Chapter 6

In C57Bl/6 mice FcγRIIB deficiency amplifies spontaneous autoimmunity caused by other loci

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SUMMARY

FcγRIIB−/− mice generated by gene targeting in 129-derived ES cells when back-crossed on C57Bl/6, but not on Balb/c background, exhibit a complex phenotype. In addition to the elevated antibody titers, increased immune complex-mediated hypersensitivity and inflammatory responses these mice are characterized by susceptibility to Collagen-Induced Arthritis and the development of severe spontaneous autoimmune disease at age. The FcγRIIB gene is located between Sle1a and Sle1b, two lupus-susceptibility loci. The 129-derived haplotype of the hypomorphic Sle1b locus causes loss of self tolerance in the context of the C57Bl/6 genome. This hampers the analysis of the specific contribution of FcγRIIB deficiency to the development of lupus in FcγRIIB KO mice generated with 129-derived ES cells. Here we demonstrate that C57Bl/6 mice deficient for the FcγRIIB gene have elevated antibody titers, increased immune complex-mediated hypersensitivity and inflammatory responses and are susceptibility to Collagen-Induced Arthritis but do not develop lethal lupus, not even in the presence of the Yaa locus. The development of lethal lupus in FcγRIIB KO mice requires synergistic epistasis between the 129-derived Sle locus and the C57Bl/6 genome indicating that FcγRIIB deficiency acts mainly as an amplifier of autoimmunity initiated by other genetic factors.
INTRODUCTION

Maintenance of immune homeostasis relies on balanced signaling from activating and inhibiting receptors. Defective negative regulation can lead to hyperactivity and autoimmunity. Fc receptors for IgG (FcγRs) are important regulators of the balance between immune activation and suppression (1;2). Functionally, the gene family of mouse FcγRs can be divided in activating, e.g. FcγRI, FcγRIII and FcγRIV, or inhibiting e.g. FcγRIIB receptors. During simultaneous triggering by immune complexes (ICs), the ratio between activating and inhibiting signals will determine whether activation occurs (2). A large body of observations suggests that defective inhibition via FcγRIIB is important in the pathogenesis of lupus (3).

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease, of complex genetic origin and is characterized by loss of tolerance against nuclear antigens (4). In humans, both a -343 G/C promoter polymorphism and a Ile232Thr transmembrane polymorphism in the FcγRIIB gene has been associated with SLE (5;6). Two functional studies have shown that lupus patients present a defective upregulation of FcγRIIB expression in late B cell developmental stages, namely, memory B cells and plasma cells (7;8). In murine SLE models, such as MRL, NZB and BXSB, deletions and polymorphisms in the promoter region and in the third intron of the FcγRIIB gene, have been associated with reduced receptor expression on germinal center B cells and macrophages (9;10). FcγRIIB-deficient mice have augmented humoral responses (35), display enhanced activity in multiple immune-complex (IC)-dependent disease models, including skin and lung Arthus reaction (11;12), IgG- and IgE-mediated systemic anaphylaxis (13) and anti-GBM antibody (Ab)-induced glomerulonephritis (14;15). In addition, FcγRIIB-deficient mice when back crossed on C57Bl/6 background are susceptible to two Ab-dependent models of inducible autoimmunity; the Collagen-Induced Arthritis (CIA) (16) and Goodpasture’s syndrome (17).

Furthermore, FcγRIIB−/− mice generated with 129-derived ES cells and subsequently backcrossed on C57Bl/6 background, but not on Balb/c background, exhibit a strong autoimmune phenotype, characterized by anti-nuclear autoAbs and IC-mediated autoimmune pathology, strongly resembling human SLE (18). Humoral autoimmunity develops in WT mice adoptively transferred with FcγRIIB−/− bone marrow (BM) (18), and restoring FcγRIIB expression by retroviral transfer in BM in lupus-susceptible strains with decreased or no FcγRIIB expression ameliorates humoral autoimmunity (19). These studies suggest that FcγRIIB expression on B cells is crucial but do not exclude a role of FcγRIIB on myeloid cells. FcγRIIB129−/− male mice with the Y chromosome-linked autoimmune accelerator (Yaa, recently identified as a Tlr-7 gene translocation and duplication event (20;21) exhibit strongly enhanced lupus-like disease with a median survival of only 4.5 months (22) suggesting a strong synergy between Yaa and the FcγRIIB−/− allele. Taken together, these observations indicate that defects in FcγRIIB function can play an important role in autoimmune disease.

However, recent results question the role of FcγRIIB in lupus. Fine-mapping placed the FcγRIIB gene outside of the Sle1b genomic region, the prime lupus-susceptibility region within Sle1 locus (23). In C57Bl/6 background the NZW-derived FcγRIIB allele (FcγRIIBNZW), with impaired expression in germinal center (GC) B cells and plasma cells, does not result in
autoAb production in the absence of the NZW-derived Sle1a and Sle1b loci (24). Wild type C57Bl/6 mice congenic for the 129-derived Chromosome 1 telomeric region (including the Sle1 locus and FcγRIIB) develop humoral autoimmunity even in the absence of any targeted mutation indicating that epistasis between the 129-derived Sle1 region and the C57Bl/6 genome is sufficient for the development of an autoimmune phenotype (25). This explains the lupus like phenotypes of SAP (Apcs) and C1q KO mice (genes closely linked to Sle1 region) (26-29).

Lupus-prone mouse strains (NZM2410, BXBS etc.) all carry identical haplotypes of the SLAM/CD2 gene family within the Sle1b region, which, in the context of the background of a non-lupus prone strain (such as C57Bl/6) causes autoimmunity (23). Remarkably the non-autoimmune 129 strain has the same Sle1b haplotype as the autoimmune prone strains. The location of the FcγRIIB gene just between two SLE susceptibility loci hampers the evaluation of the FcγRIIB intrinsic contribution to the development of autoimmune disease in FcγRIIB KO mice derived from 129 ES cells.

Here we define the intrinsic role of FcγRIIB in the development of autoimmunity independently of the 129-derived Sle locus using newly generated FcγRIIB−/− mice (FcγRIIB B6−/− mice) generated with C57Bl/6-derived ES cells. FcγRIIB B6−/− mice developed elevated antibody titers after immunization, enhanced IC-induced inflammation and were susceptible to CIA similar to FcγRIIB 129−/− mice. However, in contrast to FcγRIIB 129−/− mice, FcγRIIB B6−/− mice exhibited only a mild form of spontaneous autoimmunity with moderate autoantibody titres and without splenomegaly and with no increase in mortality. Compared to the autoantibody titres in the Yaa and FcγRIIB B6−/− parental strains, autoantibody titres were somewhat increased in FcγRIIB B6−/−.Yaa male mice of the same age suggesting synergism between Yaa and the FcγRIIB KO locus. However, mortality was not increased within the first 8 months, while it is reported that FcγRIIB 129−/−.Yaa males show a median survival of only 4.5 months (22). Together these observations suggest that the strongly increased mortality of FcγRIIB 129−/−. Yaa males is mainly based on synergism between the 129-derived Sle1 locus and the yaa locus and not on synergism between FcγRIIB and Yaa. In conclusion our results provide strong direct evidence that FcγRIIB deficiency, in itself, is not sufficient for the development of severe, lethal lupus in C57Bl/6 mice. In FcγRIIB 129−/− mice FcγRIIB deficiency acts as an amplifier of autoimmunity controlled by other genes of the Sle1 locus.
MATERIALS AND METHODS

Mice
FcγRIIB<sub>129</sub>-/- mice, generated on 129.C57Bl/6 background were a gift of Dr. T. Takai and backcrossed in our facility onto C57Bl/6 background for 8 generations. FcγRIII-/- mice have been described previously (30). The generation of FcγRIIB<sub>B6</sub><sup>fl/fl</sup> and FcγRIIB<sub>B6</sub>-/- mice is described in the result section. The EIIαCre deleter strain (n=12 on C57Bl/6 background, was kind gift of Dr. Heiner Westphal (31). C57Bl/6.Jico control mice were purchased from Charles River, the Netherlands. Mice were housed and experiments were performed at the SPF animal facilities of the Leiden University Medical Center. All experiments were approved by the local ethical committee. 6-12 weeks old mice were used for the experiments unless stated otherwise. Mice were routinely checked for their genotype by PCR and cytofluorimetry.

Micro-satellite and SNP analysis
Genotyping of the mice was carried out using polymorphic microsatellite markers, a standard polymerase chain reaction and either 4% MetaPhor agarose (Cambrex Bioscience Rockland, Rockland, ME, USA) or 16% polyacrylamide gels stained with ethidium bromide. The positions and sequences were determined from the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org). The list of markers used is available on request. Single nucleotide polymorphisms (SNPs) were analyzed by PCRing over the SNP reported to be different in 129Ola and C57Bl/6. The PCR product was purified and sequenced using one of the PCR primers.

Immunization with TNP-KLH
Mice were immunized subcutaneously in the tail base with 100 µg 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 7, 14, 21, 49 and 56 after the primary immunization. The antibody response was determined by ELISA. Microtiter plates (Greiner Bio-one) were coated with TNP-KLH (20 µg/ml) in carbonate buffer overnight at 4 °C. After washing with PBS/0.1%BSA/0.05%Tween 20, plates were blocked with PBS/1%BSA for 1 hour at room temperature and samples, diluted in PBS (1:16000), were incubated overnight at 4 °C. Plates were washed 5 times with PBS/0.05%Tween 20 and incubated with rabbit-anti-mouse IgG HRP (1:2000) or rabbit-anti-mouse IgG1 HRP (1:1000) in PBS/1%BSA/0.01%Tween 20 for 2 hours at room temperature. Plates were developed using 2, 2-azino-bis- 3-ethylbenzothiazoline-6-sulfonic acid (Sigma) and read at 405nm. Samples were compared to a standard curve prepared from the pooled sera.

In vitro phagocytosis assay
Bone marrow-derived macrophages (BMDM) were cultured from mice in 15 % L-cell conditioned medium (L929) for 8 days in Petri dishes (Greiner Bio-one), pretreated overnight with 20 ng/ml recombinant IL-4, to increase FcγRIIB expression, and subsequently incubated at 37 °C for 1 hour with TNP conjugated sheep red blood cells (SRBC) opsonized with anti-TNP IgG1 Ab. The ingested SRBCs were counted using a light microscope.
Passive Cutaneous Anaphylaxis
Mice were injected intradermally (i.d.) in the ear with 20 µl of varying concentrations (3-10-30 µg) of IgG1 anti-TNP mAb, and 2 hours later given an intravenous (i.v.) injection of 500 µg HSA-TNP in 100 µl PBS with 1% Evans blue. After 30 minutes, extravasation was visualized by blue staining of the ear as an indication of a positive anaphylactic response.

Monitoring the development of spontaneous autoimmunity
Mice were monitored up to the age of 12 months and mortality was recorded. Blood was collected periodically via the retro-orbital plexus and serological analyses performed as described below. After sacrificing the mice spleens were removed and weighted.

Serological analyses
Anti-nuclear Abs
Serum levels of anti-nucleosome Abs were determined by ELISA. Microtiter plates (Greiner Bio-one) were coated overnight with S2-fraction of oligonucleosomes isolated from L1210 cells. After blocking with PBS with 5% FCS, 0.05% Tween 20, the plates were incubated with serially diluted serum (1:100, 1:200, 1:400) for 2 hours. Bound Abs were detected using goat-anti mouse Ig-HRP. Plates were developed with 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and read at 415 nm. Titres were calculated by comparing to a known concentration of anti-nucleosome sample.

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. For anti-dsDNA and anti-ssDNA ELISAs microtitre plates (Greiner Bio-one) were either sensitized with streptavidin (1 µg/ml, Sigma) and then coated with biotinylated dsDNA (200 ng/ml) or coated directly with ssDNA (10 µg/ml). For the anti-chromatin and anti-histone ELISAs, plates were coated with chromatin (0.5 mg/ml) or histones (0.5 mg/ml) respectively. Samples were diluted 1:100 in PBS with 2 % BSA, 0.05% Tween 20, 0.02 % NaN₃. Bound Abs were detected using alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Sigma). Results were expressed as arbitrary units relative to a standard positive sample derived from an MRL/Mp-lpr/lpr mouse pool.

Assessment of renal pathology
Kidneys were dissected for histologic analysis, fixed in 10 % formalin in PBS and embedded in paraffin. 4 µm thick sections were cut and stained with Periodic Acid–Schiff (PAS) stain or hematoxylin and eosin (H&E), examined and photographed with a DC200 Leica camera and Leica DMLB microscope (Leica Microsystems Inc.). Histological sections from the kidneys of aged mice of both FcγRIIB<sub>B6</sub><sup>-/-</sup> and FcγRIIB<sub>129</sub><sup>-/-</sup> mice and C57BL/6 controls were scored by an experienced observer for the following parameters of glomerulonephritis: glomerular cellularity, glomerular basement membrane thickness, active lesions, interstitial fibrosis, tubular casts and glomerular segmental sclerosis as described previously (32).

Flow cytometry
Spleens were isolated, single cell suspensions prepared and labeled using specific Abs for analysis by flow cytometry (FACSScan or LSRII, Beckton Dickinson). Data was analyzed
using Cell Quest software. All Abs were from BD Pharmingen unless stated otherwise: CD3-PE, B220-PE, CD4-APC, CD8β-PE, CD69-FITC, 2.4G2-FITC, K9.361-Alexa488 anti-Ly17.2 (clone K9.361, B6-type FcγRIIB-specific, gift from Dr. Shozo Izui, Geneve).

**Induction of arthritis**

*Collagen-Induced Arthritis*

Mice were immunized by intraperitoneal (i.p.) injection of 100 µg bovine type II collagen (bCII, MD Biosciences) in complete Freund’s adjuvant (CFA, DIFCO) in the tail base followed by a boost 21 days later in the tail base with 100 µg bCII in IFA (DIFCO). Mice were monitored for the development of clinical signs of arthritis 3 times a week and scored using an extensive scoring system (0-60). In brief, each limb was assigned a score of 0-15 on the basis of the number of joints affected, giving a possible maximum score of 60 per mouse. An arthritic toe and knuckle was scored as 1, with a maximum of 10 per paw. An affected ankle or mid paw was given a score of 5. Mice with two legs reaching the maximal score were euthanized and their end score was carried forward in the analysis (33). Serum was collected for the measurement of anti-CII Ab titres on days 14, 35 and 60 after the primary immunization by bleeding the mice via the retro-orbital plexus.

*K/BxN serum-induced arthritis*

K/BxN serum pools were collected from arthritic mice generated from a cross between KRN transgenic mice and Bl10g7 congenic mice (carrying the NOD derived g7 MHC allele) by bleeding the mice via the retro-orbital plexus at the age of 6 weeks. Due to the robust nature of this model, a relative increase in arthritis severity becomes only apparent when suboptimal amount of serum is injected. Therefore a lower amount of K/BxN serum (7.5 µl/g body weight) has been injected that induced only mild arthritis in control mice. Arthritis was induced in the recipient strain by intraperitoneal injection of K/BxN serum at days 0 and 2. Ankle thickness as a parameter of arthritis was measured up to 14 days after the first injection using a caliper and compared to baseline values.

**Induction of experimental nephritis**

For the induction of accelerated nephrotic nephritis (NTN) mice were immunized subcutaneously with 200 µg of purified sheep IgG (Sigma) in complete Freund’s adjuvant (Sigma) followed five days later by intravenous injection of sheep nephrotic serum (NTS). Preparation of NTS has been described previously (34). Mice were assessed daily for development of proteinuria (Haema Combistix).

**Histological studies**

For light microscopy kidneys were fixed overnight in 10 % formalin and embedded in paraffin and stained with periodic acid Schiff (PAS) reagent. Glomerular histology was graded as follows: grade 0, normal; grade I, segmental hypercellularity in 10-25 % of the glomeruli; grade II, hypercellularity involving >50 % of the glomerular tuft in 25-50 % of glomeruli; grade III, hypercellularity involving >50 % of the glomerular tuft in 50-75 % of glomeruli; grade IV, glomerular cellularity in >75 % or crescents in >25 % of glomeruli. Glomerular thrombosis was assessed by grading the degree of PAS-positive material per glomerular cross-section as follows: grade 0: no glomeruli with PAS-positive material; grade 1, 0-25%; grade 2, 25-50%; grade 3, 50-70%; and grade 4, 75-100%. Fifty glomeruli were scored
per section and the mean score was calculated for each mouse. For immunofluorescence kidneys were embedded in OCT, snap-frozen in isopentane cooled with liquid nitrogen and stored at -70 °C. Frozen sections were cut at a thickness of 5 µm and fixed in acetone for 10 minutes. FITC-conjugated goat anti-mouse IgG (Fc-specific, Sigma), FITC-conjugated goat anti-mouse C3 (ICN Biomedicals) and FITC-conjugated goat anti-sheep IgG were used for immunofluorescence studies. All Abs were used at a dilution of 1:200 (except anti-C3 used at 1:100), and incubated for one hour at room temperature. For quantitative immunofluorescence, sections were examined at 100x magnification. The mean intensity of 20 glomeruli for each sample was recorded in arbitrary fluorescence units (AFU). All light microscopic analysis and quantitative immunofluorescence were performed in a blinded manner.

Assessment of renal function
Mice were housed in metabolic cages overnight for urine collection. The albumin concentration was measured by radial immune-diffusion as previously described (34).

Statistics
Parametric data were represented as mean ± SEM. Multiple groups were compared by analysis of variance and further evaluated using Bonferroni or unpaired student’s t test. Nonparametric data were represented by medians and multiple groups compared using a Kruskal-Wallis test or Mann-Whitney U test. A P value of 0.05 was considered statistically significant.
RESULTS

Generation of the conditional C57Bl/6 FcγRIIB KO mouse model

For the generation of FcγRIIB<sup>−/−</sup> mice on C57Bl/6 background (FcγRIIB<sub>B6</sub><sup>−/−</sup>) a targeting vector was constructed based on a 12 kb fragment derived from the BAC clone RPCI23-87B18 of the RPCI 23 Female (C57Bl/6J) mouse BAC genomic library (Pieter J. de Jong). 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 intron 5 (Figure 1A). Gene targeting was performed in C57Bl/6-derived ES cells (Bruce4). Clones in which homologous recombination occurred were identified by Southern blotting (Figure 1B) and subsequently injected in albino C57Bl/6 blastocysts. The obtained chimeras were crossed with albino C57Bl/6 mice and the F1 positive for the FcγRIIB targeted allele was crossed with the EIIaCre deleter strain and the resulting F2 with C57Bl/6. The EIIaCre negative F3 were intercrossed resulting in the following homozygous recombinant FcγRIIB strains: FcγRIIB<sub>B6</sub>neo/<sup>−</sup>neo, the hypomorph strain with the neo selection marker still in place; the FcγRIIB<sub>B6</sub>fl/fl with the floxed FcγRIIB gene without neo selection marker gene and the FcγRIIB<sub>B6</sub>−/−, the FcγRIIB null mutant, in which the exons 4 and 5, encoding the second extracellular ligand binding (EC2) and transmembrane (TM) domains, are deleted (Figure 1A). Cre-mediated recombination was analyzed with PCR and Southern blot (Figure 1C and D). The phenotype of FcγRIIB<sub>B6</sub>−/− mouse was analyzed in a series of in vitro and in vivo assays and compared with the phenotype of the FcγRIIB<sub>129</sub>−/− mouse (generated with 129-derived ES cells and subsequently backcrossed 8 generations on C57Bl/6 background). FcγRIIB<sub>B6</sub>fl/fl and C57Bl/6 mice, which responded similar in all assays were used as controls. The phenotype of the FcγRIIB<sub>B6</sub>neo/<sup>−</sup>neo mice, which expressed ~2% of WT levels of FcγRIIB was indistinguishable from the phenotype of FcγRIIB<sub>B6</sub>−/− mice (data not shown).

FcγRIIB<sub>B6</sub>−/− and FcγRIIB<sub>B6</sub>fl/fl mice developed normally and showed normal breeding characteristics. No gross abnormalities were observed in these mice. The expression of FcγRIIB on splenic B cells from FcγRIIB<sub>B6</sub>fl/fl and C57Bl6 mice was similar, while no FcγRIIB expression was observed in FcγRIIB<sub>B6</sub>−/− mice (Figure 2A). Similar results were obtained with B cells and myeloid cells isolated from peripheral blood, bone marrow and peritoneal cavity (data not shown).

Increased antibody titres and antibody-dependent effector cell functions in FcγRIIB<sub>B6</sub>−/− mice

FcγRIIB<sub>129</sub>−/− mice show enhanced immune functions in a number of in vivo and in vitro experimental systems (2;35). To determine T cell-dependent immune responses, FcγRIIB<sub>B6</sub>−/− mice were immunized with TNP-KLH. FcγRIIB<sub>B6</sub>−/− mice, similar to FcγRIIB<sub>129</sub>−/− mice, exhibited elevated TNP-KLH-specific IgG titers after immunization compared to C57Bl/6 mice (p<0.001) (Figure 2B). It has previously been reported that phagocytosis of opsonized sheep red blood cells (SRBC) by FcγRIIB<sub>129</sub>−/− macrophages is elevated (36). Like bone-marrow derived macrophages (BMDMs) from FcγRIIB<sub>129</sub>−/− mice, BMDMs from FcγRIIB<sub>B6</sub>−/− mice showed increased phagocytic activity compared to BMDMs from C57Bl/6 mice (Figure 2C)
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**Figure 1. Generation of FcγRIIB<sup>−/−</sup> mice**

(A) From top to bottom: schematic representation of the mouse FcγRIIB wild-type gene, the targeting construct, the targeted allele and the genomic configurations of the gene in mice obtained after the Cre-mediated *in vivo* deletion of the Neo selection cassette, the FcγRIIB<sup>Neo<sub>fl/fl</sub></sup>, or exon 4 and 5 are marked in accordance to the functional domains they encode: S1 and S2 signal peptide; EC1 and EC2 the extracellular immunoglobulin-like domains; TM/C, transmembrane-cytoplasmic tail region. Restriction sites are as follows: SphI (S), HindIII (H), XbaI (X). Coding parts depicted as closed boxes, non-coding parts as open boxes.

(B) Southern blot analysis of targeted ES cells using a 3’ probe and a 5’ probe in combination with HindIII and SphI digestion of the genomic DNA respectively. Locations of the probes are indicated with filled squares on panel A.

(C) PCR screening of recombination events after the Cre-mediated *in vivo* deletion. Locations of the primers are depicted on panel A with arrows, P1 to P6.

(D) Southern blot analysis of genomic DNA digested with XbaI from mice homozygous for three different configurations after Cre-mediated *in vivo* recombination. The location of the probe that maps on the exon C1 is indicated with an open square on panel A.

(**** p<0.001, Bonferroni’s multiple comparison test). Passive cutaneous anaphylaxis induced by IgG1-containing immune complexes (ICs) is augmented in FcγRIIB<sup>129<sub>−/−</sub></sup> mice because in the mast cells of these mice the FcγRIIB-dependent down regulatory signal counterbalancing...
the FcγRIII mediated activation by immune complexes is absent (35). Similarly, FcγRIIB<sup>B6</sup>-/- mice mounted an effective anaphylactic reaction at 10 fold lower concentration of antigen than C57Bl/6 mice (Figure 2D). Taken together, FcγRIIB<sup>B6</sup>-/- mice exhibited increased antibody titres and antibody effector cell functions, similar to FcγRIIB<sup>129</sup>-/- mice.
Development of spontaneous autoimmunity in FcγRIIB<sup>B6</sup><sup>−/−</sup> mice

FcγRIIB<sup>129</sup><sup>−/−</sup> mice when back-crossed onto C57Bl/6, but not onto Balb/c background, develop spontaneously, SLE-like autoimmune disease starting from 3-4 months of age and die prematurely due to glomerulonephritis (18). To investigate the development of spontaneous autoimmunity in FcγRIIB<sup>B6</sup><sup>−/−</sup> mice, cohorts of mice were followed during a period of at least one year.

**FcγRIIB<sup>B6</sup><sup>−/−</sup> mice showed normal lifespan**

Mortality of female FcγRIIB<sup>B6</sup><sup>−/−</sup> mice at 6, 9 and 12 months (0% (0/51), 3.9% (2/51), 13.0% (6/46)) was not increased compared to C57Bl/6 mice (0% (0/12), p=0.299 at 6 months; 0% (0/12), p=0.662 at 9 months; 0% (0/7), p=0.459 at 12 months, Fisher’s exact test) (Figure 3A). In contrast, mortality of female FcγRIIB<sup>129</sup><sup>−/−</sup> mice reached 12.2% (5/41) at 6 months, 40% (10/25) at 9 months and 83.3% (10/12) at 12 months, a significant increase compared to C57Bl/6 and FcγRIIB<sup>B6</sup><sup>−/−</sup> mice confirming results from literature (p<0.05; p<0.01; p<0.01, compared to FcγRIIB<sup>B6</sup><sup>−/−</sup> mice).

**FcγRIIB<sup>B6</sup><sup>−/−</sup> mice develop autoAbs with a more restricted specificity, lower titres and lower incidence compared to FcγRIIB<sup>129</sup><sup>−/−</sup> mice**

A major hallmark of SLE is loss of tolerance against nuclear antigens characterized by the occurrence of nuclear autoAbs (4). Several types of autoAbs have been found in aged FcγRIIB<sup>129</sup><sup>−/−</sup> mice (18). Therefore Ab titres against nucleosome (NS), ssDNA, dsDNA, histone and chromatin from serum of 6/7 and at 8/9 months were analyzed using ELISA. FcγRIIB<sup>B6</sup><sup>−/−</sup> mice at 6/7 months exhibited elevated titres against ssDNA, dsDNA and chromatin, but not against nucleosome or histone structures. At 8/9 months FcγRIIB<sup>B6</sup><sup>−/−</sup> mice had elevated titres against nucleosomes and ssDNA but not dsDNA, chromatin or histones (Figure 3B to F). FcγRIIB<sup>129</sup><sup>−/−</sup> mice displayed enhanced titres against all nuclear structures analyzed at both 6/7 months and 8/9 months (Figure 3B to F, * p<0.05, ** p<0.01, *** p <0.001, Mann-Whitney U). Penetrance of autoAbs was calculated using the mean plus 2 x SD of C57Bl/6 titres at 6/7 months as a cut off (Table 1). In addition to anti nuclear autoAbs FcγRIIB<sup>129</sup><sup>−/−</sup> mice also develop low but significant titres of anti-erythrocyte autoAbs. In contrast anti-erythrocyte autoAbs were undetectable in FcγRIIB<sup>B6</sup><sup>−/−</sup> mice (data not shown). In conclusion, although FcγRIIB<sup>B6</sup><sup>−/−</sup> mice developed spontaneously autoAbs indicating that tolerance can be broken in these mice, specificity was more restricted and both titres and penetrance were significantly lower compared to the same characteristics of the autoAbs in FcγRIIB<sup>129</sup><sup>−/−</sup> mice.

**FcγRIIB<sup>B6</sup><sup>−/−</sup> mice failed to develop splenomegaly**

Splenomegaly has been described in FcγRIIB<sup>129</sup><sup>−/−</sup> mice (18). In FcγRIIB<sup>B6</sup><sup>−/−</sup> mice the average spleen size was normal both at 8/9 and 10/12 months of age (Figure 3G). In contrast, as expected, the average spleen size of FcγRIIB<sup>129</sup><sup>−/−</sup> mice was increased compared to both C57Bl/6 controls at 8/9 and 10/12 months (p<0.05 and p<0.001) and FcγRIIB<sup>B6</sup><sup>−/−</sup> mice at both time points (p<0.01 and p<0.001) (Figure 3G).

**FcγRIIB<sup>B6</sup><sup>−/−</sup> mice have normal splenic populations**

Since FcγRIIB<sup>B6</sup><sup>−/−</sup> mice did not exhibit splenomegaly further analysis of the spleen was performed by flow cytometric analysis of splenocytes from 10/12 month old mice. Cytofluorimetric analysis revealed significantly increased percentages of activated T cells (CD3<sup>+</sup>CD69<sup>+</sup>) in FcγRIIB<sup>129</sup><sup>−/−</sup> (48.0 ± 5.6%) but not in FcγRIIB<sup>B6</sup><sup>−/−</sup> (35.2 ± 2.9) mice compared
FcγRIIB in autoimmunity

Figure 3. FcγRIIB<sub>B6</sub><sup>−/−</sup> mice only develop mild form of spontaneous autoimmunity
(A) C57Bl/6 (n=7), FcγRIIB<sub>129</sub><sup>−/−</sup> (n=12), and FcγRIIB<sub>B6</sub><sup>−/−</sup> (n=46) female mice were followed to calculate the cumulative mortality at the indicated ages. Values are plotted as the cumulative mortality of the population of the given month.
(B to F) Anti-NS, anti-ssDNA, anti-dsDNA, anti-chromatin and anti-histone titres at 6/7 and 8/9 months in C57Bl/6, FcγRIIB<sub>129</sub><sup>−/−</sup>, and FcγRIIB<sub>B6</sub><sup>−/−</sup> mice. Horizontal bar represents median, dotted line represents the cut-off level; determined as two times the standard deviation above the mean level found in C57Bl/6 serum.
(G) Spleen weights of female mice at 6/7, 8/9 and 10/12 month of age (* P<0.05, ** P<0.01, *** P<0.001, unpaired t-test or Mann-Whitney-U test).

to FcγRIIB<sub>B6</sub><sup>−/−</sup> mice (26.9 ± 1.4) (Bonferroni’s, p<0.001) (Table 2). Influx of CD11b<sup>+</sup> myeloid cells was significantly increased in FcγRIIB<sub>129</sub><sup>−/−</sup> spleens (14.4 ± 2.5) compared to FcγRIIB<sub>B6</sub><sup>−/−</sup> spleens (8.3 ± 0.6, p<0.01) or C57Bl/6 (6.6 ± 0.4, p<0.01) (Bonferroni). The Th/Tc ratio (CD4<sup>+</sup>/CD8<sup>+</sup>) was increased significantly in FcγRIIB<sub>129</sub><sup>−/−</sup> (3.1 ± 5.2) but not in FcγRIIB<sub>B6</sub><sup>−/−</sup>.
mice (2.42 ± 0.24) compared to FcγRIIB<sub>B6</sub><sup>fl/fl</sup> mice (1.95 ± 0.1, p<0.05) or to C57Bl/6 mice (Bonferroni) (Table 2). Taken together, flow cytometric analysis of splenocytes did not reveal an activated lymphocytic compartment in FcγRIIB<sub>B6</sub>-/- mice as found in FcγRIIB<sub>129</sub>-/- mice.

Table 1. Penetrance of autoantibodies

FcγRIIB<sub>B6</sub>-/- mice developed less severe kidney pathology compared to FcγRIIB<sub>129</sub>-/- mice. FcγRIIB<sub>B6</sub>-/- mice when back-crossed onto a C57Bl/6, die prematurely due to severe glomerulonephritis (18). IC deposition in the kidney initiates inflammation via activating FcR and the complement system (37). FcγRIIB-deficiency results in enhanced disease activity (38). FcγRIIB<sub>129</sub>-/- kidneys displayed clear signs of severe glomerulonephritis confirming previous reports (Figure 4A-D) (18).

Table 2. Analysis of populations in the spleen

Percentage of hematopoietic cell populations in spleens was determined by flow cytometric analysis. Results represent mean and SEM of 12/14 months old mice (m/f). Statistical significance was analyzed by Bonferroni test, asterisk indicate significance compared to C57Bl/6 mice (*p<0.05, **p<0.01), to FcγRIIB<sub>B6</sub>-/- mice (#p<0.05, ###p<0.001) or to FcγRIIB<sub>129</sub>-/- mice ($$p<0.01). Percentages indicate cell population per total spleen cells or per total B or T cell population.
Surprisingly, most parameters of glomerulonephritis were also substantially increased in FcγRIIB<sub>B6</sub>-/- mice when compared to age-matched C57Bl/6 mice (Figure 4B-D). Taken together, FcγRIIB<sub>B6</sub>-/- mice developed characteristic kidney damage, described for FcγRIIB<sub>129</sub>-/- mice, although with lower numbers of active lesions (Figure 4D).

Since FcγRIIB<sub>129</sub>-/- mice die prematurely and FcγRIIB<sub>B6</sub>-/- mice do not we concluded that
the substantial kidney pathology observed in FcγRIIB<sub>B6</sub>−/− mice is not sufficient to increase mortality in these mice.

**Weak synergism between the FcγRIIB<sup>−/−</sup> allele and Yaa locus in the development of autoimmunity**

FcγRIIB<sub>129</sub>−/−.Yaa male mice exhibit strongly enhanced lupus-like disease with a median survival of only 4.5 months (22). In contrast, FcγRIIB<sub>B6</sub>−/−.Yaa mice were still alive with no sign of disease at the age of 8 months. Their anti-dsDNA and anti-chromatin autoAb titers were significantly increased compared to the anti-dsDNA and anti-chromatin autoAb titers of the FcγRIIB<sub>B6</sub>−/− parental strain and the C57Bl/6 control but this increase was not significant compared to the anti-dsDNA and anti-chromatin autoAb titers of the Yaa parental strain (Figure 5A and B) (Kruskall–Wallis, Dunn’s test). Monocytosis, as determined by CD11b+ cells in PBMCs (39), was significantly increased in FcγRIIB<sub>B6</sub>−/−.Yaa mice compared to the monocytosis in Yaa mice at 8 month 32.5 ± 11.4 compared to 11.2 ± 4.5 (Figure 5C) (ANOVA, Kruskall–Wallis).

These data show that on C57Bl/6 background FcγRIIB-deficiency and the Yaa locus act synergistically in the development of monocytosis without increasing mortality. The development of autoAbs, mainly determined by the presence of the Yaa locus, is hardly amplified by FcγRIIB-deficiency. The reported strongly increased susceptibility of FcγRIIB<sub>129</sub>−/−.Yaa male mice to lupus-like disease (22) compared to the susceptibility for lupus of FcγRIIB<sub>129</sub>−/− mice can be explained by epistatic interactions of the 129-derived Sle1 locus and the Yaa locus.

**FcγRIII<sup>−/−</sup>** mice display an autoimmune phenotype similar to that of C57Bl/6 mice congenic for the 129-derived Sle1 region

From the direct comparison of the phenotypes of the FcγRIIB<sub>B6</sub>−/− and FcγRIIB<sub>129</sub>−/− mice presented here we concluded that the first strain is hardly and the later one is strongly...
susceptible to the development of spontaneous autoimmune disease indicating that epistasis between the C57Bl/6 genome and the 129-derived Sle1 locus present in FcγRIIB-/- but absent in FcγRIIBB6-/- mice plays a dominant role in the disease process. In order to confirm the dominant role of epistasis between the 129-derived Sle1 locus and the C57Bl/6 genome in the development of spontaneous autoimmunity aged FcγRIII KO mice generated with 129-derived ES cells and subsequently backcrossed 12 generations to C57Bl/6 (FcγRIII129-/-) were examined for the presence of autoAbs and splenomegaly. No increase in mortality was observed in these mice (data not shown). Between the age of 8 and 12 months the average spleen size of FcγRIII129-/- mice was significantly increased compared to the spleen size of age-matched C57Bl/6 controls (318.3 ± 204.0 versus 125.7 ± 25.82 (p<0.001, t-test) (Figure 6A). The same FcγRIII129-/- mice displayed elevated autoAb titres for anti-nucleosome (p<0.01, Mann-Whitney U), anti-ssDNA (p<0.05, Mann-Whitney U), anti-dsDNA (p<0.05, Mann-Whitney U) and anti-histone Abs compared to C57Bl/6 controls (p<0.05, Mann-Whitney U) (Figure 6B to F). Although FcγRIII129-/- mice also displayed elevated anti-chromatin Ab titres the difference was not significant (p=0.230, Mann-Whitney U).

From these observations we concluded that the presence of the 129-derived Sle1 region in FcγRIII129-/- mice causes autoimmunity in these mice.

Figure 6. Autoimmunity in FcγRIII129-/- mice
(A) Spleen sizes (mg) of female aged mice (** P<0.01, unpaired t test)
(B to F) AutoAb titres in aged female mice. C57Bl/6 values are from 12 month old, FcγRIII129-/- values are from mice aged 6 to 12 months. Horizontal line indicates mean.
**FcγRIIB\[^{B6}\]^-/-** mice develop Collagen-Induced Arthritis (CIA) with lower incidence compared to **FcγRIIB\[^{129}\]^-/-** mice

FcγRIIB\[^{B6}\]^-/- mice, unlike C57Bl/6 mice, develop CIA when immunized with bovine type II collagen (bCII) (16). This susceptibility is partially attributed to the anti-bCII titers that FcγRIIB\[^{129}\]^-/- mice exhibit after immunization. In order to define the specific contribution of FcγRIIB-deficiency and the 129-derived Sle1 locus in the susceptibility to CIA, both FcγRIIB\[^{129}\]^-/- and FcγRIIB\[^{B6}\]^-/- mice were immunized with bCII. FcγRIIB\[^{129}\]^-/- mice developed CIA with an incidence of 95.5% (21 out of 22 mice) (Figure 7A) similar to published data. C57Bl/6 mice were protected from CIA development; 0 out of 10 developed arthritis (Figure 7A). FcγRIIB\[^{B6}\]^-/- mice developed CIA with an incidence of 61.9% (13 out of 21) at the end of the experiment (day 60), which was significantly lower compared to the incidence of FcγRIIB\[^{129}\]^-/- mice (p<0.05, Fisher’s exact test). However, the severity of arthritis as determined by mean maximum severity of the clinical scores was not different in FcγRIIB\[^{129}\]^-/- (40.7 ± 3.8) and FcγRIIB\[^{B6}\]^-/- mice (38.2 ± 5.3) (means ± SD; p = 0.696, t-test) (Figure 7B). There was no difference observed in the onset of the disease: FcγRIIB\[^{129}\]^-/- (25.7 ± 9.4; n=21) and FcγRIIB\[^{B6}\]^-/- mice (27.9 ± 2.1; n=21) (means ± SD; p = 0.469, t-test). Histological examination of knee sections from arthritic FcγRIIB\[^{129}\]^-/- and FcγRIIB\[^{B6}\]^-/- mice revealed thickened synovium and severe destruction of cartilage and bone, indistinguishable between the two groups. No signs of inflammation or destruction of the cartilage-bone structure was seen in control groups (Figure 7C). Anti-bCII IgG titers in the serum of both FcγRIIB\[^{129}\]^-/- and in FcγRIIB\[^{B6}\]^-/- mice were elevated compared to C57Bl/6 mice at both day 35 (p<0.001 and p<0.05 for FcγRIIB\[^{129}\]^-/- and FcγRIIB\[^{B6}\]^-/-, respectively) and at 60 days after immunization (p<0.05, t-test) (Figure 7D). Although the anti-bCII IgG titers in the serum of FcγRIIB\[^{B6}\]^-/- mice were lower compared to the anti-bCII IgG titers in FcγRIIB\[^{129}\]^-/- mice the difference is not significant. Taken together, these results show that deficiency of FcγRIIB is sufficient to render C57Bl/6 mice susceptible for CIA, although with a lower incidence compared to FcγRIIB\[^{129}\]^-/- mice. Once arthritis develops in FcγRIIB\[^{B6}\]^-/- mice, the pathology is indistinguishable from that of FcγRIIB\[^{129}\]^-/- mice.

<table>
<thead>
<tr>
<th>AutoAb</th>
<th>C57Bl/6</th>
<th>FcγRIIB[^{129}]^-/-</th>
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<tbody>
<tr>
<td>Anti-NS</td>
<td>0 % (0/8)</td>
<td>83.3 % (5/6)</td>
</tr>
<tr>
<td>Anti-ssDNA</td>
<td>0 % (0/4)</td>
<td>57.1 % (4/7)</td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>0 % (0/4)</td>
<td>42.9 % (3/7)</td>
</tr>
<tr>
<td>Anti-chromatin</td>
<td>0 % (0/4)</td>
<td>57.1 % (4/7)</td>
</tr>
<tr>
<td>Anti-histone</td>
<td>0 % (0/4)</td>
<td>71.4 % (5/7)</td>
</tr>
</tbody>
</table>

**Table 3.** Penetration of autoantibody titres in FcγRII\[^{129}\]^-/- mice

Penetration based on cut off mean + 2xSD of C57Bl/6 females at 12 months.
Figure 7. FcγRIIB<sup>−/−</sup> mice are susceptible to Collagen-Induced Arthritis (CIA)
(A) Incidence and (B) severity scores of mice after CIA induction. Data are combined from three independent experiments. Incidence in FcγRIIB<sub>129</sub>−/− and FcγRIIB<sub>B6</sub>−/− groups are significantly higher compared to B6 mice, p<0.001 and p=0.012 respectively (Fisher’s exact test). End point incidence in FcγRIIB<sub>B6</sub>−/− mice is lower than in FcγRIIB<sub>129</sub>−/− mice (Fisher’s exact test: p<0.01). Both FcγRIIB<sub>B6</sub>−/− and FcγRIIB<sub>129</sub>−/− mice have a significantly increased incidence compared to C57Bl/6 mice.
(C) Histological presentation of CIA. Representative knee cross-sections from a normal C57Bl/6 mice show normal cartilage-bone structure. In contrast arthritic mice from FcγRIIB<sub>B6</sub>−/− and FcγRIIB<sub>129</sub>−/− groups exhibit thickened synovium together with severe destruction of cartilage and bone. Original magnifications: 100x.
(D) Anti-collagen type II total IgG Abs serum titers as determined by ELISA pooled from two independent experiments. Arbitrary titres compared to a pooled reference sera and the mean ± SEM is shown. Values are compared to levels of B6 mice (Students unpaired t-test * p<0.05, *** p<0.001).

FcγRIIB<sub>B6</sub>−/− mice develop milder K/BxN serum-induced arthritis compared to FcγRIIB<sub>129</sub>−/− mice

Since FcγRIIB<sub>B6</sub>−/− mice showed a decreased incidence in the development of CIA compared to FcγRIIB<sub>129</sub>−/− mice but exhibited no significant difference in anti-bCII titres, we turned to a passive IC-mediated arthritis model, the K/BxN serum-induced arthritis, in order to determine whether this difference in incidence was due to differences in the downstream effector phase. In the K/BxN model the disease is induced by passive transfer of serum from K/BxN mice that develop spontaneous arthritis (40;41). Arthritis is initiated by serum IgG that recognize GPI in the serum (42). Previous studies have shown increased joint inflammation in FcγRIIB<sub>129</sub>−/− mice compared to WT controls in models of arthritis in...
which disease is induced by passive injection of pathogenic antibodies (43-45). Whilst ankle thickening in FcγRIIB129−/− mice was significantly increased compared to FcγRIIBB6fl/fl mice from day 3 to day 14 (p<0.01 and p<0.001), it only reached significance on days 6-7 and 12-14 in FcγRIIBB6−/− mice (Bonferroni’s multiple comparison test, p<0.05). Mean maximum ankle thickening (AT) in FcγRIIB129−/− mice (1.53 ± 0.06 mm) was significantly elevated compared to FcγRIIBB6fl/fl mice (0.59 ± 0.14 mm) (Bonferroni’s multiple comparison test, p<0.001) (Figure 8A and B). This significant increase in mean maximum ankle thickening was not observed in FcγRIIBB6−/− mice. These results show that FcγRIIB controls IC-induced joint inflammation also on a C57Bl/6 background and suggest that 129-derived alleles may contribute to the enhanced severity of the downstream effector phase of arthritis observed in the FcγRIIB129−/− mice compared to the severity in FcγRIIBB6−/− mice.

**FcγRIIBB6−/− mice developed Nephrotoxic Nephritis with an intermediate severity compared to the severity in FcγRIIB129−/− and C57Bl/6 controls**

Since in the CIA model in FcγRIIBB6−/− mice tolerance can still be broken but with a lower incidence we turned to the accelerated nephrotoxic nephritis model (NTN) in order to determine whether there is a difference between FcγRIIB129−/− and FcγRIIBB6−/− mice when a non-autoimmune inflammatory disease is induced. In the accelerated NTN model mice are pre-sensitized therefore the autologous phase is initiated at the same time the nephrotoxic serum (NTS) is administered. It is known that the autologous phase of NTN is FcγR dependent (14;46) and FcγRIIB is reported to play an inhibitory role in accelerated NTN (47). Analysis of daily urine sample revealed detectable proteinuria from day 1 and day 2 after NTS administration in both FcγRIIB129−/− and FcγRIIBB6−/− mice, but not in C57Bl/6 mice (data not shown). Haematuria increased strongly in FcγRIIB129−/− mice from day 2 (data not shown), consequently the experiment was closed on day 5. Albuminuria measurements from end point (Day 5) urine samples revealed significantly reduced albumin clearance in FcγRIIB129−/− mice but not in FcγRIIBB6−/− mice (Figure 9A) (Kruskal-Wallis, p<0.01). Histological analysis

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**Figure 8. Enhanced severity of passive K/BxN serum-induced arthritis in FcγRIIBB6−/− mice**

(A) Suboptimal volumes of K/BxN sera (2x100 ul) were injected in different mouse strains and arthritis development was followed by measurement of ankle thickening. Values represent ankle thickening (in mm) vis-à-vis to the baseline value.

(B) Summary of K/BxN serum-induced arthritis data. AT: ankle thickening (data compared to FcγRIIBB6fl/fl mice (* or # p<0.05, ** p<0.01, *** p<0.001, Student’s unpaired t-test). Results are representative of two independent experiments performed. No significant difference was observed between FcγRIIBB6−/− and FcγRIIBB6−/−.
of kidneys showed a slight increase in glomerular cellularity in both FcγRIIB<sup>129</sup>-/- and FcγRIIB<sup>B6</sup>-/- mice compared to C57Bl/6 mice, reaching significance for FcγRIIB<sup>B6</sup>-/- mice only (Kruskal-Wallis, p<0.05) (data not shown). Glomerular thrombosis was significantly higher in FcγRIIB<sup>129</sup>-/- mice and FcγRIIB<sup>B6</sup>-/- mice compared to C57Bl/6 (Figure 9B) (Kruskal-Wallis, p<0.001 and p<0.05 respectively). Although FcγRIIB<sup>129</sup>-/- mice displayed higher levels of thrombosis compared to FcγRIIB<sup>B6</sup>-/- mice, the difference was not significant (p>0.05, Kruskal-Wallis). Both C3 and mouse IgG deposition were increased in both FcγRIIB<sup>129</sup>-/- and FcγRIIB<sup>B6</sup>-/- mice compared to C57Bl/6 mice (Figures 9C and D, Kruskal-Wallis). Total anti-sheep IgG titres in the end point sera were enhanced in FcγRIIB<sup>129</sup>-/- mice (0.626 ± 1.06) compared to FcγRIIB<sup>B6</sup>-/- and C57Bl/6 mice (0.243 ± 0.04 and 0.214 ± 0.04, respectively) although this difference did not reach significance. IgG2b is reported to be the principle isotype in this model of nephrotoxic nephritis (47); however, no significant differences were observed between FcγRIIB<sup>B6</sup>-/- and FcγRIIB<sup>129</sup>-/- mice, in the IgG1, IgG2b or IgG3 response (Table 4). FcγRIIB<sup>B6</sup>-/- mice showed increased IgG2a titres (1.137 ± 0.29) compared to C57Bl/6 mice (0.844 ± 0.07, p<0.001, one way ANOVA).
Table 4. Anti-sheep IgG titres in NTN model
Mean OD ± standard deviation for anti-sheep total IgG, IgG1, IgG2a, IgG2b and IgG3 (* p<0.001, one way ANOVA, compared to C57/Bl6).

<table>
<thead>
<tr>
<th>Anti-sheep IgG</th>
<th>C57Bl/6</th>
<th>FcγRIIB(^{129,-})-/-</th>
<th>FcγRIIB(^{B6,-})-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgG</td>
<td>0.214 ± 0.04</td>
<td>0.626 ± 1.06</td>
<td>0.243 ± 0.04</td>
</tr>
<tr>
<td>IgG1</td>
<td>0.171 ± 0.03</td>
<td>0.239 ± 0.09</td>
<td>0.185 ± 0.02</td>
</tr>
<tr>
<td>IgG2a</td>
<td>0.844 ± 0.07</td>
<td>1.137 ± 0.29 *</td>
<td>0.955 ± 0.13</td>
</tr>
<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>
</tr>
</tbody>
</table>

In conclusion both FcγRIIB\(^{129\,-}\)-/- and FcγRIIB\(^{B6\,-}\)-/- mice showed enhanced disease compared to C57Bl/6 mice as revealed by accelerated onset of disease, increased renal injury, albuminurea and increased kidney deposition of mouse IgG and C3 (Figure 9C and D). FcγRIIB\(^{B6\,-}\)-/- mice showed disease with an intermediate severity between the disease severity in FcγRIIB\(^{129\,-}\)-/- and C57Bl/6 controls, which is perhaps due to the lower IgG2a levels observed in these mice compared to FcγRIIB\(^{129\,-}\)-/- mice.
CONCLUDING REMARKS

Despite intense investigations to define the complex role of FcγRIIB in immunity, the contribution of FcγRIIB to the development of SLE remains controversial. Several studies show that FcγRIIB acts as a major regulator in the development of lupus (7;19;48). Yet other, mainly genetic analyses question the relevance of FcγRIIB in SLE (23;24). In the present study we have dissected the complex phenotype of the FcγRIIB129−/− mice by generating FcγRIIB KO mice on C57Bl/6 background (FcγRIIBB6−/− mice) and redefined the role of FcγRIIB in autoimmunity. We provided direct evidence that the 129-derived Sle1 region, directly linked to the FcγRIIB gene, strongly contributes to the severe lethal autoimmune phenotype of FcγRIIB129−/− mice. In FcγRIIBB6−/− mice, in which the 129-derived Sle1 region is absent, the FcγRIIB KO allele causes only mild autoimmunity. However, FcγRIIBB6−/− mice are susceptible to CIA indicating that FcγRIIB plays a role in maintaining tolerance. Moreover, FcγRIIBB6−/− mice exhibit elevated antibody titers after immunization and increased IC-mediated inflammatory responses indicating a crucial background-independent regulatory role for FcγRIIB in antibody production and antibody dependent effector cell functions. Although FcγRIIBB6−/− mice did not show increased mortality, kidney damage in these mice is more often seen than it could be expected from the penetrance of circulating autoAbs. One possible explanation is that the titres of the autoantibodies with the relevant specificity were not determined. In addition, the histology scores of kidneys from FcγRIIBB6−/− mice were comparable to the histology scores of kidneys from FcγRIIB129−/− mice except for ‘active lesions’ which were twice as high in the FcγRIIB129−/− mice. However, at the age the kidneys were collected (9-11 months) the majority of the FcγRIIB129−/− mice had died (70-90%), most likely due to kidney failure, and the kidneys of the dead mice have not been included in the histological analysis. In contrast, within one year mortality was not increased in FcγRIIBB6−/− mice compared to mortality in controls. From these data it is impossible to conclude whether there is a difference in kidney inflammation between the two strains.

Our analysis of splenocyte populations of aged mice did not reveal changes in FcγRIIBB6−/− mice that are characteristic for the splenocyte populations in FcγRIIB129−/− mice, such as increased numbers of monocytes and activated T cells (Table 2). However a more detailed analysis of the different subpopulations of splenocytes is required to draw conclusions about the differences between FcγRIIBB6−/− and FcγRIIB129−/− mice at this point.

The lower incidence of CIA development in FcγRIIBB6−/− mice compared to the incidence of CIA in FcγRIIB129−/− mice suggests that it is more difficult to break tolerance in FcγRIIBB6−/− mice. It would be interesting to see whether C57Bl/6 mice congenic for the NZW-type FcγRIIB alone (24) or for the complete Sle1-region (25) already develop increased anti-collagen titres after immunization with bovine collagen type II.

Interestingly, IC-induced inflammation was consistently elevated in FcγRIIB129−/− mice compared to FcγRIIBB6−/− mice (NTN, K/BxN) suggesting that 129-derived alleles may also influence antibody effector functions. The mild autoimmunity syndrome in FcγRIIB129−/− mice shows that the presence of Sle1 region is sufficient for the development of mild autoimmunity, confirming previous data (25). Splenomegaly was also present in FcγRIIB129−/− mice and therefore can be fully attributed to epistasis between the 129-derived Sle1 locus and the
C57Bl/6 genome. However, it should be noticed that these mice carry the NZW-type FcγRIIB allele, present in the 129-derived Sle1 region, with disturbed expression in macrophages and mature B cells, which may contribute to their autoimmune phenotype. FcγRIIB plays an inhibitory role at multiple levels during the development of lupus (3). Our data show that FcγRIIB plays only a minor role in the loss-of-tolerance, but rather acts as a distal checkpoint in lupus. In the FcγRIIB\textsubscript{129}\textsuperscript{-/-} mice the initial event, loss of tolerance to nuclear antigens, is mediated mainly by epistasis between unidentified gene(s) in the 129-derived Sle1 region and the C57Bl/6 genome. These genes most likely play a role in the control of central tolerance in B cells. The allelic variant of Ly108 present in the NZW-type autoimmune prone Sle1b sublocus, which shows a strong shift in the ratio between two splice isoforms compared to the Ly108 gene in C57Bl/6 mice resulting in a dramatic change in a variety of B cell responses upon triggering the B cell receptor, is a promising candidate lupus susceptibility gene (49).

After B cell tolerance is lost FcγRIIB dysfunction aggravates lupus pathogenesis at several levels. Not only the titers, isotype or specificity of autoAbs may be modified by the lack of FcγRIIB but also their downstream effector mechanisms as suggested by the severe, lethal phenotype of FcγRIIB\textsubscript{129}\textsuperscript{-/-} mice compared to the relatively benign autoimmune phenotype of B6.Sle1 mice (23;25). The role of FcγRIIB as an amplifier is further supported by our observation that FcγRII/III\textsubscript{129}\textsuperscript{-/-} mice have elevated autoAb titers compared to FcγRII\textsubscript{129}\textsuperscript{-/-} (data not shown).

The presence of the NZW-type FcγRIIB allele, although showing disturbed function, is not sufficient for the development of full disease in combination with the 129-derived Sle1 locus. In contrast, the ‘null’ allele of FcγRIIB in combination with the 129-derived Sle1 locus is sufficient. In conclusion, there is a crucial difference between the impaired NZW-type FcγRIIB allele, and the ‘null’ allele with respect to lupus development. Whether this difference is present in another cell type (DCs, FDCs) or is it at another developmental stage in B cells is not yet known. It seems that there is a more substantial loss of tolerance in the FcγRIIR KO in the presence of the 129-derived Sle1 locus (FcγRIIB\textsubscript{129}\textsuperscript{-/-} mice) suggesting that the repertoire of autoantibodies is larger compared to that repertoire in the FcγRIIB\textsubscript{B6}\textsuperscript{-/-} mice. Therefore the chance to develop an autoantibody with a relevant specificity (against a kidney constituent) is much higher in the FcγRII\textsubscript{129}\textsuperscript{-/-} mice compared to this chance in FcγRIIB\textsubscript{B6}\textsuperscript{-/-} mice.

Despite equivalent titers of ANA FcγRIIB\textsubscript{129}\textsuperscript{-/-}.lpr mice are protected from disease progression (22). In contrast, Yajima et al. found enhanced lupus-like disease in FcγRIIB\textsubscript{129}\textsuperscript{-/-}.lpr mice with penetrance reaching 100% (50). This discrepancy can be explained by genetic differences between the FcγRIIB\textsubscript{129}\textsuperscript{-/-}.lpr mouse strains both research groups used. After backcrossing the FcγRIIB\textsubscript{129}\textsuperscript{-/-} strain onto the lpr background in the two independent FcγRIIB\textsubscript{129}\textsuperscript{-/-}.lpr strains different parts of the 129-derived Sle1 locus linked to the FcγRIIB\textsubscript{129}\textsuperscript{-/-} allele were maintained. Unknown genetic variation might also contribute to the difference between the phenotypes of the FcγRIIB\textsubscript{B6}\textsuperscript{-/-} and FcγRIIB\textsubscript{129}\textsuperscript{-/-} mouse strain. The Bruce4 ES cell line is derived from a C57Bl/6 congenic for the NZB-derived Thy-1 locus and therefore contains areas of heterogeneity originating from NZB background (51). In addition, during the generation of the FcγRIIB\textsubscript{B6}\textsuperscript{-/-} line non-C57Bl/6-derived genomic fragments from the EIIaCre deletor line (FVB/N, n=13 C57Bl/6) may have been introduced.
Micro-satellite marker analysis of genomic regions on chr12 and 17 of the FcγRIIB−/− mice, from which is reported that they play a role in the epistasis between the FcγRIIB−/− allele and the C57Bl/6 genome required for the development of autoimmunity (22;52), confirmed their C57Bl/6 origin (data not shown). Additional SNP analysis confirmed that at least 2 Mb of the genomic region telomeric and 1 Mb centromeric from fcgr2b gene was of 129Sv origin in FcγRIIB129−/− mice, whereas all SNPs of that region were of C57Bl/6 origin in FcγRIIBB6−/− mice (data not shown).

In different studies, using the 56R transgenic model it has been shown that FcγRIIB acts at a peripheral checkpoint either to inhibit the secretion of IgG autoAb by autoreactive B cells (48) or by preventing B cells to enter into the GC reaction (53). To exclude a contribution of the linked 129-derived Sle1 locus in these processes these studies should now be repeated in the FcγRIIBB6−/− mice described here.

In conclusion our results suggest that each locus (129-derived SLE, FcγRIIB and Yaa) lowers the threshold for the development of autoimmunity. The cascade of events probably starts with Sle1 and/or Yaa, and is further amplified by FcγRIIB deficiency. FcγRIIB129−/−.Yaa mice that have all three loci show very severe lupus with a median survival of 4.5 months (22). However, all three, Sle1, Yaa and FcγRIIB seem to be involved directly in the maintenance of tolerance, as single factors they all show mild autoimmunity with age. This synergy between these three disease-causing loci indicates the complexity of the human SLE. Our results help to define more accurately the contribution of FcγRIIB to autoimmunity.

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Reference list


