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Siglec-G Deficiency Leads to Autoimmunity in Aging C57BL/6 Mice

Jennifer Müller,* Benjamin Lunz,* Inessa Schwab,* Andreas Acs,* Falk Nimmerjahn,* Christoph Daniel,† and Lars Nitschke*

Siglec-G, a member of the sialic acid–binding Ig-like lectin (Siglec) family, is expressed on B cell and dendritic cell surfaces. It acts as an inhibitory coreceptor and modulates B cell activation, especially on B1 cells, as Siglec-G–deficient mice show mainly a B1 cell–restricted phenotype resulting in increased B1 cell numbers. Although higher B1 cell numbers are discussed to be associated with autoimmunity, loss of Siglec-G does not result in autoimmune disease in BALB/c mice. However, there is evidence from Siglec-G × CD22 double-deficient mice and Siglec-G−/− mice on an autoimmune-prone MRL/lpr background that Siglec-G is important to maintain tolerance in B cells. In this study, we analyzed the role of Siglec-G in induction and maintenance of B cell tolerance on C57BL/6 background and in the FcγRIIb-deficient background. We find that aging Siglec-G–deficient and Siglec-G × FcγRIIb double-deficient mice develop an autoimmune phenotype with elevated autoantibody levels and mild glomerulonephritis. Aging Siglec-G−/− mice have elevated numbers of plasma cells and germinal center B cells, as well as a higher number of activated CD4 T cells, which likely all contribute to autoantibody production. Additional loss of the inhibitory receptor FcγRIIb in Siglec-G−/− mice does not result in exacerbation of disease. These results indicate that Siglec-G is important to maintain tolerance in B cells and prevent autoimmunity.

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Activation of B cells and Ab production need to be tightly regulated to prevent the generation of autoantibodies during immune responses. There are several tolerance mechanisms that sense autoreactive B cells, deplete them from bone marrow or the periphery, and protect from autoimmune diseases. The most important mechanisms of central B cell tolerance, that is, receptor editing, B cell deletion, and anergy induction, are dependent on BCR signaling strength after self-antigen encounter (1).

ITIM-containing inhibitory receptors on B cells are essential for down-modulation of the BCR signal and are therefore key regulators for the maintenance of B cell tolerance (2). Ag binding by the BCR induces phosphorylation of ITIMs on inhibitory receptors by the tyrosine kinase Lyn and recruitment of the negative regulator of BCR calcium (Ca2+) signaling, the Src homology region 2 domain–containing phosphatase (SHP)-1 (3). Deficiencies in inhibitory receptors, such as CD72, PIR-B, or FcγRIIb, can lead to autoimmunity and to systemic lupus erythematosus (SLE)–like disease in mice (4–6). Additionally, the loss of the downstream signaling molecules Lyn or SHP-1 results in strongly elevated BCR signaling and development of autoreactive B cells (7, 8). In contrast, deficiency in the sialic acid–binding Ig-like lectin (Siglec) family members CD22 (on C57BL/6 [B6] background) or Siglec-G (on BALB/c background), which are two other important inhibitory receptors on B cells, does not lead to autoimmunity in mice (9–12). There appears to be a functional redundancy of both molecules for B cell tolerance induction, as Siglec-G × CD22 double-deficient mice do show an autoimmune phenotype at old age with high levels of IgG autoantibodies (with specificities including ssDNA, dsDNA, and RNA) and development of glomerulonephritis (13). However, also the single loss of one of these Siglecs can contribute to development of autoimmunity, as Siglec-G−/− mice crossed to the autoimmune prone MRL/lpr background or CD22−/− mice crossed to the Y chromosome–linked autoimmune accelerator (yaa) background show an autoimmune phenotype (14, 15). Furthermore, murine B cells lacking the Siglec ligand–modifying enzyme sialic acid acetylaselate, which affects CD22 and Siglec-G sialic acid binding, develop a lupus-like autoimmune disease (16). Defective sialic acid acetylaselate function is also genetically linked to a variety of human autoimmune diseases (17).

Siglec-G (or the human ortholog Siglec-10) is constitutively expressed on B cell surfaces and acts as an inhibitory coreceptor of the BCR by negatively modulating its Ca2+ response (9). The extracellular ligand-binding domain of the transmembrane protein Siglec-G recognizes exclusively α2,3- and α2,6-linked sialic acids, terminal carbohydrates abundantly expressed in higher vertebrates and found on many soluble and cell surface–bound glycoproteins (18). The cytoplasmic signaling domain of Siglec-G carries an ITIM- and an ITIM-like motif. For Siglec-G, it was shown that it binds and activates the phosphatase SHP-1, whereas Siglec-10 binds both SHP-1 and SHP-2 phosphatases. These tyrosine phosphatases can dephosphorylate positive regulators of the BCR signaling cascade and cause a dampening of the BCR signal (19, 20). Although Siglec-G is expressed on all B cell subsets (19, 21),
deficiency of Siglec-G in mice causes a strong enlargement of the B1 cell population, but not of B2 cells, indicating that Siglec-G has mainly a B1 cell–restricted inhibitory function. Additionally, Siglec-G–deficient B cells are hyperreactive and exhibit a highly elevated Ca\(^{2+}\) flux as well as a prolonged live span only in B1 but not in B2 cells (9). Recently it was demonstrated that the binding of Siglec-G to its ligand is crucial for its inhibitory function, as the Siglec-G–IgM association was ablated on B cells expressing Siglec-G with a mutated ligand-binding domain. B1 cell surfaces show a higher expression of α2,3-linked sialic acids, which is the preferential ligand for Siglec-G. It is therefore likely that the differential abundance of Siglec-G ligands on B1 and B2 cells leads to this specific phenotype in Siglec-G–deficient mice (21). FcγRIIB, an inhibitory IgG-binding Fc receptor, which is constitutively expressed on B cells, has a variety of functions in humoral immunity. In contrast to BCR stimulation alone, coligation of FcγRIIB and the BCR by IgG immune complexes, consisting of IgG and Ag, reduces Ca\(^{2+}\) signaling and proliferation in B cells (22). Crosslinking of FcγRIIB with the BCR induces phosphorylation of the intracellular ITIM and recruitment of SHIP-1 or SHP-2, which then inhibit positive regulators of BCR signaling (23). On plasma cells, which do not express BCR molecules anymore, crosslinking of FcγRIIB by immune complexes induces apoptosis through an proapoptotic pathway, which does not require ITIMs or SHP-1 (24-26). Because IgG is the major Ab isotype responsible for tissue inflammation during autoimmune diseases, FcγRIIB function is highly important for the inhibition of IgG-mediated activation of B cells and prevention of autoantibody production by B cells (27). FcγRIIB-deficient mice on a mixed 129Sv/B6 background (FcγRIIB\(^{129-/-}\)) develop severe, lethal lupus with high titers of IgG autoantibodies, whereas FcγRIIB-deficient mice on a pure B6 background (FcγRIIB\(^{B6-/-}\)) exhibit a hyperactive phenotype, but no lupus-like autoimmune disease (4, 28). The FcγRIIB gene, located on chromosome 1 of the mouse genome, is next to the Sle1b locus, which is associated with a strong autoimmune phenotype in mixed 129/B6 backgrounds (29). Within this locus the SLAM cluster, consisting of genes for stimulatory and inhibitory signaling molecules, was identified to cause autoimmunity in mixed strains. The fact that the 129Sv-derived Sle1b haplotype causes loss of tolerance in combination with the B6 genome explains the much stronger autoimmune phenotype of FcγRIIB\(^{129-/-}\) mice compared with FcγRIIB\(^{B6-/-}\) mice. Although FcγRIIB deficiency does not result in autoimmunity, it could be demonstrated that loss of FcγRIIB in mice amplifies spontaneous autoimmune in the context of autoimmune susceptibility loci, such as yaa or Sle1b (28). In humans, both a FcγRIIB promoter polymorphism and an allelic FcγRIIB 1232T variant are associated with a higher risk of SLE development (30–32). Siglec-G deficiency on the BALB/c background does not result in autoimmune disease (9). In this study we addressed the question of whether Siglec-G deficiency in a B6 genome can lead to a break of tolerance and to development of spontaneous autoimmunity. We therefore backcrossed Siglec-G–deficient BALB/c mice to the B6 genome (Siglec-G\(^{B6-/-}\)) and tested for the development of autoimmune disease both in the Siglec-G–deficient as well as in the FcγRIIB\(^{B6-/-}\) × Siglec-G\(^{B6-/-}\) double-deficient background. Our results show that the loss of Siglec-G in the B6 genome causes a break in tolerance with or without deficiency of FcγRIIB, when compared with wild-type (WT) littermate controls. Siglec-G–deficient mice develop high autoantibody titers and mild glomerular kidney damage. However, the combined loss of both inhibitory receptors does not lead to a stronger autoimmune phenotype compared with Siglec-G deficiency alone.

**Materials and Methods**

**Mice**

Siglec-G–deficient BALB/c mice (9) were backcrossed to the B6 background (Siglec-G\(^{B6-/-}\)) by marker-assisted speed congenics (tested in the N\(_2\) and N\(_9\) generations), followed by two random backcrosses to the B6 background. The resulting Siglec-G–deficient generation (N\(_5\)) was crossed to FcγRIIB–deficient mice, which were derived from B6 embryonic stem cells (33) (all mice were then an N\(_9\) backcross to B6). These mice were then intercrossed to obtain Siglec-G\(^{B6-/-}\) × FcγRIIB\(^{B6-/-}\) double-deficient, or control mice. To determine the backcrossing efficiency, eight mice of all genotypes from the intercrossed N\(_9\) generation were tested with 69 genomic single nucleotide polymorphism (SNP) markers (LGC Genomics, KASP genotyping) covering all mouse chromosomes. This analysis showed that the intercrossed N\(_9\) animals had on average 94.6% B6 homozygosity. WT controls were littermates of heterozygous breedings or age-matched from the same (N\(_6\)) background. Animal experiments were approved by a local ethics committee (Regierung Mittelfranken).

**ELISA**

We measured Ig serum levels from naive mice by standard ELISA methods (34). Sera and standard were applied to the plates in serial 1:3 dilutions in 1% (v/w) BSA, 0.05% (v/w) sodium azide in PBS and incubated overnight at \(3^\circ C\). Isotype-specific Abs coupled to alkaline phosphatase (AP; SouthernBiotech, Birmingham, AL) were used for detection. Plates were measured at 405-nm wavelength in the ELISA reader (VerasMaxPLUS) and analyzed using Softmax Pro software. The OD values of the serial dilutions were fit to a curve using the four-parameter fit.

**Rheumatoid factor IgM.** For detection of rheumatoid factor IgM titers, MaxiSorp plates were precoated with 0.01% poly-l-lysine diluted in H\(_2\)O. Precoated plates were coated with 20 μg/ml calf thymus dsDNA or ssDNA. ssDNA was obtained by incubation of dsDNA at 98°C for 25 min. A sera pool of aged MRL/lpr mice served as standard. Goat anti-mouse IgG (1 μg/ml) coupled to AP was used for detection.

**Anti-dsDNA Abs.** For detection of anti-dsDNA Abs, 96-well Polysorp ELISA plates (Nunc) were coated with 1 μg/ml goat anti-mouse isotype-specific Abs (SouthernBiotech). Mouse Ig isotype mAb (1 μg/ml) served as standard (SouthernBiotech). Goat anti-mouse secondary AP-coupled Abs (1 μg/ml) were used for detection.

**Autoimmune encephalitis (EAE).** We first tested for detection of the disease using ELISPOT assay. Ninety-six–well MaxiSorp plates were coated with goat anti-mouse IgG or IgA and saturated with 1% gelatin in PBS. Bone marrow or spleen cells (1 × 10\(^6\)) were cultured overnight at 37°C with 1:1000 diluted anti-CD45 Ab and simultaneously stimulated with Mycobacterium alternarii (Sigma-Aldrich). A sera pool of aged MRL/lpr mice served as standard. Goat anti-mouse IgG or IgM (1 μg/ml) coupled to AP was used for detection.

**Detection of anti-nuclear Abs**

Anti-nuclear Abs (ANA) were detected by the use of ImmunoGlo ANA HEp-2 substrate (Immco Diagnostics) according to the manufacturer’s instructions. Slides were incubated with 1:300 diluted sera for 30 min at room temperature. Anti-nuclear total IgG was detected with Alexa Fluor 488–conjugated rabbit anti-mouse IgG (Invitrogen). The slides were analyzed with x10 magnification on a fluorescence microscope. Fluorescence intensity of cells compared with a standard of pooled MRL/lpr serum was determined with ImageJ64.

**Blood urea nitrogen**

An enzymatic blood urea nitrogen (BUN) kit (Stanbio Laboratory) was used according to the manufacturer’s instructions to determine the urea nitrogen content in blood. Sera were diluted 1:100 in enzymatic reagent on 96-well MaxiSorp plates. After incubation in color reagent, the samples were measured at 600-nm wavelength in the ELISA reader (VerasMaxPLUS).
and analyzed using Softmax Pro software. Values of <35 mg/dl were considered normal.

Immunohistochemistry

Spleens and kidneys were harvested and snap-frozen in TissueTek OCT Cryomold (Sakura). Three consecutive 8-μm cryostat sections were fixed on poly-L-lysine slides with ice-cold acetone for 5 min and rehydrated with 1× PBS for 5 min. Spleen sections were saturated with 20% horse serum, 20% FCS, 1% anti-CD16/32 (2.4G2, own hybridoma) in 1× PBS and kidney sections were saturated with 20% horse serum in 1× PBS. Spleen cryosections were stained with anti-IgD-bio (eBioscience), anti-peanut agglutinin (PNA)-FITC (Vector Laboratories), anti-IgM-FITC (own hybridoma, clone 29-11), anti-IgG-Alexa Fluor 488 (Invitrogen), and streptavidin-Cy3 (GE Healthcare). Spleen cryosections were stained for immunocomplex depositions with anti-Ig-G Alexa Fluor 488 (Invitrogen), anti-IgG1-FITC (BD Biosciences), anti-IgG2b-FITC (BD Biosciences), anti-IgG2c-FITC (SouthernBiotech), and anti-IgG3-FITC (SouthernBiotech). All stained sections were fixed with mounting medium and analyzed on a fluorescence microscope with the same exposure time the next day. Fluorescence intensity was determined with ImageJ64.

Histology

Kidneys were fixed in 4% formalin overnight and stored in 70% ethanol. Sections, stainings, and scoring were performed as previously described (14).

Flow cytometry

Single-cell suspensions of bone marrow, spleen, kidney, and peritoneal cavity were treated with Gey’s solution to deplete erythrocytes. Kidneys were predigested with collagenase D (1 mg/ml; from Clostridium histolyticum, Roche) and DNase I (100 ng/ml; DNase I from bovine pancreas, Roche) in RPMI 1640 medium for 30 min at 37°C. Staining was done with the following Abs (conjugated with allopbyocyanin, biotin, FITC, PercP-Cy5, or PE): anti-B220 (RA3-6B2; E Bioscience), anti-GL7 (GL7; E Bioscience), anti-IgM (11-61/4; our hybridoma), anti-IgD (11-26C; our hybridoma), anti-CD1d (213; our hybridoma), anti-CD4 (GK1.5; BD Biosciences), anti-CD21 (2396; our hybridoma), anti-CD23 (B3B4; E Bioscience), anti-CD95 (Jo2; BD Pharmingen), and anti-CD138 (281-2; BD Biosciences). Sections, stainings, and scoring were performed as previously described (14). Where feasible, we analyzed whether loss of one or both inhibitory receptors in single-deficient mice resulted in altered B cell numbers. Flow cytometric analysis in bone marrow and spleen of 12 wk-old Siglec-G FcγRIIB B6 mice revealed no gross changes in total B cell numbers for typical developmental stages compared with WT littermate controls, apart from elevated IgM numbers in B6 mice compared with WT controls in aged Siglec-G FcγRIIB B6 mice (9, 36). Additionally, the peritoneal B1b/B2 cell population was significantly increased compared with WT controls in aged Siglec-G FcγRIIB B6 mice and Siglec-G FcγRIIB B6 double-deficient mice. The other changed B lineage populations were increased numbers of plasma cells and plasma blasts in Siglec-G FcγRIIB B6 double-deficient mice (Table I). B cells of aged Siglec-G FcγRIIB B6 and Siglec-G FcγRIIB B6 double-deficient mice did show slightly increased expression of the activation marker CD86 (Fig. 2A).

Because both Siglec-G and FcγRIIB are expressed on B lymphocytes, we analyzed whether loss of one or both inhibitory receptors in single-deficient mice results in altered B cell numbers. Flow cytometric analysis in bone marrow and spleen of 12 wk-old Siglec-G FcγRIIB B6 mice revealed no gross changes in total B cell numbers for typical developmental stages compared with WT littermate controls, apart from elevated IgM numbers in B6 mice compared with WT controls in aged Siglec-G FcγRIIB B6 mice (9, 36). Additionally, the peritoneal B1b/B2 cell population was significantly increased compared with WT controls in aged Siglec-G FcγRIIB B6 mice and Siglec-G FcγRIIB B6 double-deficient mice. The other changed B lineage populations were increased numbers of plasma cells and plasma blasts in Siglec-G FcγRIIB B6 double-deficient mice (Table I). B cells of aged Siglec-G FcγRIIB B6 and Siglec-G FcγRIIB B6 double-deficient mice did show slightly increased expression of the activation marker CD86 (Fig. 2A).

Because Siglec-G is also expressed on dendritic cells (DCs) (19, 21), we analyzed DC numbers in aging mice and also determined activation markers such as MHC class II and CD86 on DCs. We did not detect any significant changes in the three knockout mouse strains (not shown). However, examination of T cell populations revealed a higher CD4+/CD8+ T cell ratio in these mice, resulting from reduced total CD8+ T cell numbers and a proportional increase of the CD4+ T cell population (Fig. 2B). Further analysis of T cell populations revealed a higher activation status of CD4+ T cells in Siglec-G FcγRIIB B6 mice, whereas no changes were found in the CD8 T cell population. Specifically, CD4+ T cells of Siglec-G FcγRIIB B6 mice expressed higher CD69 levels (Fig. 2C). There was a tendency of higher T follicular helper cell numbers (identified as CD4+CXCR5+PD-1hi) in Siglec-G FcγRIIB B6 mice (Fig. 2D). In about half of the Siglec-G FcγRIIB B6 mice we also noted an increased CXCR5+PD-1hi population (Fig. 2D), which may be T follicular helper precursors (37). Furthermore, a higher number of effector memory CD4 T cells (CD62LloCD44hi) was detected in Siglec-
GB6
2
/2
mice (Fig. 2E). This higher activation status of CD4
T cells in Siglec-G B6
2
/2
mice may depend on a higher Ag presentation of DCs, which lack the inhibitory protein Siglec-G.

Aged Siglec-G–deficient and Siglec-G B6
2
/2
FcγRIIbB6
2
/2
double-deficient mice show increased autoantibody titers

**FIGURE 1.** Dysregulated Ig production and higher plasma and germinal center cell numbers in aged Siglec-G
/−/−, FcγRIIb
/−/−, or Siglec-G B6
/−/− × FcγRIIbB6
/−/−
double-deficient mice. (A) Serum Ig levels of 12- and 48-wk-old WT littermates, Siglec-G
/−/−, FcγRIIb
/−/−, and Siglec-G B6
/−/− × FcγRIIbB6
/−/−
double-deficient mice were measured by ELISA. Each symbol represents the serum of a single mouse. (B) IgM (upper row) and IgG (middle row) plasma cells (green) in spleens of 70-wk-old WT, Siglec-G
/−/−, FcγRIIb
/−/−, and Siglec-G B6
/−/− × FcγRIIbB6
/−/−
double-deficient mice were analyzed on cryosections with fluorescent-conjugated Abs against IgM and total IgG. Germinal center B cells (green) were detected with anti-PNA Ab (lower row). B cell follicles were detected with anti-IgD Abs (red). Numbers of germinal centers per area were quantified by counting PNA
+ follicles per section. Original magnification ×10. Each symbol represents analysis of one spleen section. Data represent typical results of one of six to eight independent experiments. (C) IgM- and IgG-producing plasma cells in bone marrow and spleens of naive 48-wk-old WT, Siglec-G
/−/−, FcγRIIb
/−/−, and Siglec-G B6
/−/− × FcγRIIbB6
/−/−
double-deficient mice were detected by ELISPOT. Each symbol represents the number of plasma cells per 1
×107 total cells isolated from bone marrow or spleen of a single mouse in four independent experiments. (D) Spleen weight of 48- and 70-wk-old animals (n = 5–10) was determined with fine scales. Mice with spleens >200 mg were defined as mice with splenomegaly.

4 SIGLEC-G DEFICIENCY AND AUTOIMMUNITY

G B6
/−/− mice (Fig. 2E). This higher activation status of CD4
T cells in Siglec-G B6
/−/− mice may depend on a higher Ag presentation of DCs, which lack the inhibitory protein Siglec-G.

**Aged Siglec-G–deficient and Siglec-G B6
/−/− × FcγRIIbB6
/−/−
double-deficient mice show increased autoantibody titers**

FcγRIIb deficiency enhances spontaneous autoimmunity in mice, which are susceptible to lupus-like diseases (28). To address the question whether Siglec-G deficiency can lead to a break of tolerance and acts to enhance autoimmunity in FcγRIIb B6
/−/− mice, respectively, we analyzed Abs titers against various autoantigens in sera of mutant mice over time. IgG autoantibodies against ssDNA and dsDNA are significantly increased in sera of 24-wk-old Siglec-G B6
/−/− and Siglec-G B6
/−/− × FcγRIIbB6
/−/−
double-deficient mice compared with WT sera and steadily rise in aging mice. Rheumatoid factor IgM is elevated in 48- and 70-wk-old
Siglec-G<sup>-/-</sup> mice but not in Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice (Fig. 3A). ANA levels were increased in sera of 24-wk-old Siglec-G<sup>-/-</sup> and Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice. At 48 and 70 wk of age, all mutant mouse strains (Siglec-G<sup>-/-</sup>, FcγRIIB<sup>-/-</sup>, and Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice) had elevated ANA levels compared with WT controls (Fig. 3B). No gender-specific differences were detected in any autoantibody levels that were analyzed. Because of the elevated B1 cell population, we also examined PC-specific Abs, which are typically produced by B1 cells. We found elevated titers of PC-specific Abs in sera of 48-wk-old Siglec-G<sup>-/-</sup> and Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice (Supplemental Fig. 2).

**Elevated immune complex depositions and mild, nonlethal glomerular damage in kidneys of Siglec-G<sup>-/-</sup> and FcγRIIB<sup>-/-</sup> mice**

Because high titers of autoantibodies against DNA and nuclear Ags were detected in sera of Siglec-G<sup>-/-</sup> and Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice, we addressed the question of whether immune complexes consisting of autoreactive Abs and autoantigen accumulate in kidneys of aged mice with defective inhibitory receptors. Glomerular nephritis results from an inflammatory response, which is caused by IgG immune complex depositions in glomeruli. Elevated depositions of total IgG, IgG1, IgG2b, IgG2c, and IgG3 immune complexes can be detected in kidney sections of 48- to 70-wk-old Siglec-G<sup>-/-</sup> and Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice (Fig. 4A). The severity of glomerular nephritis can be determined by the BUN concentration and level of proteinuria. Sera of aged MRL/lpr mice served as positive control, as these mice exhibit severe kidney damage by immune complex depositions. BUN levels of all mutant mice were in a range of 25–35 mg/dl and therefore normal, when compared with WT controls (Fig. 4B). A further method to measure kidney damage is to determine the amount of protein in the urine. All tested mice did not show elevated protein contents with >300 mg/dl in urine (not shown). Histopathological evaluation of aging healthy WT mice showed mild glomerular alterations, including mesangial matrix expansion as a sign of senescence (Fig. 5, WT). The strongest pathological alterations were found in kidneys of Siglec-G<sup>-/-</sup> mice showing renal injury typical for experimental lupus nephritis, including intracapillary thrombus formation, abnormal glomerular basement membrane, mesangial cell proliferation, and matrix expansion (Fig. 5, Siglec-G<sup>-/-</sup>). FcγRIIB-deficient mice showed similar alterations as identified in Siglec-G<sup>-/-</sup> mice, but intracapillary thrombus formation was rarely found in these animals (Fig. 5, FcγRIIB<sup>-/-</sup>). Surprisingly, renal alterations in Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice were less pronounced compared with kidney biopsies from Siglec-G<sup>-/-</sup> or FcγRIIB<sup>-/-</sup> mice (Fig. 5, Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup>). Female Siglec-G<sup>-/-</sup>, FcγRIIB<sup>-/-</sup>, and Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice animals showed significantly higher glomerular alterations compared with WT controls than did male animals (Fig. 5, lower panels). Tubulointerstitial infiltration with inflammatory cells, tubular atrophy, and fibrosis were rarely found in all investigated groups and not dependent on the genotype. To determine whether kidney alterations were also accompanied by lymphocyte infiltration, lymphocyte populations in kidneys of 48-wk-old mice were examined. Elevated B cell and plasmablast numbers were found in Siglec-G<sup>-/-</sup> and Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice compared with littermate controls, whereas plasma cell and T cell numbers were normal in these mice (Supplemental Fig. 3).

**Discussion**

Siglec-G is an inhibitory coreceptor of the BCR and modulates the B cell activation strength after Ag encounter, especially on B1 cells. In this study we reveal that loss of Siglec-G in mice on the B6 background results in autoimmunity characterized by autoantibody and enhanced plasma cell production as well as by development of mild, nonlethal glomerular damage in the kidney. Combined deficiency of Siglec-G and the inhibitory Fc receptor FcγRIIB does not lead in general to increased severity of autoimmunity compared with Siglec-G deficiency alone. The numbers of B1 and B2 cells in the peritoneal cavity, germinal center B cells in the spleen, as well as IgM and IgG plasma cells in spleen and bone marrow are increased in aged Siglec-G<sup>-/-</sup>-deficient and Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice. The elevation of plasma cell numbers in these mice is reflected by higher titers of serum IgM and some IgG subclasses. Also, Siglec-G<sup>-/-</sup> mice show an enhanced CD4<sup>+</sup> T cell activation. Additionally, higher levels of autoantibodies against ssDNA, dsDNA, and rheumatoid factor can be detected in both aged Siglec-G<sup>-/-</sup> and Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice. A mild form of glomerulonephritis with a significantly elevated glomerular alteration score

| Table I. Absolute cell numbers and surface marker expression of lymphocyte populations in 48-wk-old WT (C57BL/6), Siglec-G<sup>-/-</sup>, FcγRIIB<sup>-/-</sup>, and Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> double-deficient mice |
|-----------------|-----------------|-----------------|
|                  | WT              | Siglec-G<sup>-/-</sup> | FcγRIIB<sup>-/-</sup> | Siglec-G<sup>-/-</sup> x FcγRIIB<sup>-/-</sup> |
| **Bone marrow**  |                 |                 |                 |                 |
| Immature cells   | 3.0 ± 1.2       | 3.1 ± 1.3       | 2.8 ± 2.1       | 3.6 ± 1.9       |
| Transitional B   | 1.1 ± 0.3       | 1.0 ± 0.5       | 1.1 ± 0.5       | 1.1 ± 0.6       |
| mature B cells   | 11.4 ± 4.4      | 9.1 ± 3.8       | 8.2 ± 5.1       | 11.1 ± 4.3      |
| Plasma cells     | 0.14 ± 0.05     | **0.25 ± 0.06** | 0.14 ± 0.08     | **0.23 ± 0.08** |
| **Spleen**       |                 |                 |                 |                 |
| Follicular B cells | 22.9 ± 10.3    | 17.6 ± 5.7      | 24.2 ± 13.5     | 21.1 ± 12.0     |
| B1a cells        | 2.0 ± 1.4       | **8.4 ± 5.0**   | **3.4 ± 1.7**   | **4.2 ± 0.9**   |
| Plasmablasts     | 0.25 ± 0.9      | **4.2 ± 1.8**   | 2.9 ± 1.1       | **3.7 ± 1.8**   |
| Plasma cells     | 0.9 ± 0.6       | **1.9 ± 1.0**   | 0.7 ± 0.3       | **2.8 ± 0.4**   |
| CD4<sup>+</sup>  | 15.1 ± 4.1      | 20.1 ± 13.1     | 13.4 ± 3.4      | 17.2 ± 7.2      |
| CD8<sup>+</sup>  | 6.5 ± 2.0       | 5.6 ± 3.0       | 4.6 ± 1.2       | **3.9 ± 1.2**   |
| CD4<sup>+</sup>/CD8 ratio | 2.5 ± 0.8   | **4.0 ± 1.7**   | **3.1 ± 1.0**   | **4.4 ± 1.4**   |
| **Peritoneal cavity** |                 |                 |                 |                 |
| B1a cells        | 2.3 ± 1.5       | **7.1 ± 4.7**   | **10.0 ± 7.0**  | **18.1 ± 16.7** |
| B1b/B2 cells     | 6.5 ± 3.8       | **11.9 ± 9.2**  | **13.0 ± 9.8**  | **15.6 ± 7.4**  |

Cell numbers and surface marker expression of indicated populations are given as means ± SD. Eight to thirteen mice were included in quantification of total cell numbers. Significant changes are shown in bold with *p < 0.05, **p < 0.005, and ***p < 0.001.
occurs in aged female Siglec-G B6–/– and FcγRIIbB6–/– deficient mice and in Siglec-G B6+/+ × FcγRIIbB6+/+ double-deficient (DKO) mice. The histogram (right panel) shows a representative result for each genotype. (B) CD4+/CD8+ T-cell ratio. The histogram (right panel) shows a representative result for each genotype. (C) CD69 gMFI of CD4+ T cells. The histogram (right panel) shows a representative result for each genotype. (D) CD4+ CXCR5hiPD-1hi T follicular helper cell numbers of 48-wk-old WT littermates, Siglec-G–/–, FcγRIIb–/–, and Siglec-G B6–/– × FcγRIIbB6–/– double-deficient mice. One representative result is shown in FACS blots for each genotype. (E) Total naive (CD44loCD62Lhi), effector memory (CD44hiCD62Llo), and central memory (CD44hiCD62Lhi) CD4+ T cell numbers of 48-wk-old WT littermates, Siglec-G–/–, FcγRIIb–/–, and Siglec-G B6–/– × FcγRIIbB6–/– double-deficient mice are shown. One representative result is shown in FACS blots for each genotype. All graphs (A–E) show the results of 12–14 mice per group. *p < 0.05, **p < 0.001 by Mann–Whitney U test and unpaired t test.

Several studies showed that mice on a mixed genetic background could develop spontaneous SLE-like symptoms without contribution of a targeted gene. The SLAM/CD2 (Sle1b) cluster on chromosome 1 was characterized to cause autoimmunity when present in a mixed background containing B6 and another genetic background such as BALB/c or 129 (29). Another study showed that this region on chromosome 1 and regions on chromosomes 3, 7, and 13 are linked to lupus-like disease in mice when present on a mixed 129/B6 background (35). Therefore, the genetic background of the B6-backcrossed Siglec-G–/– mice was carefully analyzed with 69 SNP markers covering all chromosomes. The overall B6 homozygosity was 94.6%. Importantly, the critical chromosome 1, containing the SLAM cluster, was determined to be 100% B6 in all mice tested. Furthermore, all tested N’o mice had a 100% B6 background on chromosomes 3 and 13. Of note, the Siglec-G–deficient mice were generated in a BALB/c embryonic stem cell line (9) and were crossed back to B6; they had never been on a 129 background. The critical region on chromosome 7 (sle3) overlaps with the Siglec gene cluster and is therefore derived from BALB/c origin in our B6-backcrossed Siglec-G–deficient mice. This region on chromosome 7 is very closely linked to the cluster of kallikrein genes, which were shown to be protective in Ab-mediated lupus nephritis and their expression is downregulated in kidneys of lupus-prone mouse strains such as NZW and also in 129 (38). The different alleles of the kallikrein locus are therefore likely to be the responsible for the chromo-
A hallmark of many autoimmune diseases is the occurrence of pathogenic autoantibodies produced by self-reactive plasma cells (39, 40). In Siglec-G<sup>B6−/−</sup> mice and Siglec-G<sup>B6−/− × FcyRIIb<sup>B6−/−</sup></sup> double-deficient mice we found increased numbers of IgM- and IgG-producing plasmablasts and plasma cells in spleen and bone marrow. Siglec-G is a modulator of BCR signaling mainly in B1 cells, and Siglec-G deficiency results in strongly elevated B1 cell numbers in the peritoneal cavity (9). In this study we found not only higher numbers of B1a cells in peritoneal cavities of all three strains, but also an increase in peritoneal B1b and B2 cell numbers. Accordingly, anti-PC Abs were also increased in aged Siglec-G<sup>B6−/−</sup>-deficient mice and Siglec-G<sup>B6−/− × FcyRIIb<sup>B6−/−</sup></sup> double-deficient mice. However, these Abs were described to be protective, for example, in atherosclerosis (41), and it is therefore not clear whether they contribute to the pathogenesis of autoimmune diseases. It is controversially discussed to what extend B1 cells can switch to IgG and contribute to development of IgG-secreting plasma cells (42, 43). Most discussed to what extend B1 cells can switch to IgG and contribute to development of IgG-secreting plasma cells (42, 43). Most discussed to what extend B1 cells can switch to IgG and contribute to development of IgG-secreting plasma cells (42, 43). Most discussed to what extend B1 cells can switch to IgG and contribute to development of IgG-secreting plasma cells (42, 43).

Some 7-linked susceptibility for lupus nephritis, but our Siglec-G<sup>B6−/−</sup> mice have a BALB/c background in this region, which is the protective background (38). We therefore conclude that our Siglec-G<sup>B6−/−</sup> mice were sufficiently backcrossed to B6 to exclude unspecific genetic background effects of the remaining 5.4% BALB/c genome.

B cells normally differentiate into plasmablasts and plasma cells after Ag encounter and T cell help in lymphatic organs in a tightly regulated manner. Recently, Siglec-G expression was confirmed on DCs, which are responsible for T cell priming during an immune response (19, 21). Therefore, loss of Siglec-G on DCs could potentially result in a enhanced activation of T cells, which in turn leads to an uncontrolled activation of Siglec-G<sup>B6−/−</sup>-deficient B cells by T cells. Because CD4 T cells of Siglec-G-deficient mice were increased and had upregulated activation markers and increased effector memory phenotype, we think that it is likely that higher activation of CD4 T cells contributes to the development of autoimmunity. Although Siglec-G-deficient mice show mainly enhanced signaling and functional consequences in B1 cells, Siglec-G can also inhibit conventional B2 cell responses, as has been demonstrated by transfections of B cell lines or by use of synthetic Siglec-G ligands (9, 19). It is therefore likely that dysregulation of T cell activation combined with overstimulation of conventional B cells contributes to the higher generation of autoantibody-producing plasma cells of IgM and IgG isotypes in Siglec-G<sup>B6−/−</sup> mice. A skewed CD4<sup>+</sup>/CD8<sup>+</sup> ratio of T cell numbers and elevation of activated T cells are reported to be typical signs of an autoimmune phenotype (28, 45). We do not know why single Siglec-G<sup>B6−/−</sup> mice showed a stronger T cell activation phenotype than did double-deficient (Siglec-G<sup>B6−/− × FcyRIIb<sup>B6−/−</sup></sup>) mice.

FcγRIlb limits the accumulation of IgG<sup>+</sup> plasma cells and modifies autoimmune susceptibility in autoimmune-prone strains...
Normal urea nitrogen levels in sera, but increased immune complex deposition, in aged Siglec-G−/−, FcγRIIb−/−, and Siglec-G−/− × FcγRIIb−/− double-deficient mice. (A) Immunecomplex depositions in kidneys of 70-wk-old mice were analyzed on cryosections with fluorescent-conjugated Abs against total IgG and IgG subclasses. Upper panels. Example of stainings of 70-wk-old WT (littermate controls), Siglec-G−/−, FcγRIIb−/−, and Siglec-G−/− × FcγRIIb−/− double-deficient kidneys. Original magnification ×20. Data represent typical results of one of three independent experiments. Lower panels. Fluorescence intensity of immune complex depositions, which was quantified with ImageJ64. Each symbol represents the mean fluorescence intensity of Ig depositions of a single kidney. The graphs show the summary of three independent experiments. (B) Urea nitrogen contents in blood of WT, Siglec-G−/−, FcγRIIb−/−, and Siglec-G−/− × FcγRIIb−/− double-deficient mice were determined with an enzymatic BUN kit. A serum pool of MRL/lpr mice served as positive control for elevated urea nitrogen levels. Each symbol represents the urea nitrogen level of a single mouse (mg/dl). The graphs show the summary of 10–14 independent experiments. *p < 0.05, **p < 0.005, ***p < 0.001 by Mann–Whitney U test.

FIGURE 5. Mild, nonlethal kidney damage in aged Siglec-G−/−, FcγRIIb−/−, and Siglec-G−/− × FcγRIIb−/− double-deficient mice. Glomerular and tubulointerstitial damage in kidneys of 70-wk-old WT littersmates, Siglec-G−/−, FcγRIIb−/−, and Siglec-G−/− × FcγRIIb−/− mice were analyzed on formalin-fixed and periodic acid–Schiff-stained kidney sections. Upper panels. Representative microphotographs of periodic acid–Schiff-stained renal biopsies of 70-wk-old WT, Siglec-G−/−, FcγRIIb−/−, and Siglec-G−/− × FcγRIIb−/− double-deficient kidneys. Lower panels. Glomerular and tubulointerstitial alterations were scored for typical changes observed in lupus nephritis. *p < 0.05, **p < 0.005, ***p < 0.001 by Mann–Whitney U test.
animals. An exception can be found in kidneys of female Siglec-G<sup>−/−</sup> mice, which have stronger elevated glomerular alteration scores than do male littermates. This is consistent with the observations of more affected female Siglec-G-deficient mice on MRL/lpr background (14), indicating that Siglec-G may be a factor regulating gender-specific progression of autoimmunity. The alterations in female Siglec-G<sup>−/−</sup> mice included thrombus formation, which is one of the histopathological changes that occur during lupus nephritis and is a consequence of endothelial damage and platelet aggregation. Thrombi in the setting of SLE are also found in humans and may be the consequence of anti-phospholipid syndrome, thrombotic microangiopathy, or SLE vasculopathy (54). We think it is unlikely that the enhanced anti-PC Abs (directed against the polar head group of phospholipids) contribute to this phenotype, as no pathological role of these Abs has been described. We propose instead that the presence of thrombus formation in Siglec-G<sup>−/−</sup> kidneys reflects increasing injury, especially on endothelial cells in these mice.

B cells or plasmablasts may contribute to autoantibody production directly in the kidneys of aged Siglec-G<sup>−/−</sup> and Siglec-G<sup>−/−</sup> × FcγRIIB<sup>−/−</sup> double-deficient mice, as they were found in elevated numbers by flow cytometry. There is evidence that plasma cells can be found in inflamed kidneys and contribute to lupus nephritis (55). However, there is no significant decrease in survival in these animals (not shown). Siglec-G<sup>−/−</sup> × FcγRIIB<sup>−/−</sup> and Siglec-G<sup>−/−</sup> × FcγRIIB<sup>−/−</sup> double-deficient animals were also analyzed on the BALB/c background. In this genetic background no autoantibodies and no glomerular damages were also analyzed on the BALB/c background. In this genetic background no autoantibodies and no glomerular damages were also analyzed on the BALB/c background. In this genetic background no autoantibodies and no glomerular damages were also analyzed on the BALB/c background.

Taken together, loss of Siglec-G on the B6 background dysregulates not only B1 cells, but also conventional B2 cells and leads to activated CD4 T cells, which together result in a loss of tolerance and development of a mild autoimmune disease. It is not clear which B cell subpopulation contributes to disease progression. Elevated B1 cells are sometimes associated with autoimmunity, which was shown by analysis of SHP-1-deficient and NZB/W mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57). However, Siglec-G-deficient mice on BALB/c mice (8, 43, 57).

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Disclosures

The authors have no financial conflicts of interest.

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Suppl. Fig. 1

Identification of IgG⁺ plasma cells by IgG/IgD/DAPI staining. Left panels: Two typical examples of a DAPI (blue), IgG (green) and IgD (red) stained 70 weeks old Siglec-G<sub>B6</sub>⁻/⁻ x FcγRIIb<sub>B6</sub>⁻/⁻ spleen cryosection. Original magnification: 63x. Right panel: typical example of a IgG (green) and IgD (red) stained 70 weeks old Siglec-G<sub>B6</sub>⁻/⁻ x FcγRIIb<sub>B6</sub>⁻/⁻ spleen cryosection. Original magnification: 10x. Data represent typical results of one of two independent experiments.
Suppl. Fig. 2

Increased levels of IgG and IgM phosphorylcholine-specific autoantibodies in aged Siglec-G^{-/-} and Siglec-G_{B6^{-/-}}x FcγRIIb_{B6^{-/-}} double-deficient mice. Anti-PC-autoantibody titer of 48 weeks old WT (littermate controls), Siglec-G^{-/-}, FcγRIIb^{-/-} and Siglec-G_{B6^{-/-}}x FcγRIIb_{B6^{-/-}} double-deficient mice were measured by ELISA. Each symbol represents a single mouse. The graphs show the summary of two independent experiments. *p<0.05; **p<0.005; ***p<0.001. Mann-Whitney test.
Suppl. Fig. 3

Elevated B-cell and plasmablasts numbers in kidneys of aged Siglec-G−/− and Siglec-GB6−/− x FcγRIIbB6−/− double-deficient mice. Bar graph shows summarized absolute cell numbers displayed as means ± SD for B-cells (B220+), plasmablasts (B220lo CD138pos), plasma cells (B220neg CD138pos), CD4+ and CD8+ T-cells. Bar graph shows the summary of 5 independent experiments. *P < 0.05; **P < 0.005. unpaired t-test; Mann-Whitney test.
Suppl. Table 1  Absolute cell numbers of lymphocyte populations in 12 week-old WT (C57BL/6), Siglec-G<sup>−/−</sup>, FcγRIIb<sup>−/−</sup> and Siglec-G<sub>B6</sub><sup>−/−</sup> x FcγRIIb<sub>B6</sub><sup>−/−</sup> double-deficient mice. Cell numbers of indicated populations are given as numbers ± SD. 8-15 mice were included in quantification of total cell numbers. Significant changes are shown in bold with *p<0.05; **p<0.005

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<th>WT</th>
<th>Siglec-G&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>FcγRIIb&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Siglec-G&lt;sub&gt;B6&lt;/sub&gt;&lt;sup&gt;−/−&lt;/sup&gt; x FcγRIIb&lt;sub&gt;B6&lt;/sub&gt;&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<td><strong>Bone marrow (x10&lt;sup&gt;5&lt;/sup&gt;)</strong></td>
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<td>Immature cells (B&lt;sub&gt;220&lt;/sub&gt;&lt;sup&gt;lo&lt;/sup&gt; Ig&lt;sub&gt;M&lt;/sub&gt;&lt;sup&gt;med&lt;/sup&gt;)</td>
<td>5.8 ± 2.9</td>
<td>5.6 ± 0.9</td>
<td>5.9 ± 2.5</td>
<td>8.2 ± 5.4</td>
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<td>Transitional B cells (B&lt;sub&gt;220&lt;/sub&gt;&lt;sup&gt;lo-hi&lt;/sup&gt; Ig&lt;sub&gt;M&lt;/sub&gt;&lt;sup&gt;hi&lt;/sup&gt;)</td>
<td>1.8 ± 0.9</td>
<td>1.6 ± 0.6</td>
<td>1.4 ± 0.7</td>
<td>1.9 ± 1.1</td>
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<td>Mature B cells (B&lt;sub&gt;220&lt;/sub&gt;&lt;sup&gt;hi&lt;/sup&gt; Ig&lt;sub&gt;D&lt;/sub&gt;&lt;sup&gt;hi&lt;/sup&gt;)</td>
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<td>3.1 ± 0.8</td>
<td>3.0 ± 1.9</td>
<td>3.2 ± 1.4</td>
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<td>Plasma cells (CD138&lt;sup&gt;hi&lt;/sup&gt; Ig κ+λ light chain&lt;sup&gt;pos&lt;/sup&gt;)</td>
<td>0.2 ± 0.07</td>
<td>0.24 ± 0.03</td>
<td>0.34 ± 0.15</td>
<td><strong>0.43 ± 0.14</strong></td>
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<td>Follicular B cells (CD21&lt;sup&gt;med&lt;/sup&gt; CD23&lt;sup&gt;hi&lt;/sup&gt;)</td>
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<td>25.5 ± 7.6</td>
<td>17.7 ± 7.9</td>
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<td>1.3 ± 0.8</td>
<td>1.7 ± 0.4</td>
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<td>1.5 ± 0.6</td>
<td>3.7 ± 2.3*</td>
<td>1.9 ± 0.4</td>
<td><strong>2.9 ± 1.3</strong></td>
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<td>1.0 ± 0.5</td>
<td>0.9 ± 0.3</td>
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<td><strong>2.6 ± 0.7</strong></td>
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<td>1.45 ± 0.29</td>
<td>1.69 ± 0.24</td>
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<td>1.9 ± 1.0</td>
<td><strong>12.1 ± 8.2</strong>**</td>
<td>1.3 ± 0.5</td>
<td><strong>6.9 ± 0.5</strong>**</td>
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