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Copresentation of Antigen and Ligands of Siglec-G Induces B Cell Tolerance Independent of CD22

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Differerntiation of self from nonself is indispensable for maintaining B cell tolerance in peripheral tissues. CD22 and Siglec-G (sialic acid–binding Ig-like lectin G) are two inhibitory coreceptors of the BCR that are implicated in maintenance of tolerance to self Ags. Enforced ligation of CD22 and the BCR by a nanoparticle displaying both Ag and CD22 ligands induces a tolerogenic circuit resulting in apoptosis of the Ag-reactive B cell. Whether Siglec-G also has this property has not been investigated in large part owing to the lack of a selective Siglec-G ligand. In this article, we report the development of a selective high-affinity ligand for Siglec-G and its application as a chemical tool to investigate the tolerogenic potential of Siglec-G. We find that liposomal nanoparticles decorated with Ag and Siglec-G ligand inhibit BCR signaling in both B1 and B2 B cells compared with liposomes displaying Ag alone. Not only is inhibition of B cell activation observed by ligating the BCR with Siglec-G, but robust tolerance toward T-independent and T-dependent Ags is also induced in mice. The ability of Siglec-G to inhibit B cell activation equally in both B1 and B2 subsets is consistent with our observation that Siglec-G is expressed at a relatively constant level throughout numerous B cell subsets. These results suggest that Siglec-G may contribute to maintenance of B cell tolerance toward self Ags in various B cell compartments.

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BPA-NeuGc, NeuGc
2-3-galactose-9-biphenyl-N-ethylamine, or NeuGc
2-6-galactose-N-ethylamine; 6
1-4-GlcAc
2-3-galactose-
-N-ethylamine; 6
1-4-GlcAc-
2-3-Galβ1-4-N-acetylglucosamine-β-ethylamine; 6
1-4-GlcAc-
2-3-Galβ1-4-N-acetylglucosamine-β-ethylamine; HEL, hen egg lysozyme; 3’-NeuGc, NeuGc2-3-Galβ1-4-GlcAc-β-ethylamine, or NeuGc2-3-Galβ1-4-GlcAc-β-ethylamine, or NeuGc2-3-Galβ1-4-GlcAc-β-ethylamine; PAMP, pathogen-associated molecular pattern; PEG2000-DSPE, pegylated disoctanoylphosphatidyl ethanolamine; STAL, siglec-engaging tolerance-inducing antigenic liposomes; WT, wild-type.

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Siglec-G plays a dominant role in peritoneal B1 cells. Mice deficient in both Siglec-G and CD22 have an even higher number of B1 cells and, in contrast to the single knockout mice, develop spontaneous autoimmunity (4). Cumulatively, these mouse genetic studies strongly suggest that CD22 and Siglec-G act in a complementary way to suppress B cell responses to self Ags.

Recent studies have shown that a nanoparticle decorated with a B cell–reactive Ag and a high-affinity ligand for CD22 is able to induce Ag-specific tolerance in mice (17, 18). Copresentation of the Ag and CD22 ligand causes juxtaposition of CD22 with the BCR, leading to a tolerogenic circuit that culminates in apoptosis of the Ag-reactive B cell. On the basis of the specificity of the ligand for CD22, tolerance induction was mainly dependent on CD22, although one report showed partial dependence on Siglec-G (17). Thus, although several studies suggest that CD22 and Siglec-G cooperatively suppress B cell responses, an independent role for Siglec-G in B cell tolerance has not been demonstrated (4, 17, 18).

To examine the potential of Siglec-G to induce B cell tolerance, we have developed a high-affinity ligand for Siglec-G that does not cross-react with CD22 and show that siglec-engaging tolerance–inducing antigenic liposomes (STALs) displaying the Siglec-G ligand give rise to robust tolerance in mice toward both T-independent and T-dependent Ags. Analysis of the initial events following stimulation of B cells with liposomes indicates that both B1 and B2 cells are equally capable of being inhibited by recruitment of Siglec-G to the BCR. This latter finding is in accordance with our finding that Siglec-G is expressed at equivalent levels in both B cell subsets, as assessed using a newly developed anti–Siglec-G mAb. We also show that Siglec-G is expressed throughout B cell development, including pre–B cells before CD22 is expressed, suggesting that it may have a unique role in regulation of BCR signaling.

Materials and Methods

Animal studies

The Institutional Animal Care and Use Committee of The Scripps Research Institute (La Jolla, CA) approved all experimental procedures involving mice. CD22KO and Siglec-GKO mice, on a C57BL/6J background, were obtained from L. Nitschke (University of Erlangen, Erlangen, Germany) and Y. Liu (University of Michigan, Ann Arbor, MI), respectively. Wild-type (WT) C57BL/6J mice were obtained from The Jackson Laboratory and crossed onto a selection marker. Other cell lines expressing murine siglecs in Chinese hamster ovary (CHO) cells were obtained from The Jackson Laboratory and grown in F12/DMEM supplemented with 10% FBS and hygromycin as described earlier (19). Myc-tagged Siglec-H–L929 cells (American Type Culture Collection) were established by retroviral transduction of murine BW5147 cells (20). Murine CD22KO and Siglec-GKO mice, on a C57BL/6J background, were obtained from The Scripps Research Institute rodent facility. A selection of congenic mice was used for this study as Siglec-G–expressing cells unless otherwise specified. Myc-tagged Siglec-G–expressing BW5147 (American Type Culture Collection) were prepared as described previously (19), and were used in this study as Siglec-G–expressing cells unless otherwise specified. Myc-tagged Siglec-G–expressing BW5147 and Myc-tagged CD22–expressing L929 cells (American Type Culture Collection) were established by retroviral transduction using the plasmids pMxS-IG-Siglec-G, pMxS-IG-Siglec-GCD3z, and pMxS-IG-Siglec-G-CD3z, as described previously (19). Myc-tagged Siglec-H–expressing BW5147 cells were prepared as described earlier (20). Murine CD22–expressing cells were prepared by stable transfection of Chinese hamster ovary (CHO) cells with a plasmid (pcDNA3.1-mCD22) encoding full-length mCD22. Cells were sorted for expression of mCD22 by FACS and maintained in F12/DMEM supplemented with 10% FBS and hygromycin as a selection marker. Other cell lines expressing murine siglecs in Chinese hamster ovary cells have been reported previously (21).

Generation of a Siglec-G–specific mAb

A total of 20 million BW5147 cells expressing Myc-tagged Siglec-G were emulsified with CFA (Difco Laboratories) (38:62, v/v) before immunization.

Two female Lewis rats were immunized in the footpad with the immunogen (100 μl per footpad), followed by two boost injections of the cells emulsified with IFA at 10-d intervals. Animals were sacrificed 3 d after the last injection, and lymphocytes isolated from common iliac lymph nodes were washed three times with serum-free RPMI 1640 medium, and then fused at a 2:1 ratio with the mouse myeloma cell line P3X63Ag.653 cells using polyethylene glycol 1500 (Roche). After the fusion, the cells were selected by hypoxanthine–aminopterin–thymidine selection. The medium for hybridoma culture was RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 μM penicillin, 100 μg/ml streptomycin, 1 mM nonessential amino acid, 1 mM sodium pyruvate, and 50 μM 2-ME, along with 2.5% Opti-Clone II–Hybridoma Cloning Factor (MP Biomedical). For the first screening, hybridoma culture supernatants were assayed for binding to the L929 cells expressing Myc-tagged Siglec-G by flow cytometry in conjunction with an anti-rat IgG secondary Ab. For the second screening, 293T cells were transfected with pcDNA3.1-Myc-His-Siglec-G, kindly provided by L. Nitschke (University of Erlangen, Erlangen, Germany). Hybridoma culture supernatants were assayed for binding to 293T cells transiently expressing Siglec-G by flow cytometry. Isotypes of the Abs were determined by flow cytometry, using biotinylated anti-Rat Ig Abs (BioLegend) followed by streptavidin-PER. Clone 4A6 generated in this study is of the IgG2a, μ isotype. For large-scale preparation of the Abs, the clones were grown 1 wk postconfluence, and the Ab in the culture supernatant was precipitated with ammonium sulfate (291 g/L), dialyzed against PBS, and purified by affinity chromatography using HiTrap Protein G HP column (GE Healthcare). Fractions containing the anti–Siglec-G Ab were dialyzed against PBS. Purified Abs were quantified by monitoring the absorbance at 280 nm. For conjugation, five equivalents of NHS-activated AF-647 (Invitrogen) were reacted with the Abs for 2 h at room temperature in sodium bicarbonate buffer (pH 8.5), followed by dialysis against PBS.

Cell preparation and flow cytometry

Single-cell suspensions of the spleen, bone marrow, and liver were prepared in HBSS containing 3% FCS. Spleen, bone marrow, and liver were ground, and the resulting cell suspension was filtered through a cell strainer (40 μm). Hepatic lymphocytes were purified by centrifugation using a 44%/66% Percoll density gradient (GE Healthcare). Peritoneal lavage from common iliac lymph nodes was performed at 10-d intervals. Animals were sacrificed 3 d after the last injection, and lymphocytes isolated from peritoneal lavage in HBSS containing 3% FCS. After erythrocyte lysis, cells were stained for 20 min at 4°C in HBSS containing 0.1% BSA and 2 mM EDTA (FACS buffer), with the respective Abs (BioLegend) at 1:200 dilution. The Siglec-G Ab was used at 3 μg/ml. Prior to staining, Fc receptors were blocked by anti-CD16/32 (BioLegend) at 1:200 dilution. Dead cells were gated out with 1 μg/ml propidium iodide. Data were acquired with an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Synthesis of siglec ligands

The CD22 ligand [9-bihexenylacetyl-N-glycolylneuraminic acid–α-2,6-galactosyl–β-1→4-N-acetylgalactosamine–β-ethylamine (6′-N9-NeuGc)] was prepared as reported earlier (22). The Siglec-G ligand [9-BPA-NeuGc2-3Galβ1-4GlcAc-β-ethylamine, or 9-bihexenylacetyl-N-glycolylneuraminic acid–α-2,6-galactosyl–β-1→4-N-acetylgalactosamine–β-ethylamine (6′-N9-NeuGc)] and the natural α2-3-sialoside NeuGc2-3Galβ1-4GlcAc-β-ethylamine, or N-glycolylneuraminic acid–α2-3-galactosyl–β-1→4-N-acetylgalactosamine–β-ethylamine (3′-NeuGc)], were prepared using a similar strategy. Briefly, 9-amino-N-glycolylneuraminic acid and N-glycolylneuraminic acid were activated with CMP synthetase from Neisseria meningitidis. Subtractively, without isolation, the resulting CMP-activated sialic acids were reacted with Galβ1-4GlcAc-β-ethylamine, using Pasteurella multocida α2-3-sialyltransferase (1.5 pmol/ml; PMST1), to yield the respective trisaccharides (23). 9-Amino-NeuGc2-3Galβ1-4GlcAc-β-ethylamine was activated with N-acetylhexosamine–activated bihexenylacetyl acetic acid (BPA) using a solvent mixture of H2O and THF (1:1) and N,N-disopropylamine (1.5 eq) as a base. Final hydrogenations with Pd/C (100 wt %) in H2O afforded 3′,6′-NeuGc and 3′-NeuGc. The preparation of antigenic liposomes and immune-liposomes containing siglec ligands was carried out as reported earlier (10). Conjugation of the siglec ligands, the nitrophenol (NP) hapten, or Alexa 647 (Invitrogen) to pegylated distearoylphosphatidanolamine (PEG2000–DSPE) was carried out by reaction of the respective compound with NHS-PEG2000–DSPE or NH2–PEG2000–DSPE. Hen egg lysozyme [(HEL) Sigma-Aldrich] and the Fab fragment of anti-IgM (Jackson Immunoresearch Laboratories) were attached to PEG2000–DSPE by modifying lysine residues with a heterobifunctional crosslinker and subsequent coupling to maleimide-PEG2000–DSPE, as described previously (18).

Preparation of liposomes

Liposomes were prepared by hydration of the lipids in PBS followed by sonication and extrusion through different filters down to a pore size of 0.1 μm.
100 nm, as described previously. All liposomes were composed of a 60:35:5 molar ratio of distearoyl phosphatidylcholine (Avanti Polar Lipids), cholesterol (Sigma-Aldrich), and the respective pegylated lipid. The total concentration of the liposomes, as defined by the molarity of the lipids, was 5 mM. Fluorescent liposomes for binding experiments contained 1% or 4% siglec ligand and 0.1% Alexa 647. Liposomes for functional assays contained 0.5% anti-IgM or 0.33–0.1% HEL and 1 or 4% siglec ligand.

**Liposome binding assays**

Murine splenocytes and siglec-expressing cells were incubated with the respective fluorescent liposomes for 40 min at 4°C in FACS buffer. Cells were washed, then stained with anti-CD19, and binding was assessed by flow cytometry.

**B cell purification**

B cells were purified from splenocytes by negative selection, using magnetic beads according to the manufacturer’s protocol (Miltenyi Biotec). The purity of isolated cells was generally ≥ 99%.

**Ca²⁺ flux**

Following preparation of single-cell suspensions of splenocytes for cells from the peritoneal cavity, cells were loaded with Indo-1 (Invitrogen), as described previously. Cells were stimulated with liposomes, and Indo-1 fluorescence was monitored by flow cytometry, as described in our previous publication.

**B cell proliferation assay and Western blotting**

Purified IgMHEL B cells were stimulated with 5 μM liposomes for 3 min, and their activation was analyzed by Western blotting. For analysis of B cell proliferation, cells were first labeled with 1 μM CellTrace Violet (Invitrogen) prior to stimulation with liposomes. For all in vitro assays, the media consisted of RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM nonessential amino acid, 1 mM sodium pyruvate, and 50 μM 2-ME.

**Immunizations and ELISA**

Mice were injected i.p. or i.v. with liposomes displaying NP or HEL with or without siglec ligand. The liposomes were delivered in 200 μl PBS at 0.5 mM total lipid concentration. Whole blood (50 μL) was collected from mice via retro-orbital bleeds. NP- and HEL-specific Abs were determined by ELISA with NP(1)-BSA–coated (20 μg/ml) MaxiSorp plates (Nunc), as described previously (18). NP(1)-BSA was prepared as described earlier. The titer was defined as the endpoint titer, which was the dilution of serum that achieved an absorbance 2-fold above background.

**Results**

Siglec-G is expressed throughout B cell differentiation

To quantitatively investigate cell surface expression of Siglec-G on murine leukocytes, we developed a mAb toward the extracellular portion of Siglec-G. The Ab shows strong specificity for Siglec-G, as highlighted by its lack of binding to cells from Siglec-G–deficient mice (Fig. 1A). Among splenic leukocytes (Fig. 1B), Siglec-G is expressed at highest levels on B cells and, to a lesser extent, on dendritic cells and a subset of macrophages (Fig. 1B). Slight expression was also observed on neutrophils, and no expression was detected on T cells or NK cells. Of interest, evaluation of B cell subsets from the spleen, bone marrow, and the peritoneal cavity revealed a consistent expression of Siglec-G on all B cell subsets examined (Fig. 1C, 1D). Notably, significant Siglec-G expression appears early on in B cell development, as early as pre-pro and immature B cells. Siglec-G expression remains at a constant level on germinal center and memory B cells, whereas no expression is observed on plasma cells (Supplemental Fig. 1A). All subsets of splenic dendritic cells showed Siglec-G expression with the highest expression on CD11b⁺ myeloid dendritic cells (Supplemental Fig. 1B). In the liver, Siglec-G expression was found on B cells and macrophages (Supplemental Fig. 1C).

**Specificity of a newly developed high-affinity glycan ligand for Siglec-G**

To evaluate the independent roles of CD22 and Siglec-G as regulators of BCR signaling, we set out to evaluate the ability of Siglec-G to induce tolerance by enforced coligation to the BCR, using nanoparticles that expressed both an Ag and a siglec ligand, which we have described as STALs. To investigate the impact of the B cell siglecs on BCR signaling, selective ligands for either siglec were required. Previously, a high-affinity and selective glycan ligand for CD22 (6'-BPA NeuGc; Fig. 2A) was described, but no equivalent ligand has been reported for Siglec-G (22). α2-6-Sialosides carrying a BPA group at the 9-position of the sialic acid (6'-BPA NeuGc) show a 100-fold increase in affinity to CD22 compared with the natural, unsubstituted ligand. Because Siglec-G is known to be capable of binding α2-3-sialoeties, whereas CD22 exhibits strict preference for α2-6-sialoieties, we tested and fortuitously found that addition of the BPA substituent to an α2-3-sialoieties results in a selective and high-affinity ligand for Siglec-G. Accordingly, the ligand 3'-BPA NeuGc was chemoselectively synthesized and incorporated into fluorescent liposomes for assessing binding to siglec-expressing cells. These 3'-BPA NeuGc liposomes showed excellent targeting to Siglec-G-expressing cells and did not bind to cells transfected with any of the other murine siglecs (Fig. 2B). Furthermore, binding of the 3'-BPA NeuGc liposomes to murine splenic B cells was completely dependent on Siglec-G, as no binding was detected in splenocytes from Siglec-G–deficient mice (Fig. 2C). Conversely, 6'-BPA NeuGc liposomes bound exclusively to CD22-expressing cells (Fig. 2B).

**Juxtaposition of Siglec-G and the BCR leads to inhibited Ca²⁺ signaling in both, B1 and B2 B cells**

In previous studies, we and others (17, 18, 25) have demonstrated that enforced colocalization of the BCR and CD22, using nanoparticles displaying both a cognate Ag and CD22 ligands, leads to strong inhibition of B cell activation. With a Siglec-G–specific ligand in hand (3'-BPA NeuGc), we set out to determine if the colocalization of BCR and Siglec-G also suppresses B cell activation and ascertain the degree to which Siglec-G regulates BCR signaling in B1a cells and B2 cells. To this end, we prepared liposomes bearing an anti-IgM Fab fragment that serves as a surrogate Ag, either with or without the Siglec-G ligand (3'-BPA NeuGc), to assess the influence of coligation of the BCR with Siglec-G. Stimulation of either B1a or B2 cells with liposomes displaying anti-IgM alone led to strong B cell activation, as monitored by calcium flux. In contrast, stimulation with anti-IgM–containing liposomes that also displayed 3'-BPA NeuGc resulted in substantial suppression of Ca²⁺ flux in both B cell populations (Fig. 3A, 3B). Although B cells from Siglec-G–deficient mice showed generally diminished responses after stimulation with the liposomes, there was no influence of the Siglec-G ligand on activation of the B cells from Siglec-G–deficient mice. Most importantly, the inhibitory activity of the Siglec-G ligand was also prevented by preincubation of the cells with the anti–Siglec-G mAb, demonstrating that the 3'-BPA NeuGc ligand exhibits its inhibitory function exclusively through Siglec-G (Supplemental Fig. 2). Moreover, anti-IgM liposomes displaying the CD22-specific ligand (6'-BPA NeuGc) suppressed activation, compared with liposomes displaying anti-IgM alone in a CD22–dependent manner in both B2 and B1a B cells (Fig. 3). These results show that the 3'-BPA NeuGc and 6'-BPA NeuGc ligands are specific for Siglec-G and CD22, respectively, and that neither siglec can independently suppress BCR activation when their ligands are incorporated into antigenic liposomes that enforce ligation of the siglec with the BCR.
Broad inhibition of B cell activation in an Shp-1–dependent manner

Although calcium flux is one hallmark of B cell activation, BCR signaling activates numerous signaling cascades that can initiate cell division and survival of B cells. To assess the impact of Siglec-G on the initial events in B cell activation, we analyzed cellular signaling pathways using phospho-specific Abs in conjunction with Western blotting. These experiments were carried out using splenic B cells from MD4 mice (26), which are reactive toward HEL. We first verified in HEL-specific B cells that liposomes displaying HEL and Siglec-G ligand suppress Ca\(^{2+}\) flux, compared with liposomes displaying HEL alone in a Siglec-G–dependent manner (Supplemental Fig. 3). Relative to liposomes displaying HEL alone, liposomes that additionally display the Siglec-G ligand suppressed phosphorylation (or degradation) of multiple BCR signaling events, including those that signal through MAPK, Erk, NF-κB, and Akt (Fig. 4A). The inhibition of B cell signaling we observe is Shp-1 dependent because the activation of B cells from Shp-1–deficient (moth-eaten) mice is not inhibited by the Siglec-G ligand (Fig. 4B). B cells from Shp-1–deficient mice contain a mixture of B1 and B2 cells. Therefore, the results suggest that Siglec-G exhibits its inhibitory function through Shp-1 in both B cell subpopulations. These results demonstrate that ligand-mediated recruitment of Siglec-G to the BCR strongly dampens both proximal and distal

FIGURE 1. Analysis of cell surface expression of Siglec-G on leukocytes. (A) The anti–Siglec-G Ab stains splenic B cells from WT but not SiglecG\(^{-/-}\) mice (anti-Siglec-G in red; isotype control in blue). (B) Siglec-G is expressed on B cells, dendritic cells, and a subset of macrophages from the spleen. (C) Siglec-G is expressed in equal amounts on CD5\(^{+}\) (B1a) and CD5\(^{-}\) (B1b+B2) B cells in the peritoneal cavity. Siglec-G is expressed at a constant level in B cell subsets of the spleen (D) and bone marrow (E).
signaling components in the BCR signaling cascade and that this inhibition is dependent on Shp-1.

Siglec-G ligands reduce B cell proliferation and survival in vitro

We next determined the longer-term fate of the HEL-reactive MD4 cells stimulated with STALs. Fluorescently labeled B cells were cultured in vitro with liposomes, and proliferation of the cells was monitored by fluorescence dilution. Cells incubated with liposomes displaying HEL alone underwent robust proliferation, as assessed at day 4 (Fig. 5A, 5B). Notably, all the cells exposed to liposomes displaying both HEL and 3-BPA NeuGc were activated and divided, but there was a pronounced diminishment in proliferation. Moreover, a significant reduction of live cells relative to cells stimulated with HEL alone (Fig. 5B) was noted. These effects were dependent on Siglec-G, as no difference was observed when Siglec-G–deficient MD4 B cells were stimulated with liposomes displaying Ag and Siglec-G ligand. Therefore, coligation of the BCR and Siglec-G leads to inhibited B cell proliferation and decreased survival, compared with stimulation by ligation of the BCR alone.

Liposomes displaying Siglec-G ligand and Ag induce tolerance in vivo

Copresentation of Ag and CD22 ligands on nanoparticles has been documented to induce Ag-specific tolerance in mice (17, 18). On the basis of the ability of liposomes bearing the Siglec-G ligand to suppress BCR signaling in vitro, we set out to determine if Siglec-G could also independently induce tolerance in vivo. Accordingly, we investigated the ability of liposomal nanoparticles carrying the Siglec-G ligand to induce tolerance to exemplary T-independent and T-dependent Ags. The i.p. injection of immunogenic liposomes displaying the T-independent Ag NP resulted in reproducible production of IgM and IgG Abs, whereas liposomes that additionally displayed 3-BPA NeuGc had greatly reduced anti-NP titers, and failed to produce Ab upon subsequent challenge with the immunogenic liposomes 2 and 4 wk later (Fig. 6A, Supplemental Fig. 4A). Similar results were obtained after i.v. administration of the liposomes (Supplemental Fig. 4B).

Similarly, i.v. administration of immunogenic liposomes displaying the T-dependent Ag HEL induced a strong IgG1 response, whereas only negligible amounts of anti-HEL Abs were produced after injection of liposomes with both HEL and 3-BPA NeuGc (Fig. 6B). Subsequent challenges with HEL liposomes demonstrated that tolerance had been induced in the group of mice given STALs in the initial injection. The identical experiment was repeated in Siglec-G–deficient mice, and no significant difference in Ab titers between the two groups was observed, demonstrating that induction of tolerance is mediated by Siglec-G (Fig. 6B).

To assess the relevance of Siglec-G–mediated tolerance to its natural function on B cells, we investigated tolerance induction by

**FIGURE 3.** Inhibition of Ca\(^{2+}\) flux in B cells by STALs displaying 3-BPA NeuGc. (A and B) Calcium flux of peritoneal cavity B1a (B220\(^{hi}\)CD5\(^{+}\)) B cells (A) or splenic (B220\(^{+}\)CD5\(^{+}\)) B2 B cells (B) from WT, Siglec\(^{−/−}\), or Cd22\(^{−/−}\) mice. Cells were stimulated at \(t = 10\) s, with liposomes displaying anti-IgM (black), anti-IgM + 3-BPA NeuGc (blue), or anti-IgM + 6-BPA NeuGc (red), and the intracellular Ca\(^{2+}\) mobilization was measured by FACS.

**FIGURE 4.** STALs displaying 3-BPA NeuGc broadly inhibit BCR signaling in a SHP-1–dependent manner. (A) Liposomes displaying HEL + 3-BPA NeuGc inhibit phosphorylation or degradation of representative signaling components of BCR signaling, compared with liposomes displaying HEL alone. Purified splenic IgMHEL B cells were stimulated with the indicated liposomes for 3 min at 37°C, then lysed, and the cell lysates were analyzed by Western blotting. Results shown are representative of two independent experiments. (B) Ca\(^{2+}\) flux in WT splenic B cells is inhibited after stimulation with liposomes displaying anti-IgM + 3-BPA NeuGc, compared with liposomes displaying anti-IgM alone, whereas no inhibition is observed in SHP-1–deficient B cells. Results are representative of two independent experiments.
antigenic liposomes displaying a natural sialoside. The sialoside chosen for this study is 3′-NeuGc, a terminal sequence commonly found on N-linked and O-linked glycans on many murine cell types. We find that even this natural ligand is capable of inducing tolerance, and it occurs in a primarily Siglec-G–dependent manner (Fig. 6C). Therefore, recruitment of Siglec-G to the immunological synapse in B cells can induce tolerance in mice, and this may represent a natural tolerogenic mechanism that operates to maintain self-tolerance.

Discussion

In this article, we demonstrate that the two murine B cell siglecs, Siglec-G and CD22, can independently regulate BCR signaling, and can induce B cell tolerance when the Ag is presented in trans with siglec ligands that enforce ligation of the siglec with the BCR. An impediment for studying the role of trans ligands in regulating the function of Siglec-G has been the lack of a suitable high-affinity and selective ligand. In this study, we circumvented this need through the development of such a high-affinity glycan ligand for Siglec-G that does not cross-react with CD22 or any other murine siglec. Incorporation of this newly described Siglec-G ligand onto immunogenic liposomes clearly revealed that recruitment of Siglec-G to the immunological synapse inhibits B cell activation, as shown by calcium flux in an Shp-1–dependent manner, signaling components of the BCR, and proliferation. Therefore, we believe that enforced ligation of Siglec-G and the BCR, resulting from a membrane displaying both Ag and Siglec-G ligand, results in inhibition of B cell activation in a manner similar to that described previously for CD22.

In B cell proliferation assays, we noticed that not only was B cell activation inhibited by liposomes displaying Ag and 3′-BPANeuGc but also the number of live B cells was greatly reduced compared with B cells stimulated with Ag alone. This finding suggested that a tolerogenic circuit is induced, resembling the one we previously described by liposomes decorated with Ag and CD22 ligand. Indeed, STALs were able to induce robust tolerance in vivo toward both T-independent and T-dependent Ag. In contrast to WT mice, Siglec-G–deficient mice did not become tolerized, which clearly demonstrated the specificity of our observations.

Of interest, we discovered that antigenic liposomes displaying a natural α2-3-sialoside could also induce tolerance in a Siglec-G–dependent manner. We believe this may relate to a natural function for Siglec-G on B cells. Tolerization of B cells autoreactive for a cell surface Ag could be aided by Siglec-G, which will be recruited to the immunological synapse by a dense layer of sialic acid. The differential specificity of CD22 and Siglec-G is interesting in this regard. Outside the nervous system, there are two predominant linkages of sialic acid to underlying glycans: α2-3- and α2-6-sialosides. It is well known that the ratio of these two forms of sialic acid varies among different cell types, in large part owing to differential expression of the appropriate 2-6- and 2-3-sialyltransferases (27–30). For instance, hepatocytes and B cells have high levels of α2-6-sialosides owing to high expression of ST6Gal1. By extension, cell types that express ST6Gal1 at lower levels will have more α2-3-sialosides. As mentioned above, CD22 exhibits a strong preference for α2-6- over α2-3-sialosides, whereas Siglec-G clearly has the ability to bind α2-3-sialosides. Thus it will be of interest to determine if CD22 and Siglec-G act cooperatively to mediate peripheral B cell tolerance to autoreactive cell surface Ag through their combined ability to recognize a broader range of sialoside sequences than either siglec alone.

As a part of these studies, we used a recently developed platform for polyclonal stimulation of B cells, which are liposomes decorated with the Fab fragment of an anti-IgM–reactive Ab (18). These were initially used to allow us to investigate both B2 and B1a B cell subtypes, because peritoneal B1a cells from MD4 mice do not express the IgM<sup>HEL</sup> transgene (31). B1a cells constitute the main population of B1 cells in the peritoneal cavity and can be

FIGURE 5. STALs displaying 3′-BPANeuGc inhibit proliferation and decrease survival of B cells in vitro. (A) Purified splenic IgM<sup>HEL</sup> B cells were incubated for 4 d with liposomes displaying HEL alone (black line) or liposomes displaying HEL + 3′-BPANeuGc liposomes, and their proliferation was determined by a fluorescence [cell trace violet (CTV)] dilution assay using FACS analysis. Unstimulated cells (gray) are shown as a control. (B) Quantitation of living cells and cell proliferation. Statistical analyses were performed by Student t test. **p < 0.01, ***p < 0.001.

FIGURE 6. Induction of in vivo tolerance with STALs displaying 3′-BPANeuGc. (A and B) Robust tolerance to (A) T-independent (NP, n = 4) and (B) T-dependent Ag (HEL, n = 8) in a Siglec-G–dependent manner. (C) STALs displaying a natural α2-3-sialoside (3′-NeuGc) also induce tolerance to NP in a Siglec-G–dependent manner (n = 4). In all studies, mice were injected at day 0 with either liposomes displaying Ag alone or liposomes displaying Ag and Siglec-G ligand. On days 14 and 28, all mice received a challenge with liposomes displaying Ag alone. Ab titers were assessed by ELISA. Data represent mean ± SEM.
distinguished from B1b cells by their surface expression of CD5. On the basis of evidence that Siglec-G–deficient mice have more B1a B cells, Siglec-G is believed to play a significant role in the development of this B cell subset. In particular, the expansion in Siglec-G–deficient mice is believed to be the result of hyperreactivity of B1a cells to BCR stimulation (4, 7). In contrast, CD22-deficient mice show enhanced BCR-simulated B cell activation in B2 cells (32–35). Using the anti-IGM liposomes, we find that Siglec-G is capable of inhibiting B cell activation to a roughly equivalent extent in B1a cells from the peritoneal cavity and B2 cells from the spleen. This finding suggests that, besides its important function in B1a cells, Siglec-G is equally capable of inhibiting BCR signaling in splenic B2 cells upon encounter of its cognate Ag in the context of Siglec-G ligands. Whereas B1a and marginal zone B cells are thought to be the primary B cell subtypes responsible for immune responses toward T-independent type II Ags (36), follicular B2 cells are more adapted for T-dependent immune responses (37). Taken together with our finding that tolerance is induced toward both T-independent type-2 and T-dependent Ags, this observation suggests an important function for Siglec-G in maintaining tolerance toward sialylated Ags in various B cell compartments.

Consistent with the hypothesis of Siglec-G playing a role in various B cell compartments, we find equivalent cell surface expression of Siglec-G in B1a cells from the peritoneal cavity and B2 cells from the spleen. Furthermore, Siglec-G is developmentally expressed at constant levels throughout B cell development in the bone marrow, even before expression of IgM in immature B cells (Fig. 1). This observation is consistent with the suggestion that Siglec-G influences the fate of B cells during early development, as evidenced by the enhanced number of B1a cells in the peritoneal cavity of Siglec-G–deficient mice (38). In contrast to Siglec-G, CD22 is only weakly expressed in immature B cells before release into the periphery, and both CD22 expression and the Lyn/CD22/SHIP regulatory access are upregulated in mature peripheral B cells (9, 39–41). Thus, CD22 is not believed to play a role in the early response against T-independent blood-borne particulate antigen. Immunity 14: 617–629.


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