mice were weighed, and organs were removed. Spleens were weighed, and kidneys were fixed in Bouin’s solution (Sigma) for 4 h, transferred to ethanol 70%, and finally embedded in paraffin, whereas spleenocytes were analyzed by flow cytometry.

Serological analyses
Antinucleosome Abs. Serum levels of anti-nucleosome Abs were determined by ELISA. Plates were coated overnight with S2 fraction of oligonucleosomes isolated from L1210 cells. After blocking with PBS/5% FCS/0.05% Tween 20, the plates were incubated with serially diluted serum (1:100, 1:200, 1:400) for 2 h at room temperature. Bound Abs were detected using goat anti-mouse Ig–HRP. Plates were developed with ABTS, and the OD was read at 415 nm. Titers were determined by comparison with a sample with a known concentration of mouse anti-nucleosome mAbs. Anti-dsDNA, anti-ssDNA, anti-chromatin, and anti-histone ELISA. Levels of anti-dsDNA, anti-ssDNA, anti-chromatin, and anti-histone Abs were measured by ELISA as described previously (16). Results were expressed as arbitrary units relative to a standard positive sample derived from an MRL/1pr/3pr mouse pool.

Detection of Coombs Abs. A flow cytometric assay was used to detect Coombs Abs using biotinylated rat anti-mouse IgM–PE (clone eB121-15F9; eBioscience), B220–PE (BD Pharmingen), IgD–FITC (clone 11-26; eBioscience), and H1.2F3; BD Biosciences), IgG and C3 deposition.

Assessment of renal pathology
Histological sections. Four-micrometer-thick kidney sections were processed and assessed as described previously (18). IgG and C3 deposition. Kidneys were embedded in OCT, snap-frozen in isopentane cooled with liquid nitrogen, and stored at −70°C. Five-micrometer-thick frozen sections were processed and incubated as described previously (18). All Abs were used at a dilution of 1:200 (except anti-C3 at 1:100). For semiquantitative immunofluorescence, all sections were examined at ×600 original magnification with an Olympus AX70 Provis microscope and 2MP slider camera (Diagnostic Instruments). The mean intensity of 20 glomeruli for each sample was recorded in arbitrary fluorescence units as 0/trace, 1+, 2+, 3+, 4+.

Proteinuria. Albumin concentration was determined in urinary samples of mice using a standard rocket immunoelectrophoresis assay. A series of diluted urinary samples (1:2, 1:4, or 1:16) in PBS alongside control samples of purified mouse albumin were run for 5–6 h at 500 V/40 mA in a 1% agarose gel in the presence of polycyonal rabbit anti-mouse albumin in Gelman buffer. The gel was dried overnight, stained with Coomassie blue, and analyzed.

Flow cytometry
Single-cell suspensions of spleens were prepared by mechanical-and-digested with specific mAbs. For flow cytometry analysis, CD4–PE (clone RM4-5; BD Biosciences), CD8–FITC (clone 53–5.8; BD Biosciences), CD19–allophycocyanin (clone IGD3; BD Biosciences), CD62L–allophycocyanin (clone MEL-14; BD Biosciences), CD69–FITC (clone H1.2F3; BD Biosciences), IgD–FITC (clone 11-26; eBioscience), and IgM–PE (clone eB121-15F9; eBioscience), B220–PE (BD Pharmingen), 2.4G2–FITC (BD Pharmingen), and K9.361–Alexa 488 anti-Ly17.2 (clone K9.361; B6/129 type FcRIIB-specific) were used. Data acquisition was performed on a BD Biosciences FACScan or LSRII. For data analysis, CellQuest software was used.

Induction of arthritis
Induction and clinical evaluation of collagen-induced arthritis (CIA) and K/B × N serum-induced arthritis was performed as described (19). In the CIA model, on days 35 and 60 after the primary immunization, serum was collected for the measurement of anti-collagen type II (CII) Ab titers by ELISA. Because of the robust nature of the K/B × N serum-induced arthritis model, the relative increase in arthritis severity in the FcγRIIB−/− mice became only apparent when a suboptimal amount of serum (7.5 μl/g body weight) was injected, which induced only mild arthritis in control mice.

Induction of experimental nephritis
Induction and clinical evaluation of accelerated nephrotic nephritis (NTN) in mice was performed as described previously (18). Mice were assessed daily for the development of proteinuria and hematuria (Haema Combistix).

Monocytosis
PBMCs were stained with M1/70 anti-CD11b and AF598 anti-CD115 mAb in the presence of saturating concentration of 2.4G2 anti-FcγRIIB/IIH mAb and analyzed with a FACS Calibur (Becton Dickinson, Mountain View, CA), as described previously (20). Percentages of CD11b+CD115+ monocytes (distinguished from polymorphonuclear leukocytes by their lower granularity, as reflected in low side-light scatter) among PBMCs were determined.

Statistics
Parametric data were represented as mean ± SEM. Multiple groups were compared by ANOVA and further evaluated using Bonferroni or unpaired Student t test. Nonparametric data were represented by median and multiple groups compared by using a Kruskal–Wallis test or Mann–Whitney U test. A p value of 0.05 was considered statistically significant. Cumulative mortality was processed by using Gehan–Breslow–Wilcoxon test.

Results
Establishing two independent FcγRIIB−/− C57BL/6 mouse strains, which differ in their FcγRIIB flanking regions
To investigate the contribution of the FcγRIIB-flanking chromosomal region to the phenotype of the FcγRIIB−/− mice, two different FcγRIIB−/− strains were generated, one with a 129-derived and the other with the B6-derived flanking region, FcγRIIB129−/− and FcγRIIBB6−/−, respectively. The FcγRIIBB6−/− mouse was generated by gene targeting in 129-derived ES cells (13) and back-crossed for eight generations into C57BL/6/J background in our laboratory. The FcγRIIBB6−/− strain was generated by gene targeting in B6-derived Brue4 ES cells and back-crossed for three generations into C57BL6/J background (Supplemental Fig. 1). The 129 and B6 origin of the flanking regions was confirmed by SNP analysis (Supplemental Table I A). In both strains, the B6 origin of 17 SSLPs with linkage to SLE, on Chr 3 (16), 9, 12, and 17 (21), was confirmed as well as the B6 origin of 8 SSLPs on Chr 7, and Chr 13 in the FcγRIIBB6−/− strain (Supplemental Table IB).

Both FcγRIIBB6−/− and FcγRIIB129−/− mice exhibit a hyperactive phenotype
FcγRIIBB6−/− and FcγRIIB129−/− mice showed a similar increase in Ab responses after immunization, in mast cell-mediated cutaneous anaphylaxis, and in phagocytosis of immune complex (IC) by macrophages compared with wild-type controls (Fig. 1A–C). FcγRIIBB6−/− mice were susceptible to CIA (Fig. 1D, 1E), although incidence was lower and onset was slightly delayed compared with incidence and onset of CIA in FcγRIIBB6−/− mice (22). An explanation might be that FcγRIIB129−/− mice developed higher anti-CII titers compared with those of FcγRIIBB6−/− mice. However, the difference is not significant due to the strong variation in anti-CII titers in FcγRIIBB6−/− mice, which might be attributed to their mixed 129/B6 background (Supplemental Fig. 2). In the passive K/B × N serum-induced arthritis model, which depends exclusively on downstream Ab effector pathways, both FcγRIIB KO strains developed more severe disease compared with...
FIGURE 1. FcγRIIB−/− mice exhibit a hyperactive phenotype. A, Increased Ag-specific serum IgG titers after immunization with TNP-KLH. Representative results from two independent experiments (10 mice per group). *p < 0.05, **p < 0.01, ***p < 0.001 (Student unpaired t test). B, Enhanced IgG-mediated passive cutaneous anaphylaxis. Mice were intradermally injected with anti-TNP IgG1 Ab and subsequently challenged by i.v. injection of HSA–TNP in Evans blue. Anaphylaxis was visualized by extravasation of the blue dye. Data are representative of three independent experiments (two to five mice per group). Original magnification ×1. C, Enhanced phagocytic activity of macrophages. Bone marrow-derived macrophages were incubated with mouse IgG1 anti-TNP opsonized SRBC–TNP and ingested SRBCs counted under a light microscope. Data are representative of two independent experiments (six mice per group). ***p < 0.001 (Bonferroni’s multiple comparison test). D and E, Susceptible to CIA. Combined data from three independent experiments (10–25 mice per group). CIA incidence (D) in FcγRIIB−/− mice was significantly higher compared with B6 mice, p < 0.001 and p = 0.012, respectively (Fisher’s exact test). End point incidence in FcγRIIB−/− mice was lower compared with that of FcγRIIB+/+ mice, p = 0.001 (Fisher’s exact test). CIA severity score (E). F–J, Enhanced accelerated NTN. Results of two independent experiments (8–10 mice per group). Experiment 1: 1:10 dilution of nephrotoxic serum inducing severe disease. Survival curve of mice with NTN (F). Experiment 2: 1:20 dilution of nephrotoxic serum inducing moderate disease. Urinary albumin (G) was measured by radial immunodiffusion in urine collected overnight from mice in metabolic cages. Glomerular thrombosis (H). Kidneys were fixed in Bouin’s solution and stained with periodic acid–Schiff (PAS) stain. Deposition of IgG (I) or C3 (J). Immunohistochemistry of snap-frozen kidneys presented as arbitrary fluorescence units (AFU). *p < 0.05, **p < 0.01, ***p < 0.001 (Kruskal–Wallis test).
wild-type B6 mice. However, severity was significantly higher in FcγRIIB129−/− mice compared with FcγRIIB6−/− mice (Supplemental Fig. 3). Both strains showed enhanced accelerated NTN compared with wild-type controls. However, onset of the disease was somewhat delayed and severity was lower in the FcγRIIB6−/− mice compared with the onset and severity of NTN in FcγRIIB129−/− mice (Fig. 1F–J). Similar to CIA, in NTN the increased severity in FcγRIIB129−/− mice compared with that in FcγRIIB6−/− mice might be explained partially by higher specific Ab titers (Table I). Taken together, both FcγRIIB6−/− and FcγRIIB129−/− mice display a hyperactive phenotype, which is more pronounced in FcγRIIB129−/− mice, suggesting a significant contribution of the 129-derived Fcyrh2 flanking region (Sl616) to this phenotype both in the effenter and afferent phase.

FcγRIIB6−/− mice do not develop spontaneous autoimmunity

Because it has been reported that FcγRIIB129−/− mice develop high ANA titers spontaneously, with high frequency with age (1), the presence of these autoantibodies in the blood of cohorts of about twenty 10-mo-old FcγRIIB6−/− and FcγRIIB129−/− female mice was analyzed. Whereas most of the FcγRIIB129−/− mice had high serum titers of autoantibodies specific for dsDNA and ssDNA, chromatin and histone, confirming the published phenotype, in the serum of FcγRIIB6−/− mice hardly any of these autoantibodies were detectable (Fig. 2A–D). This lack of autoantibodies was neither caused by a delay in onset nor restricted to ANA. At the age of 12 mo, at which the mice were sacrificed, ANA Abs were still undetectable (data not shown), whereas anti-erythrocyte Abs present in FcγRIIB129−/− mice were also absent in FcγRIIB6−/− mice (Fig. 2E). Moreover, FcγRIIB6−/− mice did not show other signs of systemic autoimmunity, such as splenomegaly (Fig. 2F) or an increase in the proportion of activated T and B cells (Table II). These results indicate that, despite their strongly increased susceptibility to CIA, FcγRIIB6−/− mice fail to develop spontaneous autoimmunity, whereas under the same experimental conditions FcγRIIB129−/− mice do. This suggests that epistatic interactions between the 129-derived Sl616 locus, present in FcγRIIB129−/− mice, and the B6 genome are decisive for the development of spontaneous autoimmunity in FcγRIIB129−/− mice.

Mild nonlethal glomerular damage in FcγRIIB6−/− mice

The presence of ANA has been strongly associated with the development of immune-complex glomerulonephritis resulting in kidney damage. A first sign of kidney failure is proteinuria. At the age of 8 mo, despite the absence of autoantibodies, 30% of the FcγRIIB6−/− mice showed a small but significant increase in the urinary albumin level (between 10 and 30 mg protein/g creatinine) compared with wild-type control mice (Fig. 3A). However, more substantial urinary albumin levels between 50 and 175 mg protein/g creatinine were detected in 22% of the FcγRIIB129−/− mice. Careful examination of kidney sections of 12-mo-old mice revealed that all genotypes developed kidney pathology ranging from very mild in wild-type B6 controls, to moderate in

Table I. Anti-sheep IgG titers in NTN model

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6 (n = 10)</th>
<th>FcγRIIB129−/− (n = 10)</th>
<th>FcγRIIB6−/− (n = 10)</th>
</tr>
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<tbody>
<tr>
<td>Total IgG</td>
<td>0.214 ± 0.04</td>
<td>0.626 ± 1.06</td>
<td>0.243 ± 0.04</td>
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<tr>
<td>IgG1</td>
<td>0.171 ± 0.03</td>
<td>0.239 ± 0.09</td>
<td>0.185 ± 0.02</td>
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<tr>
<td>IgG2a</td>
<td>0.694 ± 0.07</td>
<td>1.137 ± 0.29*a</td>
<td>0.955 ± 0.13</td>
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<tr>
<td>IgG2b</td>
<td>0.127 ± 0.05</td>
<td>0.430 ± 0.82</td>
<td>0.145 ± 0.03</td>
</tr>
<tr>
<td>IgG3</td>
<td>0.155 ± 0.03</td>
<td>0.233 ± 0.17</td>
<td>0.234 ± 0.24</td>
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Mean OD ± SD for anti-sheep total IgG, IgG1, IgG2a, IgG2b, and IgG3. *p < 0.01 (one-way ANOVA, compared with C57BL/6).

Table II. No increase in proportion of activated T and B cells in FcγRIIB6−/− mice

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>FcγRIIB129−/−</th>
<th>FcγRIIB6−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDS6C2D2Llow</td>
<td>20.2 ± 1.2</td>
<td>55.4 ± 9.1*</td>
<td>16.9 ± 4.2</td>
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<tr>
<td>CDS4C2D6hlow</td>
<td>47.2 ± 4.5</td>
<td>89.5 ± 1.4***</td>
<td>66 ± 4.9</td>
</tr>
</tbody>
</table>
| CDS4C2D6hphlow|19.3 ± 1.3|46.7 ± 3.9***|26.9 ± 2.6*
| CD19C2D69hphlow|1.9 ± 0.1|3.9 ± 1.0***|1.4 ± 0.2|
| CD19IgMlowIgDlow|2.4 ± 0.2|5.5 ± 1.1|5.6 ± 0.7|

Percentage of splenic T and B cell subpopulations as determined by flow cytometry. Results represent mean ± SEM of 12-mo-old mice. *p < 0.05, ***p < 0.01 (significance compared with C57BL/6 mice; statistical significance was analyzed by ANOVA and Bonferroni test).
Fc\textsubscript{g}R\textsuperscript{IIIB}\textsubscript{6/2} mice, to severe in Fc\textsubscript{g}R\textsuperscript{IIIB}\textsubscript{129/2} mice (Fig. 3B). The pathology score in arbitrary categories from 0 to 4 correlated with increased mortality. Twenty-two percent of the Fc\textsubscript{g}R\textsuperscript{IIIB}\textsubscript{129/2} mice developed fatal disease between the ages of 8 and 12 mo, whereas neither the Fc\textsubscript{g}R\textsuperscript{IIIB}\textsubscript{6/2} nor the wild-type control mice developed fatal disease even at the age of 12 mo (Fig. 3D). These results show that the Sle16-driven nonlethal autoimmune features culminate in fatal lupus in the absence of functional Fc\textsubscript{g}R\textsuperscript{IIIB}, suggesting epistatic interactions between the Sle16 locus and the Fc\textsubscript{g}R\textsuperscript{IIIB} gene. Moreover, the significant increase of IgG and C3 depositions in the kidneys of 12-mo-old Fc\textsubscript{g}R\textsuperscript{IIIB}\textsubscript{6/2} mice (Fig. 3B, Table III) in the absence of substantial autoantibody titers suggests that in the absence of Fc\textsubscript{g}RIB, the clearance of IgG–IC is impaired. This is in agreement with our previous observations in a model of Ag-induced arthritis (23) and might point to an important role of Fc\textsubscript{g}RIB on resident mesangial cells, on which Fc\textsubscript{g}RIB is the most abundantly expressed Fc\textsubscript{g}R (24), in the clearance of IgG–IC in the kidney.

**Spontaneous autoimmunity in Fc\textsubscript{g}RIB\textsubscript{129/2} mice with 129-derived Sle16 locus**

To confirm further the contribution of the Sle16 locus to lowering the threshold for breaking tolerance in B6 mice, the development

Table III. IgG and C3 deposition in kidneys of Fc\textsubscript{g}RIB\textsubscript{129/2} and Fc\textsubscript{g}RIB\textsubscript{6/2} mice

<table>
<thead>
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<th>Genotype</th>
<th>Mouse No.</th>
<th>Pathology Score</th>
<th>IgG</th>
<th>C3</th>
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<td>12</td>
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<td>+</td>
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<td>17</td>
<td>2</td>
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<td>Fc\textsubscript{g}RIB\textsubscript{129/2}</td>
<td>22</td>
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<td>26</td>
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<td></td>
<td>56</td>
<td>3</td>
<td>+++</td>
<td>++</td>
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IgG and C3 deposition was graded on a scale from negative (−) up to strong positive (+++).
FIGURE 4. Spontaneous autoimmunity in FcγRIII129−/− mice with 129-derived Sle16 locus. A–E, Anti-chromatin (A), anti-dsDNA (B), anti-ssDNA (C), anti-histone (D), and anti-nucleosome (E) autoantibody titers in aged (6–12 mo old) FcγRIII129−/− female mice and 12-mo-old C57BL/6 female control mice as determined by ELISA and presented in arbitrary ELISA units (AEU). F, Spleen weights of aged female FcγRIIB129−/− and C57BL/6 control mice. Horizontal bar indicates median (four to eight mice per group). *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired t test or Mann–Whitney U test).

of spontaneous autoimmunity was analyzed in 12-mo-old female mice of a KO strain generated by targeting the FcyR3 gene, closely linked to the FcyR2b gene, in 129-derived ES cells (FcγRIII129−/− mice) and back-crossed 13 generations into B6 background. The presence of the 129-derived flanking Sle16 region was confirmed by SNP analysis (data not shown). FcγRIII129−/− mice developed a high incidence of autoantibodies specific for dsDNA and ssDNA, nucleosome, chromatin, and histone (Fig. 4A–E, Table IV) and showed other signs of systemic autoimmunity, such as splenomegaly (Fig. 4F). In addition, in FcγRIII129−/− mice the proportion of activated T and B cells was increased (Table V), which is characteristic for the highly autoimmune FcγRIIB129−/− mouse. These results demonstrate the strong contribution of the 129-derived Sle16 region in the development of spontaneous autoimmunity in B6 background (12). However, increased mortality was not observed. The absence of FcγRIII, one of the activating FcγR expressed on circulating myeloid effector cells, which are indispensable for the development of glomerulonephritis (25), most likely increases resistance to lupus.

The 129-derived FcγRIIB flanking Sle16 region is a dominant lupus susceptibility locus

To investigate whether the 129-derived Sle16 locus is a recessive or dominant lupus susceptibility locus in B6 mice deficient for FcγRIIB, the development of lupus was analyzed in F1 offspring from the cross between FcγRIIB129−/− and FcγRIIB6−/− mice. The F1 offspring (FcγRIIB129×B6−/−), containing one copy of the 129-derived flanking region (Sle16) and one copy of the B6-derived flanking region, developed splenomegaly and high ANA titers (Fig. 5A–E), although titers and incidence were decreased compared with titers and incidence in FcγRIIB129−/− mice (Figs. 2A–D, 5A–D) suggesting a gene dose effect. This is in agreement with observations in Sle1 congenic strains. Kidney pathology was significantly increased in F1 mice compared with wild-type B6 mice (Fig. 5F, 5G), whereas mortality was comparable between F1 and FcγRIIB129−/− mice; 25 and 22%, respectively, died before the age of 1 y (Figs. 3D, 5H). These results show that the 129-derived Sle16 locus is a dominant lupus susceptibility locus and explain the finding that heterozygosity of FcγRIIB129−/− mice is sufficient for the development of rheumatoid factor (26). In addition, it demonstrates that the lack of autoimmunity disease in FcγRIIB6−/− mice is not caused by the presence of an unidentified, dominant SLE-suppressor locus.

Positive epistasis between the FcγRIIB6−/− allele and the Yaa locus in lupus

FcγRIIB129−/− male mice carrying the Yaa locus develop severe autoimmune disease characterized by striking splenomegalgy, proteinuria with 90% penetrance, and a median survival of only 4 mo (21). This severe autoimmune phenotype has been explained by strong synergism between the Yaa locus and the FcγR2b KO allele. However, synergism between the 129-derived flanking Sle16 region, with its NZW haplotype, and the B6 genome could not be excluded because strong synergism between Sle1NZW and Yaa has been demonstrated (27). To investigate the putative synergism between the Yaa and FcγR2b KO allele in the absence of the 129-derived Sle16 locus, the FcγRIIB6−/− strain was crossed with a Yaa B6 congenic strain, and the development of autoimmune disease in the FcγRIIB6−/−.Yaa male offspring was analyzed. At the age of 10 mo, the male mice of the Yaa B6 congenic strain developed low but significantly increased anti-chromatin and anti-dsDNA autoantibody titers compared with those of males of the B6 control and FcγRIIB6−/− strain (Fig. 6A, 6B). The penetrance and the titers were further increased in the FcγRIIB6−/−.Yaa male mice (Fig. 6A, 6B), indicating that the absence of FcγRIIB enhanced the mild autoimmune phenotype of the C57BL/6.Yaa males. The FcγRIIB6−/−.Yaa males displayed a moderate lupus phenotype with age characterized by splenomegalgy, monocytosis, and substantial kidney pathology, but only 30% died between the ages of 10 and 12 mo (Fig. 6C–F, Table VI). These results confirm

Table IV. Penetrance of autoantibody titers in FcγRIII129−/− mice

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>% C57BL/6 (n)</th>
<th>% FcγRIII129−/− (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-nucleosome</td>
<td>0 (0/8)</td>
<td>83.3 (5/6)</td>
</tr>
<tr>
<td>Anti-ssDNA</td>
<td>0 (0/4)</td>
<td>71.4 (5/7)</td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>0 (0/4)</td>
<td>57.1 (4/7)</td>
</tr>
<tr>
<td>Anti-chromatin</td>
<td>0 (0/4)</td>
<td>71.4 (5/7)</td>
</tr>
<tr>
<td>Anti-histone</td>
<td>0 (0/4)</td>
<td>85.7 (6/7)</td>
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</table>

Penetrance based on cutoff mean + 2 × SD of C57BL/6 females at 12 mo.
synergism between the Fcγr2b KO allele and the Yaa locus, which in B6 mice is sufficient for the development of fatal lupus in a minority of the male mice after the age of 10 mo. However, FcγRIIBB62/2.Yaa male mice show a moderate lupus phenotype compared with the published highly severe phenotype of the FcγRIIB1292/2.Yaa male mice. This indicates that in the latter one, in addition to synergism between the Fcγr2b KO allele and the Yaa locus, synergism between the Yaa locus and the 129-derived Sle16 locus and the B6 genome strongly contributes to its very severe autoimmune phenotype.

Discussion
Our comparison of two FcγRII KO strains on B6 background, one with 129-derived (FcγRIIB1292/2) and the other with B6-derived (FcγRIIBB62/2) Fcγr2b flanking regions, revealed that in full B6 mice, FcγRIIB deficiency is not sufficient to cause lupus despite its pleiotropic effect affecting not only B cells and macrophages but also dendritic cells (28, 29). In FcγRIIB1292/2 mice, the development of lupus is initiated by epistatic interactions between the 129-derived Fcγr2b flanking Sle16 region and the B6 genome, causing loss of tolerance resulting in the development of autoreactive B cells. This is confirmed by the nonlethal autoimmune phenotype of FcγRIII KO mice back-crossed into B6 background, which contain the same Sle16 region. In FcγRIIB1292/2 mice, the contribution of these autoreactive B cells to the disease process is amplified by the loss of FcγRIIB turning the nonlethal autoimmune phenotype into a fatal lupus phenotype. Our observations support genetic studies based on congenic dissection of the

### Table V. Analysis of splenocyte populations of FcγRIII129−/− mice

<table>
<thead>
<tr>
<th>CD8+CD62Llow</th>
<th>CD4+CD62Llow</th>
<th>CD4+CD69high</th>
<th>CD19+CD69high</th>
<th>CD19+IgMlowIgDlow</th>
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<tbody>
<tr>
<td>25.70 ± 0.4</td>
<td>29.65 ± 0.25</td>
<td>10.55 ± 0.75</td>
<td>0.79 ± 0.22</td>
<td>1.49 ± 0.24</td>
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<td>65.10 ± 4.9</td>
<td>88.08 ± 2.6</td>
<td>32.30 ± 3.4</td>
<td>1.98 ± 0.22</td>
<td>6.68 ± 1.5</td>
</tr>
</tbody>
</table>

Percentages of splenic T and B cells. Results represent mean ± SEM of 10-mo-old mice (M/F). *p < 0.05, **p < 0.01, ***p < 0.001 (significance compared with FcγRIIBB6 fl/fl mice; statistical significance was analyzed by unpaired t test).
Sle1 locus derived from the autoimmune-prone NZW strain showing that Sle1b\textsuperscript{NZW} congenic B6 mice develop ANA independently from the presence of the Fc\textsubscript{g}r2b\textsuperscript{NZW} allele, whereas Fc\textsubscript{g}r2b\textsuperscript{NZW} congenic B6 mice do not develop autoimmunity despite impaired Fc\textsubscript{g}RIIB expression. In contrast, the absence of autoimmunity in Fc\textsubscript{g}RIIB\textsuperscript{B6/2}/\textsuperscript{2} mice seems to be conflicting with the results of a recent functional study using Ig gene cloning from single isolated B cells (30). However, because the Fc\textsubscript{g}RIIB\textsuperscript{129/2}/\textsuperscript{2} mouse was used, most likely Sle16-dependent mechanisms are responsible for the observed development of autoreactive B cells.

Taken together, our results show that Fc\textsubscript{g}RIIB is an autoimmune disease modifier and not, as has been postulated, a “single overriding factor,” which may ultimately dictate whether the disease progresses or not (31). Defining accurately the relative contribution of an individual disease modifier, such as Fc\textsubscript{g}RIIB, to the development of a complex autoimmune disease is very difficult. The model of “threshold liability” can explain a substantial part of the highly severe phenotype of Fc\textsubscript{g}RIIB\textsuperscript{129/2}/\textsuperscript{2}.Yaa male mice. The penetrance of the autoimmune phenotype increases in relation to the increasing number of the susceptibility genes, Yaa, Sle16, and Fc\textsubscript{g}r2b\textsuperscript{2}/\textsuperscript{2}, present in the genome of these male mice. However, in addition to the inheritance in a simplistic additive fashion, epistatic interactions might modify the inheritance of the autoimmune phenotype in a complex fashion that may not be additive (2). This is demonstrated by our observations that in B6 mice, besides the reported positive epistatic interactions between the Sle1b and Yaa loci (27), also epistatic interactions between Fc\textsubscript{g}r2b and yaa resulted in the development of lethal lupus. According to this “multiplicative model,” the interactions of all susceptibility and suppressor alleles present in its genome determine the susceptibility of an individual. As a consequence, the contribution of an individual gene to the development of autoimmune disease can vary depending on all other susceptibility and suppressor loci present in the genome. In addition, environmental conditions also play an important role, as illustrated by the significant differences in kidney pathology between the two genetically

\begin{table}[h]
\centering
\begin{tabular}{llll}
\hline
 & Fc\textsubscript{g}RIIB\textsuperscript{B6/2}/\textsuperscript{2} & Fc\textsubscript{g}RIIB\textsuperscript{B6/2}/\textsuperscript{2}.Yaa & \textit{p} Value \\
\hline
CD4\textsuperscript{+} & 14.4 \pm 2.0 & 14.3 \pm 1.0 & 0.92 \\
CD4\textsuperscript{+}CD62L\textsuperscript{+} & 37.7 \pm 9.5 & 17.2 \pm 9.7* & 0.02 \\
CD4\textsuperscript{+}CD69\textsuperscript{+} & 17.8 \pm 3.9 & 35.7 \pm 10.1* & 0.02 \\
CD8\textsuperscript{+} & 9.8 \pm 2.0 & 8.2 \pm 2.7 & 0.37 \\
CD8\textsuperscript{+}CD62L\textsuperscript{+} & 75.2 \pm 7.6 & 72.1 \pm 7.7 & 0.59 \\
B220\textsuperscript{+} & 53.0 \pm 8.8 & 58.6 \pm 4.7 & 0.31 \\
B220\textsuperscript{+}IgM\textsuperscript{+} & 42.9 \pm 7.0 & 40.0 \pm 5.4 & 0.54 \\
B220\textsuperscript{+}CD69\textsuperscript{+} & 2.1 \pm 0.6 & 6.5 \pm 3.1* & 0.03 \\
\hline
\end{tabular}
\caption{Splenic phenotype of Fc\textsubscript{g}RIIB\textsuperscript{B6/2}/\textsuperscript{2}.Yaa}
\end{table}

Percentages of splenic cell populations. Results represent mean \pm SEM of 10-mo-old mice (M/F) (\textit{n} = 4).

*\textit{p} < 0.05 (significance compared with Fc\textsubscript{g}RIIB\textsuperscript{B6/2} mice; statistical significance was analyzed by unpaired \textit{t} test).
identical aging cohorts of FcγRIIB−/− mice maintained in different facilities (Figs. 3C, 6E).

The absence of autoimmunity in FcγRIIB−/− mice together with the increase in IgG and C3 deposition and pathology in the kidney independent from the development of autoantibodies suggest a minor role of FcγRIIB in maintaining tolerance in the effenter phase but an important role in regulating downstream Ab effector pathways. In humans, autoantibodies develop long before the clinical onset of SLE, suggesting that unknown additional changes are required to trigger the development of the disease (32). We hypothesize that in FcγRIIB−/− mice, FcγRIIB deficiency results in impaired IC clearance and enhanced myeloid effector cell responses, which lower the threshold for the induction of fatal glomerulonephritis by autoantibodies.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References