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The Ligand-Binding Domain of Siglec-G Is Crucial for Its Selective Inhibitory Function on B1 Cells

Stefan Hutzler,* Lamia Ö zgör,* Yuko Naito-Matsui, † Kathrin Kläsener, ‡,§,¶ Michael Reth,*§,¶ and Lars Nitschke*

Siglec-G is an inhibitory receptor on B1 cells. Siglec-G–deficient mice show a large B1 cell expansion, owing to higher BCR-induced Ca^{2+} signaling and enhanced cellular survival. It was unknown why Siglec-G shows a B1 cell–restricted inhibitory function. With a new mAb we could show a comparable Siglec-G expression on B1 cells and conventional B2 cells. However, Siglec-G has a different ligand sialic acid–binding pattern on peritoneal B1 cells than on splenic B cells, and its sialic acid ligands are expressed differentially on these two B cell populations, suggesting that cis-ligand binding plays a crucial role on B1 cells. This observation was further studied by generation of Siglec-G knockin mice with a mutated ligand-binding domain. These mice show increased B1 cell numbers, increased B1 cell Ca^{2+} signaling, better B1 cell survival, and changes in the B1 cell Ig repertoire. These phenotypes are very similar to Siglec-G–deficient mice. The mutation of the ligand-binding domain of Siglec-G strongly reduces the Siglec-G–IgM association on the B cell surface. Thus, Siglec-G sialic acid–dependent binding to the BCR is crucial for the B1 cell–restricted inhibitory function of Siglec-G and is regulated in an opposite way to that of the related protein CD22 (Siglec-2) on B cells. The Journal of Immunology, 2014, 192: 5406–5414.

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iglec-G binds to \( \alpha_2,3Sia \) and \( \alpha_2,6Sia \), which are abundantly expressed on glycoproteins of cellular surfaces, including those of B cells (11). Therefore, as is true for all Siglecs, Siglec-G may be engaged in sialic acid binding in cis, binding to other glycoproteins on the same cellular surface, or in trans, to ligands on other cells, leading to cell–cell interactions. For CD22 (Siglec-2), a related B cell–specific Siglec, it has been

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shown that CD22 binding to cis-ligands has a crucial function. The binding to α2,6Sia on neighboring CD22 molecules on the B cell surface prevents the CD22 association to the BCR and thereby affects the strength of inhibition (12–14). Because the Siglec-G inhibitory protein is expressed throughout the B cell lineage and in all subpopulations of B cells, it is unclear why it shows a B1 cell–restricted inhibitory effect. To explain this, two hypotheses can be raised: either the Siglec-G surface expression is significantly higher on B1 cells than on conventional B2 cells, or Siglec-G cis-ligands control the association of the inhibitory protein to the BCR differentially on B1 cells than on B2 cells.

We developed a novel Siglec-G–specific mAb and could show with this tool that Siglec-G surface expression is only marginally higher on B1 cells than on B2 cells. Thus a possible higher expression pattern of Siglec-G on B1 cells appears unlikely as a reason for the B1 cell–restricted inhibitory function. However, Siglec-G showed a different cis-ligand binding pattern on B1 cells than on B2 cells, suggesting that differential cis-ligand binding can explain the functional differences of Siglec-G on B1 cells and B2 cells. To follow up on this finding, a Siglec-G knockin mouse with a mutated ligand-binding domain was generated. This knockin mouse showed a very similar phenotype to that of Siglec-G–deficient mice with increased Ca2+ responses and a large expansion of the B1 cell population. This finding indicates that Siglec-G cis-ligand binding on B1 cells is crucial for the B1 cell–restricted inhibitory function of Siglec-G.

Materials and Methods

Generation of an anti–Siglec-G Ab

For the generation of anti–Siglec-G Ab, Siglec-G–deficient mice were twice immunized i.p. at day 0 and 6 wk later with an Alum-precipitated Siglec-G–Fc protein as a carrier antigen. Three– to 5-month-old male mice with the Siglec-G knock-out genetic background were obtained. Control mice were age-matched wild-type (WT) mice from heterozygous matings or BALB/c WT breedings. Experiments were performed in accordance with the German law for protection of animals, after approval by the animal welfare committee.

ELISA

Total Ig titers from naive mice were determined by ELISA on Polysorp plates (Nunc) coated with isotype-specific Abs (Southern Biotechnology Associates, Birmingham, AL). Monoclonal Ig isotype Abs served as standards (Southern Biotechnology Associates). For all ELISA, samples were measured in a serial dilution ranging from 1:3 to 1:1000000. Experiments were performed in triplicate, and the absorbance was measured at 405 nm using a microplate reader (Luminex, San Diego, CA). Standard curves were plotted using a 4-parameter logistic curve fit.

Cell preparation and flow cytometry

Single-cell suspensions of bone marrow, spleen, thymus, or isolated peritoneal lavage cells were prepared in PBS and 5% FCS. Blood was obtained from tail vein and used with PBS containing 10% heparin (Roche, Basel, Switzerland). After erythrocyte lysis, cells were washed and incubated for 20 min at 4°C with 4C11 antibodies. Cells were washed and PBS was added. Flow cytometric analysis was performed using a FACSCalibur (Becton Dickinson, San Jose, CA); subsequently, cells were analyzed using FlowJo software (TreeStar, Ashland, OR). For intracellular cytokine staining, cells were stimulated with ionomycin and PMA for 4 h in the presence of monensin/propidium iodide. After stimulation, cells were fixed and permeabilized (BD Pharmingen, San Diego, CA). Intracellular cytokine staining was performed in PBS with 0.1% (w/v) sodium azide and their concentration was quantified against the standard curve. For these experiments, isotype-specific Abs coupled to alkaline phosphatase (Southern Biotechnology Associates) were used for detection.

Calcium mobilization assays

Cells from peritoneal cavity and spleen were loaded with Indo-1 (Molecular Probes, Eugene, OR), as described (3), and stained extracellularly with anti-B220 and anti-CD8. Baseline fluorescence was measured for 50 s using an LSR II (Becton Dickinson, San Jose, CA); subsequently, cells were stimulated by IgM cross-linking (B7.6 clone, our hybridoma), and calcium influx was measured for an additional 3 min. The loading efficiency was determined by separate ionomycin stimulation.

Proximity ligation assay

Abs and chemicals for proximity ligation assay (PLA) probes, the following Abs were used: IgM (SB 1140-01, clone 2-3Galβ1-4GlcNAc-O(CH2)2-PAA, 1 MDa size]; and 2-6Sia on neighboring CD22 molecules on the B cell surface prevents the CD22 association to the BCR and thereby affects the strength of inhibition (12–14). Because the Siglec-G inhibitory protein is expressed throughout the B cell lineage and in all subpopulations of B cells, it is unclear why it shows a B1 cell–restricted inhibitory effect. To explain this, two hypotheses can be raised: either the Siglec-G surface expression is significantly higher on B1 cells than on conventional B2 cells, or Siglec-G cis-ligands control the association of the inhibitory protein to the BCR differentially on B1 cells than on B2 cells.

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1B4B1), Siglec-G (SH2.1), and CD22 (Cy34.1). The Ab used for BCR stimulation was IgM(Fab)\(^2\) (Southern Biotechnology). Pervanadate was freshly prepared for each experiment with equal molar amounts of orthovanadate and peroxide. All Fab fragments specific for IgM-hc, Siglec-G, and CD22 were prepared with the Pierce Fab Micro Preparation Kit (Thermo Scientific), using immobilized papain or ficin according to the manufacturers’ protocol. After desalting (Zeba Spin Desalting Columns; Thermo Scientific), Fab fragments were coupled with PLA Probemaker PLUS or MINUS, according to the manufacturer’s instructions (Olink Bioscience). Detection of all the fluorescence microscopy experiments was performed with a Carl Zeiss 780 Meta confocal microscope (×63 oil-immersion objective). At least five images were captured from different regions and processed with Zen (2011) software. For each experiment ≥500 cells from several images were analyzed by single-cell analysis. Average numbers of PLA dots per cell from at least three independent repeated experiments were then normalized and analyzed using Graphpad Prism software (version 5). All the statistical analysis works were performed in GraphPad Prism software (version 5). The statistical analyses were performed using Prism software. The Mann–Whitney \(U\) test was used to evaluate the statistical significance of differences in mean Ig levels determined by ELISA; for other data, the unpaired \(t\) test was used. Statistical data are presented as mean ± SD unless stated otherwise.

**Cell sorting and T cell depletion**

Peritoneal cavity cells were negatively sorted with anti-CD90 beads and anti-CD23–PE (eBioscience)/anti-PE Abs coupled to magnetic beads (Miltenyi Biotec, Auburn, CA) and subsequently positively sorted with anti-CD5 beads (Miltenyi Biotec) to obtain B1a cells. The achieved purity was 90%. For T cell depletion, erythrocyte-depleted splenic cells were incubated with anti-CD4, -CD8, and -CD90 Abs (our hybridomas); T cells were lysed with baby rabbit complement (Cedarlane, Burlington, NC). For PLA, splenic B2 cells were purified by depleting all other cells by anti-CD43 beads.

**In vitro apoptosis test**

Purified B1a cells from peritoneal cavity or splenic B cells obtained by T cell complement lysis were cultured in 5% FCS RPMI 1640 medium supplemented with nonessential amino acids (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 U/ml Pen-Strep (Life Technologies), 1.2 mM L-glutamine (Life Technologies), and 50 mM 2-mercaptoethanol (Life Technologies). Each day, samples were extracellularly stained for CD5 and B220 and fixed with 2% paraformaldehyde in PBS for 10 min at room temperature. At the end of the experiment, cells of all time points were permeabilized by incubating overnight in 1 ml ice-cold 70% methanol in PBS and intracellularly stained with 10 mg DAPI/ml PBS + 0.1% Triton-X. Cells with subG1 DNA content were considered apoptotic.

**Western blot analysis**

Purified B1a cells from peritoneal cavity or splenic B cells were lysed by Brij lysis buffer, and proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk powder in 0.05% Tween-PBS and incubated with rabbit anti–Siglec-G (3). Ab binding was visualized using HRP-conjugated secondary Abs and ECL.

**Results**

**Siglec-G is a protein with expression on the surface of B lineage cells and DCs**

To address the question whether the B1 cell–specific inhibitory function of Siglec-G is due to a higher expression of the protein on the surface of B1 cells than on conventional B2 cells, a new mAb was developed. For this purpose, Siglec-G–deficient mice were immunized with a Siglec-G–Fc protein containing the first three Ig domains of Siglec-G fused to the Fc part of the human IgG H chain. After three immunizations, splenic cells of immunized Siglec-G–deficient mice were fused with myeloma cells and the resulting hybridoma supernatants were screened on chicken DT40 cells transfected with Siglec-G–GFp (3). Three anti–Siglec-G mAbs (SH1, SH2, and SH3) were developed, which all showed a Siglec-G–specific staining pattern, as they did not bind to untransfected DT40 cells and all were of the IgG1 isotype (Supplemental Fig. 1). All three mAbs showed a similar staining of mouse cells. The hybridoma of SH2 was subcloned, and the subclone SH2.1 was used for all further experiments as a biotinylated Ab.

The anti–Siglec-G mAb SH2.1 stained B lineage cells in the bone marrow from the proB/preB to the mature B cell stage, with a slightly higher Siglec-G expression detected on proB/preB and immature than on mature B cells (Fig. 1A). In the spleen, marginal zone B cells had a slightly higher Siglec-G level on the surface than did follicular B cells (Fig. 1B). Peritoneal B1a cells showed only marginally higher surface expression of Siglec-G than did B2 cells, whereas splenic B1 cells showed the same level of expression as splenic B2 cells (Fig. 1C, 1D). Siglec-G is not expressed on T cells (Supplemental Fig. 2A). On myeloid cells, Siglec-G is expressed on both myeloid and lymphoid DCs (Supplemental Fig. 2B, 2C). However, it is absent on monocytes, macrophages, and granulocytes (Supplemental Fig. 2D, 2E). Siglec-G is also expressed on the surface of plasmacytoid DCs and eosinophils (Supplemental Fig. 2F, 2G). We conclude that Siglec-G is expressed throughout the B cell lineage and that the B1 cell–restricted inhibitory function of Siglec-G cannot be attributed to a much higher expression pattern of Siglec-G on B1 cells than on B2 cells.

**Siglecs on peritoneal B1 cells show differential sialic acid binding compared with Siglecs on conventional B2 cells of the spleen**

A second hypothesis for the B1 cell–specific inhibitory function of Siglec-G was that its binding to the BCR is regulated differently on the surface of B1 cells than on B2 cells. Siglec-G can bind to both \(\alpha_2,3\text{Sia}\) and \(\alpha_2,6\text{Sia}\) ligands, as has previously been shown by staining B cells of WT and Siglec\(^{-/−}\) mice with probes containing \(\alpha_2,3\text{Sia}\) and \(\alpha_2,6\text{Sia}\) on high m.w. PAA particles (11). However, a comparison of B1 cells and B2 cells with such probes has not been done previously. To analyze the ligand binding of Siglec-G, B1 and B2 cells from either the peritoneal cavity or the spleen were stained with \(\alpha_2,3\text{Sia}-\text{PAA}\) and \(\alpha_2,6\text{Sia}-\text{PAA}\) probes. CD22-deficient, as well as Siglec-G/CD22–double-deficient mice were included in the analysis. As expected, on splenic B cells \(\alpha_2,6\text{Sia}-\text{PAA}\) stained WT B cells well, and staining was lower in both Siglec\(^{-/−}\) and CD22\(^{-/−}\) B cells, and more strongly reduced in Siglec-G/CD22–double-deficient mice, suggesting that both receptors contribute to binding of \(\alpha_2,6\text{Sia}\) ligands (Fig. 2A). Of interest, this staining gave very different results for B cells in the peritoneal cavity. B1a cells from the peritoneal cavity were stained well with \(\alpha_2,6\text{Sia}-\text{PAA}\), when the cells were obtained from WT or CD22-deficient mice. In contrast, this staining was completely lost on Siglec-G–deficient or Siglec-G/CD22–double-deficient B1a cells (Fig. 2A). A similar staining was found for the population of B220\(^+\text{CD}^{5−}\) B cells in the peritoneal cavity, which contains both B2 cells and B1b cells. These results suggest that Siglec-G is the only receptor that is accessible to external \(\alpha_2,6\text{Sia}\)-containing probes on peritoneal B1 cells. CD22 is not accessible to these probes, maybe because it is blocked by endogenous \(\alpha_2,6\text{Sia}\) cis-ligands with higher affinity. Staining with \(\alpha_2,3\text{Sia}\) probes did show a Siglec-G–dependent binding with no differences for B1 or B2 cells in both organs (Fig. 2B).

To address this further, the sialic acid expression pattern was compared between B1 and B2 cells from the two different locations by lectin stainings. Although \(\alpha_2,6\text{Sia}\) was quite similarly expressed on B1a and B2 cells, a marked differential expression of \(\alpha_2,3\text{Sia}\) was noted. This form of sialic acid was found to have higher expression on B1a cells than on B2/B1b cells, both from spleen and peritoneal cavity (Fig. 2C). Because \(\alpha_2,3\text{Sia}\) can be bound only by Siglec-G, but \(\alpha_2,6\text{Sia}\) can be bound by both CD22...
and Siglec-G, these results suggest a different regulation of cis-ligand binding for the two Siglecs, Siglec-G and CD22, on peritoneal B1 cells versus splenic B cells. The cis-ligand binding of Siglecs may affect association to the regulated activating receptor, that is, the BCR on B cells.

Siglec-G knockin mice with a mutated ligand-binding domain show a B1 cell phenotype similar to that of Siglec-G–deficient mice

To further examine the function of the ligand-binding domain of Siglec-G by a genetic approach, a point mutation was introduced into the mouse Siglecg gene by gene targeting, coding for an exchange from Arg120 to Glu in the first Ig domain of Siglec-G (Supplemental Fig. 3). Arg120 is a highly conserved amino acid within the Siglec family, and is required for sialic acid binding, as has been shown for many Siglecs (1). The mutation of the conserved Arg (positively charged) to Glu (negatively charged) in the Siglec family destroys ligand binding in all known cases (14). The Siglec-G knockin mice (referred to as Siglec-G–R120E mice hereafter) showed a surface expression of Siglec-G similar to that of WT B cells (Fig. 3A). Functional loss of ligand binding was demonstrated in B cells of Siglec-G–R120E mice, to the same extent as in B cells of Siglec-G–deficient mice (Fig. 3B). We conclude that we have introduced a mutation in the mouse Siglecg gene, which leads to a normally expressed Siglec-G protein with a functional loss of Siglec-G ligand binding.

Because Siglec-G is important for B1 cell homeostasis, we first examined whether the R120E mutation affected B1 cell numbers. Of note, Siglec-G–R120E mice showed an increase of B1a cell numbers similar to that in Siglec-G–deficient mice (3). In the peritoneal cavity, B1a cells were increased 5-fold in total numbers, whereas other cell populations were not affected (Fig. 4A). Siglec-G–deficient mice showed a 10-fold increase of B1a cells. Similarly, numbers of B1 cells in the spleen were increased 2-fold in Siglec-G–R120E mice and ~4-fold in Siglec-G–deficient mice (Fig. 4B). Thus mice with a mutated Siglec-G binding domain have a similar, but somewhat weaker, phenotype than that of Siglec-G–deficient mice, suggesting a strong, but not complete, loss of function. B cell populations other than B1 cells were not affected (Supplemental Table I). We also did not detect any changes in non–B cell populations.

Siglec-G knockin mice with a mutated ligand-binding domain show increased Ca2+ signaling in B1 cells owing to impaired Siglec-G association to the BCR

Next we tested whether the loss of ligand binding also affected the inhibitory potential of Siglec-G. Loss of the Siglec-G protein leads to highly increased BCR-induced Ca2+ responses in B1a cells, but not in B2 cells (3). In Siglec-G–R120E expressing B1a cells, we also detected increased BCR-induced Ca2+ responses, yet not to the same level as in Siglec-G–deficient B1a cells (Fig. 5). B2 cells were not affected and showed similar Ca2+ responses in all three
types of mice. These data suggest that the reduction of cis-ligand binding affects the inhibitory function of Siglec-G for BCR signaling. To test whether the Siglec-G association to the BCR was affected, a PLA was performed. This assay measures protein associations in situ by Fab fragments of Abs directed against Siglec-G and IgM with attached oligonucleotides that can hybridize only when the two proteins are in close proximity to each other and are detected by a rolling circle PCR (14, 17). The PLA showed that in the Siglec-G–R120E mutant the IgM-Siglec-G association was disturbed, as many fewer molecules of Siglec-G were found to be associated with IgM in the mutant, both in the resting and in the stimulated stages of peritoneal B1a cells (Fig. 6A) or splenic B2 cells (Fig. 6B). In addition, stimulated peritoneal B1a cells showed higher Siglec-G/IgM association than did splenic B2 cells (Fig. 6C). These data demonstrate that the sialic acid binding property of Siglec-G is needed for association to IgM and that this interaction is more pronounced on B1 than on B2 cells.

Siglec-G–R120E mice have increased IgM serum levels and a change in the Ig repertoire, similar to that in Siglec-G–deficient mice

The higher B1a cell numbers in Siglec-G–deficient mice were shown to be due to a lower rate of apoptosis, compared with WT B1a cells (4). When cultivated in medium without cytokines, Siglec-G–R120E cells (Fig. 6B). In addition, stimulated peritoneal B1a cells showed higher Siglec-G/IgM association than did splenic B2 cells (Fig. 6C). These data demonstrate that the sialic acid binding property of Siglec-G is needed for association to IgM and that this interaction is more pronounced on B1 than on B2 cells.

FIGURE 2. Differential α2,6Sia binding by Siglecs and differential Sia expression on peritoneal B1 versus splenic B2 cells. (A and B) B cells of the indicated genotypes and organs were stained with high m.w. (1 MDa) biotinylated PAA conjugated with α2-6Neu5Gc (2,6Sia-PAA) (A) or α2-3Neu5Gc (2,3Sia-PAA) (B). Background staining by streptavidin alone is shown in gray. (C) 2,6Sia and 2,3Sia expression on B1a cells and B2/B1b cells from two organs of WT mice is analyzed by lectin stainings. SNA is 2,6Sia specific, whereas MAA detects 2,3Sia. Results of each are representative of three independent experiments.

FIGURE 3. Generation of Siglec-G–R120E mice. Intact Siglec-G surface expression and defective ligand binding. (A) Flow cytometry analysis of peritoneal B220⁺ B cells from WT, Siglec-G–R120E, and Siglec-G–deficient mice. The appropriate genotypes are indicated. Mean fluorescence intensities (mfi) of WT and Siglec-G–R120E mice are shown ±SD (n = 4). (B) Splenic B cells of the indicated genotype were stained with high m.w. (1 MDa) biotinylated PAA-conjugated α2-3Neu5Gc and α2-6Neu5Gc (as indicated by symbols). Background staining by streptavidin alone is shown in gray. Results are representative of three independent experiments.
B1a cells showed a resistance to apoptosis similar to that in Siglec-G–deficient cells (Fig. 7A). In contrast, splenic B2 cells showed an apoptosis rate in both Siglec-G mutants similar to that in WT B2 cells (Fig. 7B). Siglec-G–deficient mice have increased IgM levels in the serum, which are largely natural Abs produced to a high extent by B1 cells. Siglec-G–R120E mice showed an increase of serum IgM similar to that of knockout mice (Fig. 7C). Both Siglec-G mutants also have decreased IgG2a levels.

B1 cells are characterized by expressing a restricted Ig repertoire containing many canonical germline-derived VDJ sequences that are important for antibacterial defenses. It was previously shown that this Ig repertoire is altered in Siglec-G–deficient mice and that

**FIGURE 4.** Expansion of the B1a cell population in Siglec-G–/– and Siglec-G–R120E mice. Numbers in FACS plots represent percentages of lymphocyte populations of B1a cells (CD5+IgM+), B2/B1b cells (CD5+IgM–), and T cells (CD5–IgM–) in peritoneal cavity (A) and spleen (B). The bar diagrams show summarized total cell numbers from nine mice each, displayed as means ±SD. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 5.** Increased calcium mobilization in B1a cells of Siglec-G–R120E mice. Calcium mobilization in peritoneal cavity B1a cells (CD5+B220low) and in splenic B2 cells (CD5–B220+) of the indicated genotypes. Stimulation with anti-IgM (clone B7.6) at indicated time (arrow) and indicated concentrations. Results are plotted as medians of bound/unbound Indo-1 over time. One of four experiments with similar results is shown.
typical canonical Ig sequences occurred less frequently in Siglec-G–deficient B1 cells (4). A prototype of a canonical Ig sequence of B1 cells is specific for phosphorylcholine. B1a cells of Siglec-G–R120E mice showed a reduced binding of phosphorylcholine to BCRs, exactly as B1a cells of Siglec-G–deficient mice (Fig. 8).

In conclusion, Siglec-G–R120E mice generally show a phenotype similar to that of Siglec-G–deficient mice, which points to the

FIGURE 6. Loss of Siglec-G association to IgM in B cells of Siglec-G–R120E mice. Association of IgM and Siglec-G in situ was analyzed by PLA with B1a cells of peritoneal cavity or splenic B2 cells. (A) Purified peritoneal B1a cells (CD5lowB220low) or (B) purified splenic B2 cells (CD5+B220+) are shown. Top, Examples of resting (R), pervanadate (PV), or anti-IgM (aIgM)–stimulated cells are shown. Both stimulations were done for 5 min. For quantification, PLA signals (dots per cell) of each sample were counted from a minimum of 500 cells. Nuclei were stained with DAPI and shown as blue signals. PLA signals are represented by red. Scale bar, 5 μm. Bottom, Means +SD. (C) Comparison of B1a cells of peritoneal cavity with splenic B2 cells of WT mice, with same data as in (A) and (B). Means ± SD from ≥500 cells per sample are shown. One of four experiments with similar results is shown.

FIGURE 7. Better B1a cell survival and higher IgM serum levels in Siglec-G−− and Siglec-G–R120E mice. Purified B1a cells (B220lowCD5+) (A) or splenic B2 cells (B220+CD5−) (B) were cultivated in medium without any cytokines, and spontaneous apoptosis was measured by DAPI staining of fixed, permeabilized cells at indicated time points in days. SubG1 phase DAPI stainings that correspond to apoptotic cells are shown in the histograms. Data are summarized from triplicates of three mice per group as means +SD. *p < 0.05. **p < 0.01. (C) ELISA of serum titers of the indicated Igs (Serum Ig). Each symbol represents one mouse. One of three experiments with similar results is shown. *p < 0.05.
importance of the ligand-binding domain for the inhibitory function of this protein.

Discussion
The purpose of this study was to explain the B1 cell–specific inhibitory function of Siglec-G. Two explanations for this phenomenon were tested: either that a higher expression of Siglec-G on the surface of B1 cells than on the surface of B2 cells is responsible, or that a preferential cis-ligand binding of Siglec-G occurs on B1 cells, but not on B2 cells. The results of this study clearly favor the second explanation. We did not find much higher expression of Siglec-G on B1 cells than on B2 cells. However, a mutation in mice, which wipes out Siglec-G sialic acid binding, affected the association of Siglec-G to the BCR and produced the same B1 cell–restricted phenotype as the complete knockout of the protein.

With the help of a newly generated anti–Siglec-G mAb, we could determine the cell surface expression pattern of this Siglec-G protein. Our study showed that Siglec-G can be found on the surface of all types of B cells. Siglec-G surface expression starts on proB cells and continues throughout B cell differentiation. Siglec-G expression on B1a cells is only marginally higher than on B2 cells in the peritoneal cavity and not at all in the spleen. The Siglec gene is under the control of the transcription factor Pax-5 (18); therefore, this B cell–restricted expression pattern was expected. Our study also showed that Siglec-G cannot be found on the surface of T cells and many myeloid cell types, such as monocytes, macrophages, and granulocytes. However, Siglec-G is expressed both on conventional DCs and on plasmacytoid DCs, as well as on eosinophils. This finding is of importance, as functions for Siglec-G on these DCs were indicated as important for preventing inflammatory responses in a liver damage model as well as for antibacterial responses (9, 10). A recent study also showed that Siglec-G was induced in macrophages by RNA viruses and that this Siglec-G upregulation was important for innate immune responses against RNA viruses (19). Whether Siglec-G can be induced on the surface of macrophages was not addressed in this study but can easily be done with the new mAb in the future. Our Ab shows a Siglec-G cellular staining pattern very comparable to that of a rat mAb recently discussed in a published paper (20).

Our studies of the Siglec-G–R120E mice show that the ligand-binding property of Siglec-G is crucial for its inhibitory function. This is because Siglec-G–R120E mice show generally the same B1 cell phenotypes as knockout mice, including a large expansion of this population and increased BCR-induced Ca$^{2+}$ signaling only of B1a cells, and increased IgM serum levels. All these similar phenotypes indicate that the sialic acid–binding property of Siglec-G must be crucial for its function. However, the R120E mutation did not fully reproduce the phenotype of the Siglec-G–deficient mice, suggesting that the ligand-binding domain is not entirely regulating the Siglec-G function. Certainly, the inhibitory intracellular domain of Siglec-G also contributes to the function and may be to some extent independent of the Siglec-G cis interactions on the surface.

The results of this study can be explained by a direct interaction of Siglec-G via cis-ligand binding with $\alpha_{2,6}$Sia- and $\alpha_{2,3}$Sia-containing ligands on IgM, as detected in the PLA experiments. This Siglec-G–IgM interaction on B1 cells is lost in the R120E mutant. We expect this interaction to be direct binding, as the PLA done with Fab fragments detects 10- to 20-nm distances between molecules and should therefore detect direct protein–protein interactions. The results of the external ligand-staining studies with $2,6$Sia-containing probes also point to a crucial cis interaction for Siglec-G on peritoneal B cells. Because CD22 cannot be stained with external $2,6$Sia-containing probes on peritoneal B cells, in contrast to Siglec-G, this could mean that CD22 is kept away from the IgM on peritoneal B cells by binding to modified high-affinity $\alpha_{2,6}$Sia-containing ligands. At the moment it is not clear why this organ-specific difference exists between peritoneal and splenic B cells. However, the finding that B1a cells in both organs express relatively more $\alpha_{2,3}$-linked sialic acids than do B2 cells is a relevant finding. As Siglec-G is the only B cell Siglec that can bind to 2,3Sia, this may directly contribute to the interaction of Siglec-G with IgM on B1 cells. Further studies are needed to analyze whether the 2,3Sia levels differ on the membrane-bound IgM molecule itself on B1 cells as opposed to B2 cells. Nevertheless, the B1 cell–specific function of Siglec-G can be explained by these findings to some extent, as the Siglec-G–IgM association detected by PLA was higher on stimulated peritoneal B1a cells than on splenic B2 cells.

The results found with the Siglec-G–R120E mice are very different from those of knockin mice with similar mutations in CD22. CD22 knockin mice with the corresponding Arg mutation (R130E) did show the opposite phenotype: decreased Ca$^{2+}$ signaling and stronger CD22–BCR association (14). In addition, mice with a loss of $\alpha_{2,6}$Sia on the surface by deficiency of the enzyme ST6GalI have reduced Ca$^{2+}$ signaling on conventional B2 cells. $St6gal1^{+/+}/Cd22^{+/+}$ mice revealed that CD22 on conventional B cells is responsible for this (12, 13). $St6gal1^{+/+}/Cd22^{+/-}$ mice had a higher association of CD22 with the BCR. Thus, cis-ligand binding for CD22 is regulated in the opposite way to Siglec-G. Whereas CD22 is kept away from the BCR by forming CD22

![Figure 8](http://www.jimmunol.org/)

**FIGURE 8.** Siglec-G$^{-/-}$ and Siglec-G–R120E mice have a lower percentage of phosphorylcholine (PC)–binding B1a cells in the peritoneal cavity. Peritoneal cavity cells of the indicated genotypes were stained with PC-BSA-FITC. The numbers in the FACS plots represent percentages of PC-binding cells ±SD of the B1a cell population. B1a cells were gated as shown. The histogram plot shows summarized percentages of PC-binding B1a cells displayed as means ±SD. These plots summarize six experiments with one mouse each. *p < 0.05.
homo-oligomers (21). Siglec-G seems to require the ligand binding on B1 cells for its association to the BCR. These findings are of importance and explain the mechanism of the inhibitory receptor Siglec-G in the mouse and potentially also its counterpart in the human, Siglec-10. Both CD22 and Siglec-G have been shown to be involved in B cell tolerance induction (11), and a deficiency in both of these receptors together leads to autoimmunity (22). Our findings are therefore highly relevant for the better understanding of B cell–driven autoimmune diseases and offer new treatment concepts for patients with these diseases. In this context, a Siglec-G ligand-dependent B cell tolerization strategy has been published recently (20).

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Disclosures

The authors have no financial conflicts of interest.

References

Suppl. Fig. 1: Flow cytometry-characterisation of the monoclonal anti-Siglec-G antibody. (A) Initial hybridoma screening on DT40 Siglec-G-GFP cells. Detection of Siglec-G positive hybridoma-supernatants by α-mIgG staining (clone SH1), representative for all anti-Siglec-G hybridomas. A negative hybridoma supernatant (sup.) is shown on the left. (B) Representative staining of the specific anti-Siglec-G binding of the hybridoma-supernatant from clone SH3 to WT and Siglec-G-/- splenic B cells. (C) Isotype analysis of the hybridoma-supernatant from clone SH1, which is representative for all obtained anti-Siglec-G hybridomas, done with WT B cells.
Suppl. Fig. 2
Suppl.Fig.2: Flow cytometry-analysis of Siglec-G expression on thymic and splenic cell-populations. Displayed populations are gated on lymphocytes (A) or on all living cells (B-G). The histograms show Siglec-G stainings with primary WT and Siglec-G -/- cells or the appropriate isotype-control as overlay of the gated populations of interest. For all anti-Siglec-G stainings the clone SH2.1 was used (biotinylated antibody). Results are representative of at least three independent experiments.
Suppl.Fig.3: Siglec-G targeting strategy and southern blot confirmation.
A) The targeting strategy with the R120E mutation (red star) located in exon 3, which codes for the ligand-binding domain of Siglec-G. A loxP-flanked neo cassette (dark grey) is placed between exon 4 and 5. The homologous integration is marked by black lines and the PstI recognition sites for the southern blot strategy are shown. The external southern blot probe is shown in light grey. At the bottom the mutant allele after targeting and CMV-cre mediated deletion is shown.
B) Southern blot of embryonic stem cell clones. PstI digestion of genomic DNA from WT or Siglec-G R120E-targeted ES cells. The size of the WT and the mutated band is shown in (A) and (B), respectively. The three positive clones were named P7E6, P1C2 and P1D6.
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<th></th>
<th>WT</th>
<th>Siglec-G/-</th>
<th>Siglec-G R120E</th>
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<tr>
<td><strong>Bone marrow</strong></td>
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<tr>
<td>Pro/pre B cells (B220&lt;sup&gt;med&lt;/sup&gt;, IgM&lt;sup&gt;neg&lt;/sup&gt;)</td>
<td>26.3 ± 14.6</td>
<td>22.0 ± 11.8</td>
<td>25.4 ± 10.4</td>
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<td>Immature cells B cells (B220&lt;sup&gt;med&lt;/sup&gt;, IgM&lt;sup&gt;med/high&lt;/sup&gt;)</td>
<td>9.9 ± 5.8</td>
<td>8.5 ± 5.8</td>
<td>8.5 ± 4.3</td>
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<td>Transitional B cells (B220&lt;sup&gt;med/high&lt;/sup&gt;, IgM&lt;sup&gt;high&lt;/sup&gt;)</td>
<td>2.4 ± 3.5</td>
<td>0.6 ± 0.5</td>
<td>1.0 ± 1.3</td>
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<td>Mature B cells (B220&lt;sup&gt;high&lt;/sup&gt;, IgD&lt;sup&gt;high&lt;/sup&gt;)</td>
<td>3.0 ± 1.0</td>
<td>1.9 ± 0.9</td>
<td>2.7 ± 1.2</td>
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<td><strong>Spleen</strong></td>
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<td>B1 cells (IgM&lt;sup&gt;high&lt;/sup&gt;, CD5&lt;sup&gt;med&lt;/sup&gt;)</td>
<td>1.4 ± 0.7</td>
<td>4.2 ± 0.6 ***</td>
<td>2.7 ± 1.3 *</td>
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<td>B2 cells (IgM&lt;sup&gt;high&lt;/sup&gt;, CD5&lt;sup&gt;neg&lt;/sup&gt;)</td>
<td>15.1 ± 5.5</td>
<td>11.2 ± 2.3</td>
<td>12.3 ± 4.4</td>
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<td>T1/MZ cells (IgM&lt;sup&gt;high&lt;/sup&gt;, IgD&lt;sup&gt;low&lt;/sup&gt;)</td>
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<td>1.6 ± 0.8</td>
<td>1.5 ± 0.6</td>
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<td>T2 cells (IgM&lt;sup&gt;high&lt;/sup&gt;, IgD&lt;sup&gt;high&lt;/sup&gt;)</td>
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<td>0.2 ± 0.1</td>
<td>0.3 ± 0.2</td>
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<td>Mature B cells (IgM&lt;sup&gt;med&lt;/sup&gt;, IgD&lt;sup&gt;high&lt;/sup&gt;)</td>
<td>5.7 ± 3.2</td>
<td>5.7 ± 2.1</td>
<td>5.4 ± 3.1</td>
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<td>Marginal zone B cells (CD21&lt;sup&gt;high&lt;/sup&gt;, CD23&lt;sup&gt;low&lt;/sup&gt;)</td>
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<td>Follicular B cells (CD21&lt;sup&gt;med/high&lt;/sup&gt;, CD23&lt;sup&gt;high&lt;/sup&gt;)</td>
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<td>12.1 ± 3.1</td>
<td>12.1 ± 4.4</td>
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<td>T cells (IgM&lt;sup&gt;reg&lt;/sup&gt;, CD5&lt;sup&gt;high&lt;/sup&gt;)</td>
<td>9.8 ± 4.8</td>
<td>9.6 ± 2.6</td>
<td>9.6 ± 2.6</td>
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<td><strong>Peritoneal cavity</strong></td>
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<td>B1a cells (IgM&lt;sup&gt;high&lt;/sup&gt;, CD5&lt;sup&gt;med&lt;/sup&gt;)</td>
<td>4.2 ± 1.5</td>
<td>42.0 ± 17.5 ***</td>
<td>20.2 ± 7.2 ***</td>
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<td>B2/B1b cells (IgM&lt;sup&gt;high&lt;/sup&gt;, CD5&lt;sup&gt;neg&lt;/sup&gt;)</td>
<td>1.8 ± 0.6</td>
<td>2.7 ± 3.2</td>
<td>1.8 ± 0.8</td>
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<td>T cells (IgM&lt;sup&gt;reg&lt;/sup&gt;, CD5&lt;sup&gt;high&lt;/sup&gt;)</td>
<td>0.7 ± 0.5</td>
<td>2.0 ± 0.8</td>
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**Suppl. Table 1:** Absolute cell numbers for 8- to 12-week old mice. Values are x 10<sup>5</sup> for bone marrow and peritoneal cavity and are x 10<sup>6</sup> for the spleen. *, p< 0.05, ***, p< 0.001. n: 9-11.