# Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo

Bao Hoa Duong,[1,2] Hua Tian,[3] Takayuki Ota,[1] Gladys Completo,[3] Shoufa Han,[3] José Luis Vela,[1,2] Miyo Ota,[1] Michael Kubitz,[1] Nicolai Bovin,[4] James C. Paulson,[3] and David Nemazee[1]

[1]Department of Immunology and Microbial Science, [2]Kellogg School of Science and Technology Doctoral Programs in Chemical and Biological Sciences, and [3]Department of Chemical Physiology, The Scripps Research Institute, La Jolla, CA 92037
[4]Shemyakin and Ovchinnokov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 117997 Moscow, Russian Federation

Autoreactive B lymphocytes first encountering self-antigens in peripheral tissues are normally regulated by induction of anergy or apoptosis. According to the "two-signal" model, antigen recognition alone should render B cells tolerant unless T cell help or inflammatory signals such as lipopolysaccharide are provided. However, no such signals seem necessary for responses to T-independent type 2 (TI-2) antigens, which are multimeric antigens lacking T cell epitopes and Toll-like receptor ligands. How then do mature B cells avoid making a TI-2–like response to multimeric self-antigens? We present evidence that TI-2 antigens decorated with ligands of inhibitory sialic acid–binding Ig-like lectins (siglecs) are poorly immunogenic and can induce tolerance to subsequent challenge with immunogenic antigen. Two siglecs, CD22 and Siglec-G, contributed to tolerance induction, preventing plasma cell differentiation or survival. Although mutations in CD22 and its signaling machinery have been associated with dysregulated B cell development and autoantibody production, previous analyses failed to identify a tolerance defect in antigen-specific mutant B cells. Our results support a role for siglecs in B cell self-/nonself-discrimination, namely suppressing responses to self-associated antigens while permitting rapid "missing self"–responses to unsialylated multimeric antigens. The results suggest use of siglec ligand antigen constructs as an approach for inducing tolerance.

CORRESPONDENCE
David Nemazee:
nemazee@scripps.edu

Abbreviations used: bNeuGc, 9-biphenylacetyl-NeuGcα2-6Galβ1-4GlcNAc; ITIM, immunotyrosine inhibitory motif; NeuGc, NeuGcα2-6Galβ1-4GlcNAc; NP, nitrophenol; PA, polyacrylamide; QM, quasimonoclonal; siglec, sialic acid–binding Ig-like lectin; TI-2, T-independent type 2; TLR, Toll-like receptor.

B lymphocytes can respond rapidly to nonself-antigens, yet even at mature stages of development can be rendered tolerant if they encounter self-antigen (Goodnow et al., 2005). How B cells distinguish self from nonself has been explained in part by Bretscher and Cohn's associative recognition ("two-signal") hypothesis (Bretscher and Cohn, 1970), which posits that B cells can only achieve activation after a second signal is delivered, the first being recognition of antigen by the BCR. Without this second signal, tolerance is induced. In response to T-dependent antigens, activated helper T cells provide this second signal. In a T-independent type 1 response, the second signal might come from the B cells' Toll-like receptors (TLRs) recognizing conserved microbial motifs attached to the anti-

gen (e.g., lipopolysaccharide; Coutinho et al., 1974). This model, however, fails to explain how T-independent type 2 (TI-2) responses occur, as TI-2 antigens require neither T cells (Mond et al., 1995) nor recognition by known innate immune receptors (Gavin et al., 2006), and can elicit antibody responses in cultures of single B cells (Nossal and Pike, 1984). Although we do not dispute contributory roles of innate immune receptors, cytokines, or accessory cells in amplifying their responses (Mond et al., 1995; Vos et al., 2000; Hinton et al., 2008), TI-2 antigens

appear to have only two surprisingly simple properties, high molecular weight and ≥20 closely spaced BCR epitopes (Dintzis et al., 1976), and are thus unlikely to have innate receptors specialized for their recognition.

Alternatively, B cells might be capable of "missing self"–recognition (Parish, 1996; Nemazee and Gavin, 2003) similar to that originally observed in NK cells (Kärre et al., 1986). In NK cell recognition, the decision to lyse a target cell depends on integration of opposing signals from activating and inhibitory receptors (Lanier, 2008). Activating receptors trigger recruitment of tyrosine kinases to immunotyrosine activating motifs of associated adapter molecules but are kept in check by inhibitory receptors recognizing classical MHC I molecules expressed on target cells (Lanier, 2008). Inhibitory receptors carry immunotyrosine inhibitory motifs (ITIMs), which serve as docking sites for phosphatases, such as SHP-1, that counteract activation (Ravetch and Lanier, 2000). Target cells that down-regulate MHC I are lysed owing to unopposed activation, hence missing self–recognition.

Extrapolating from this model, we hypothesize that besides their BCR epitopes, self-antigens carry self-markers that can engage inhibitory receptors on B cells, preventing antiself TI-2–like responses and rendering activation dependent on second signals. The concept that self-markers might facilitate self-tolerance was first suggested many years ago by Burnet and Fenner (1949) but has garnered little experimental support with respect to lymphocyte tolerance. According to our model, antigens that simultaneously cross-link the BCRs and inhibitory receptors should prevent or blunt B cell responses. Conversely, antigens that bind only the BCR and not inhibitory receptors are predicted to elicit a TI-2 response, provided that they carry the appropriate number and spacing of epitopes. This missing self–model of self-/nonself-discrimination would explain why B cells constitutively express so many inhibitory receptors that recognize ubiquitous self-components, and why null mutations in those receptors or their signaling machinery can lead to autoantibody formation (Nishimura et al., 1998; Pan et al., 1999; Ravetch and Lanier, 2000; Nemazee and Gavin, 2003).

In this study, we chose to test if self-/nonself-discrimination is regulated by self-markers through the roles of the sialic acid–binding Ig-like lectins (siglecs) CD22 and Siglec-G in B cells. The siglec family consists of 9 members in mice and 13 members in humans (for review see Crocker et al., 2007). In mice, mature B cells express CD22 (Siglec 2) and Siglec-G, which bind to host sialic acids carried on glycans of glycoproteins and glycolipids and have properties of inhibitory receptors. They carry ITIMs capable of recruiting the tyrosine phosphatase SHP-1 and attenuating BCR signaling (Campbell and Klinman, 1995; Doody et al., 1995; Cornall et al., 1998). Mice carrying null mutations in either *CD22* or *Siglecg* exhibit B cell hyperactivity, variable responses to T-independent antigens, and a tendency toward autoantibody formation (O'Keefe et al., 1996; Otipoby et al., 1996; Sato et al., 1996; Nitschke et al., 1997; Cornall et al., 1998; O'Keefe et al., 1999; Ding et al., 2007; Hoffmann et al., 2007). Mouse CD22 exhibits a strong prefer-

ence for sialoside ligands with the disaccharide sequence NeuGcα2-6Gal (Collins et al., 2006a; Crocker et al., 2007), whereas Siglec-G, before this study, has had an unknown ligand specificity. Their disaccharide ligands represent terminal sugars commonly carried on N- and O-linked glycans of glycoproteins and are found on virtually all cells, including B cells (Crocker et al., 2007). It is well documented that CD22 binds to glycans on endogenous B cell glycoproteins in cis, and masks the ligand binding site from binding synthetic polymeric ligands (Hanasaki et al., 1995; Razi and Varki, 1998; Razi and Varki, 1999; Collins et al., 2002; Han et al., 2005). Yet, CD22 is able to recognize native ligands on glycoproteins of apposing cells in trans, causing it to redistribute to the site of cell contact (Lanoue et al., 2002; Collins et al., 2004). Although mutations of the ligand binding domain of CD22 (Jin et al., 2002; Poe et al., 2004) and ablation of enzymes involved in the synthesis of its glycan ligands (Hennet et al., 1998; Poe et al., 2004; Collins et al., 2006b; Ghosh et al., 2006; Grewal et al., 2006; Naito et al., 2007; Cariappa et al., 2009) document the importance of siglec ligands in the regulation of CD22 function, a unifying role for CD22 ligand interactions in B cell biology has not yet emerged (Crocker et al., 2007; Walker and Smith, 2008). Although less is known about the specificity of Siglec-G and its interaction with ligands, it is assumed that similar concepts regarding cis and trans ligands will apply to the modulation of Siglec-G function (Hoffmann et al., 2007; Chen et al., 2009).

Because siglecs see sialylated glycans that are usually absent from microbes, with the notable exceptions of some pathogenic microbes (Crocker et al., 2007; Carlin et al., 2009a), one possible role of siglecs is to discriminate self from nonself. Though CD22 and Siglec-G have been implicated to play roles in B cell tolerance, evidence has been indirect, inferred from the facts that they possess ITIMs able to recruit SHP-1 and dampen $Ca^{2+}$ signaling (Otipoby et al., 1996; Sato et al., 1996; Nitschke et al., 1997; O'Keefe et al., 1999; Ding et al., 2007; Hoffmann et al., 2007). Hypomorphic or null alleles of *CD22* and SHP-1 (*Ptpn6*) have been correlated with anti-DNA production and development of lupus erythematosus (Shultz et al., 1993; O'Keefe et al., 1999; Mary et al., 2000). *CD22* mutations also lead to increased in vivo B cell proliferation and turnover (Otipoby et al., 1996; Nitschke et al., 1997; Poe et al., 2004; Haas et al., 2006; Onodera et al., 2008). However, studies designed to directly assess tolerance induction in antigen-specific *CD22*$^{-/-}$ or SHP-1 mutant B cells found, paradoxically, a more robust tolerance relative to unmutated controls (Cyster and Goodnow, 1995; Cornall et al., 1998; Ferry et al., 2005). This suggests that the autoimmune phenotypes of siglec and SHP-1 null mutants could be caused by abnormal B cell selection and development rather than failure of tolerance. It is generally assumed that physical association of CD22 with the BCR will allow CD22 to exert a maximal inhibitory response (Pezzutto et al., 1987; Doody et al., 1995; Lanoue et al., 2002; Courtney et al., 2009), but evidence to support this has been garnered only from in vitro experiments (Ravetch and Lanier,

2000; Lanoue et al., 2002; Tedder et al., 2005; Courtney et al., 2009). In this paper, we show in wild-type mice with unaltered B cell selection and development that decorating a TI-2 antigen with siglec ligands not only prevents its immunogenicity but can also tolerize B cells to subsequent challenges with the unsialylated, immunogenic form. The results suggest that one function of B cell inhibitory receptors like siglecs is to assist B cells in distinguishing self from nonself.

## RESULTS
### Specificity of CD22 and Siglec-G for multivalent sialosides linked to polyacrylamide (PA)
Because our goal was to design copolymers of siglec ligands and a TI-2 antigen that would recruit siglecs to the hapten-specific BCR, we characterized highly multivalent sialylated glycans linked to PA for their ability to compete with cis ligands and bind to CD22 and Siglec-G on native B cells. We and others previously showed that synthetic trans ligands of CD22 (NeuGcα2-6Gal) compete poorly with endogenous cis ligands unless they were displayed on highly multivalent polymers and included hydrophobic substituents at the 9 position that increase affinity (Razi and Varki, 1998; Collins et al., 2002; Collins et al., 2006a). Therefore, we evaluated binding of highly multivalent PA conjugates (1,000 kD) carrying sialosides (n ≈ 400) and biotin tags to facilitate their detection. Each was analyzed for its binding to B cells from wild-type, CD22-deficient, Siglec-G–deficient, and CD22/Siglec-G double-deficient mice (Fig. 1). The PA–sialosides with NeuAcα2-6Galβ1-4GlcNAc–containing glycans showed no binding to mouse B cells, consistent with the known preference of mouse CD22 for NeuGcα2-6Galβ1-4GlcNAc (NeuGc; Fig. 1, A and D; Crocker et al., 2007). The conjugate containing the preferred native ligand of CD22, NeuGc, was bound well by wild-type cells (Fig. 1 B). Surprisingly, however, binding to this glycan by both $CD22^{-/-}$ and $Siglecg^{-/-}$ single-deficient B cells was also significantly reduced, whereas $CD22^{-/-};Siglecg^{-/-}$ double-deficient B cells completely failed to bind, indicating that both siglecs contribute to binding (Fig. 1 B). Thus, the glycan previously described as the natural ligand for CD22 (Pezzutto et al., 1987; Collins et al., 2002; Blixt et al., 2003; Crocker et al., 2007) is also a ligand of Siglec-G, and both siglecs are required for optimal binding of this ligand. Similarly, the PA–9-biphenyl-acetyl-NeuGcα2-6Galβ1-4GlcNAc (PA–bNeuGc) conjugate, previously shown to have high affinity for mouse CD22 (Collins et al., 2006a), was bound strongly by B cells, and whereas binding to CD22 was dominant, both CD22 and Siglec-G contributed to the association (Fig. 1 C). B cells from $CD22^{-/-};Siglecg^{-/-}$ mice exhibited residual binding to PA–bNeuGc (~6%), suggesting that an additional receptor might also recognize this ligand. Importantly, binding to wild-type B cells of sialoside with NeuGc in α2-3 linkage to Galβ1-4GlcNAc was also observed (Fig. 1 E). However, in this case binding was exclusively caused by Siglec-G, for there was little change in binding to $CD22^{-/-}$ B cells. We conclude that Siglec-G and CD22 have distinct and overlapping specificities,

and together bind native sialosides containing both the NeuGcα2-6 and NeuGcα2-3 linkages to Galβ1-4GlcNAc found as terminal sequences on glycans of most, if not all, mouse cells.

### In vivo responses to TI-2 conjugates carrying siglec ligands
To see if incorporating siglec ligands into TI-2 antigen affects B cell in vivo responses, PA was conjugated with nitrophenol (NP) hapten and one of two glycans found to be ligands of CD22 and Siglec-G. We selected the natural sialoside NeuGc and the high affinity siglec ligand bNeuGc to ensure that adequate competition with cis ligands would be achieved. As an immunogenic control, a corresponding conjugate (NP–PA) was prepared with a glycan lacking sialic acid (Galβ1-4GlcNAc). The resulting polymers contained ~200 NP haptens and 400 glycan moieties (Fig. 2 A).

Because haptens appropriately displayed on high molecular weight PA should behave as TI-2 antigens (Dintzis et al., 1976), we anticipated that NP–PA would elicit an anti-NP antibody response, whereas the response to NP–PA–NeuGc and NP–PA–bNeuGc might be blunted by recruitment of siglecs to the BCR binding the antigen. As anticipated, robust IgM and IgG3 responses against NP were detected in mice immunized with NP–PA (Fig. 2, B and C). In contrast, NP–PA–bNeuGc failed to induce a significant anti-NP response (Fig. 2 B) and NP–PA–NeuGc elicited a significantly reduced response (Fig. 2 C). The results indicated that presentation of siglec ligands reduced or prevented the antibody response to the antigen, and the extent of the reduction correlated with their affinity to CD22 and Siglec-G.

### Contributions of siglecs in down-regulation of antigen responses by native glycan ligands
As shown in Fig. 1, both Siglec-G and CD22 combine for optimal binding of the native ligand (NeuGc) incorporated into the NP–PA–NeuGc construct. To assess the roles of these siglecs in the altered response, we compared NP antibody responses to NP–PA–NeuGc and NP–PA in $CD22^{-/-}$, $Siglecg^{-/-}$, and wild-type mice (Fig. 3). NP–PA–NeuGc elicited a significantly reduced response compared with NP–PA in wild-type mice. $CD22^{-/-}$ mice also responded poorly to sialylated compound, though the response to unsialylated conjugate was slightly lower than in wild-type mice (Fig. 3, A and B), consistent with previous studies (Otipoby et al., 1996; Nitschke et al., 1997). In contrast, the presence of sialylated glycans completely failed to reduce the response in $Siglecg^{-/-}$ mice (Fig. 3 C). Thus, consistent with the binding data (Fig. 1 B), Siglec-G rather than CD22 is predominately responsible for the reduced response to natively sialylated conjugate under the conditions of immunization used in this study.

### Tolerance induction by TI-2 antigen carrying siglec ligands
We considered three possibilities for the effects of antigen sialylation on B cell response: (1) it dampened the response, (2) it dampened the response and actively induced tolerance, and (3) it rendered antigen invisible. The robust immunogenicity

of NP–PA–NeuGc in the absence of Siglec-G recognition showed that the poor response elicited to this compound in wild-type and *CD22*^{−/−} mice was not caused by hapten inaccessibility, arguing against the third possibility. To further investigate the possibility of tolerance induction, a series of experiments was performed whereby mice previously injected with sialylated NP conjugates were subsequently challenged with immunogenic NP–PA.

Because NeuGc-carrying NP conjugates appeared to suppress anti-NP responses less efficiently than those with bNeuGc, we first generated additional conjugates with similar amounts of native siglec ligand but less NP, and tested them in C57BL/6 mice for their ability to elicit anti-NP responses and to modulate a secondary challenge with $NP_{200}$–PA. Optimal results were obtained with $NP_{65}$–PA–$NeuGc_{400}$. Remarkably, the sialylated antigen not only elicited significantly lower primary IgM and IgG3 responses than the corresponding immunogenic $NP_{65}$–PA control but also led to reduced responses upon subsequent immunization with unsialylated $NP_{200}$–PA antigen (Fig. 4 A). The results demonstrated the ability of natively sialylated TI-2 antigen to suppress antibody responses and to promote tolerance, provided that the BCR signal did not outweigh siglec-mediated inhibition.

Even more striking biological effects were seen in mice injected with the conjugate containing the higher affinity siglec ligand NP–PA–bNeuGc and then subsequently challenged with NP–PA. Remarkably, a single dose of NP–PA–bNeuGc rendered mice tolerant to NP–PA for at least 1 mo, as indicated by the near absence of anti-NP responses after two challenges with the unsialylated antigen 14 and 31 d later (Fig. 4 B). Control experiments showed that PA–bNeuGc lacking NP had no negative effect on the subsequent NP response to NP–PA and that tolerance induction by NP–PA–bNeuGc took about 2 wk to be fully established (Fig. S1, A and B). Furthermore, conjugates carrying identical amounts of Galβ1-4GlcNAc (nonsiglec binding) and decreasing amounts of NP did not induce tolerance to NP but instead simply showed decreasing potency in eliciting an initial anti-NP response (Fig. S2), thus ruling out the trivial possibility that glycan modifications hinder recognition of NP because of steric interactions. These results further supported the notion that antigen sialylation did not prevent its recognition by B cells but led to a distinct biological response. We conclude that attachment of high affinity siglec ligands to TI-2 antigen promoted antigen-specific tolerance rather than immunity, whereas a lower affinity ligand significantly blunted but did not always completely prevent the antibody response.

To test the robustness of the tolerance, we asked if nonspecific activators of inflammation could prevent tolerance induction by sialylated antigen. Accordingly, mice were challenged
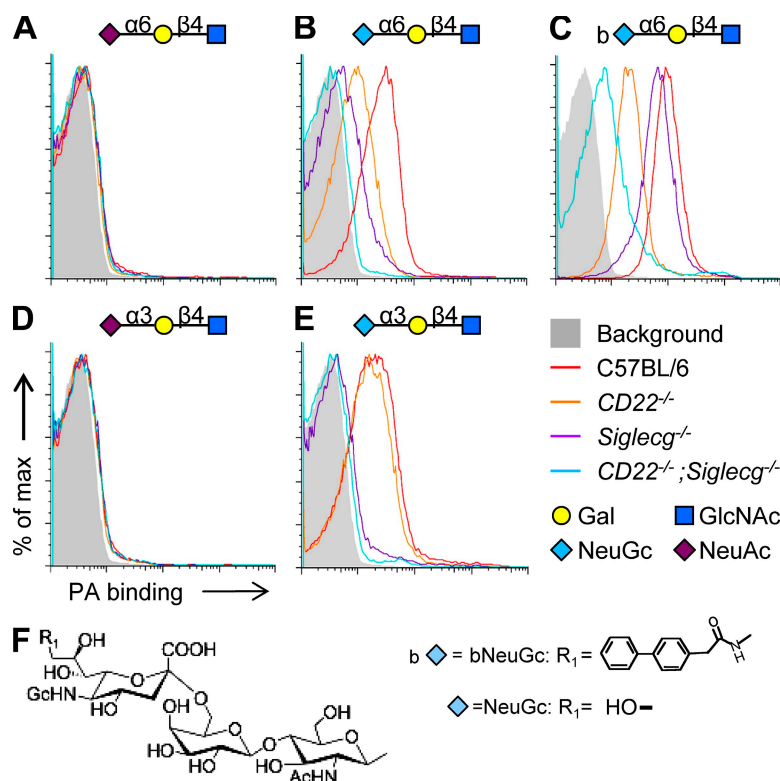


**Figure 1. Binding analysis of various sialylated PA–glycan conjugates to wild-type compared with siglec mutant B cells.** Results are representative of three independent experiments. (A–E) B cells of the indicated genotypes were stained with biotinylated PA conjugated with the following glycans: (A) NeuAcα2-6Galβ1-4GlcNAc, (B) NeuGc, (C) bNeuGc, (D) NeuAcα2-3Galβ1-4GlcNAc, and (E), NeuGcα2-3Galβ1-4GlcNAc. Binding was revealed using fluorescent streptavidin. Background staining by streptavidin alone is shown in gray. (F) Chemical structures of the NeuGc-containing sialosides.

with NP–PA–bNeuGc mixed with Ribi, an adjuvant containing TLR4 ligand and other proinflammatory signals. This treatment was only able to rescue IgM and IgG3 responses to 10–20% of the response to NP–PA given with Ribi (Fig. 5 A), though it also rescued subsequent responses to rechallenge with NP–PA 18 d later (Fig. 5 B). Thus, although inflammatory signals provided by adjuvant overrode tolerance, siglec ligands could still significantly suppress their amplification of the TI-2 response.

Analysis of the siglec dependence revealed that both CD22 and Siglec-G were required for optimal induction of tolerance by NP–PA–bNeuGc. Even though neither $CD22^{-/-}$ nor $Siglecg^{-/-}$ mice made an anti-NP response to initial treat-

ment with NP–PA–bNeuGc, both strains generated a strong IgM response upon subsequent immunization with NP–PA (Fig. 4, C and D, top). However, in both mutants subsequent IgG3 responses to NP–PA were still notably deficient. Overall, these data indicated that tolerance induced by sialylated antigen was impaired in $CD22^{-/-}$ and $Siglecg^{-/-}$ mice, and therefore was partly dependent on both CD22 and Siglec-G. A preliminary experiment immunizing $CD22^{-/-};Siglecg^{-/-}$ mice gave similar results to the single mutants (Fig. S3), suggesting that there may be another siglec on B cells compensating for their function (see Discussion).

### In vivo visualization of the B cell response

To follow cellular aspects of anti-NP B cell responses in vivo, splenic NP-specific B cells were isolated from quasimonoclonal (QM) anti-NP BCR-transgenic mice (Cascalho et al., 1996), transferred to $Rag1^{-/-}$ hosts, and challenged with conjugates given i.p., and recipient splenocytes were analyzed 7 d later. Mice were administered BrdU throughout to label cells that proliferated during this period. Significant differences in proliferation were seen in response to NP–PA compared with NP–PA–bNeuGc (P = $8.5 \times 10^{-6}$; Fig. 6 A, left two columns). In recipients immunized with NP–PA, 90% of $B220^+$ cells were BrdU labeled during the 7-d period, whereas proliferation was lower in response to NP–PA–bNeuGc (50%), PA–bNeuGc (20%), or saline (22%; Fig. 6 C). Similar results were obtained when QM B cells were transferred along with nontransgenic B cells or transferred into unirradiated, RAG-sufficient hosts (Fig. S4 and not depicted). A large fraction of the $BrdU^+$ cells in recipients challenged with NP–PA appeared to be follicular phenotype B cells, as they expressed CD23 and IgD. Importantly, the generation of NP-specific plasma cells and serum antibody was robust in NP–PA–immunized mice but extremely poor in other treatment groups (Fig. 6, B and D). The increased proliferative response to NP–PA–bNeuGc, compared with PA–bNeuGc or saline challenge, was attributable to NP recognition but was not correlated with an antibody response. We conclude that tolerance induction to sialylated antigen is associated with suboptimal B cell proliferation without plasma cell differentiation and likely death of abortively activated cells.

### Responses of Bcl2-transgenic B cells

To assess the possibility that NP–PA–bNeuGc promoted tolerance through an apoptotic mechanism, we challenged mice carrying the Eμ-Bcl2-22 transgene (*Bcl2 Tg*; Strasser et al., 1991), which have enforced Bcl2 expression in B cells and are known to have impaired peripheral B cell tolerance (Lang et al., 1997). NP–PA–bNeuGc challenge failed to prevent *Bcl2 Tg* mice from making a subsequent response to NP–PA, whereas similarly treated wild-type mice were tolerized (Fig. 7, condition C). These data suggest that NP–PA–bNeuGc challenge led to apoptosis of NP-reactive B cells in wild-type mice but not in *Bcl2 Tg* mice. It is noteworthy that enforced expression of Bcl2 did not permit B cells to respond by antibody production to NP–PA–bNeuGc (condition A), whereas
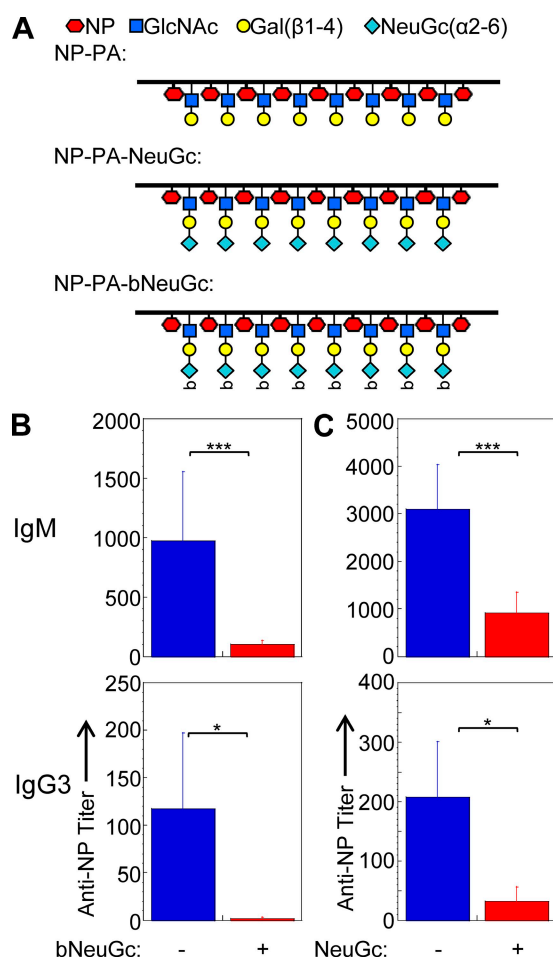


**Figure 2.** **Design of sialylated and unsialylated TI–2–like conjugates and comparison of initial antibody responses elicited in C57BL/6 mice.** (A) Structure of immunogenic and tolerogenic conjugates. Shown schematically are PA conjugates carrying both NP and the nonsiglec-binding carbohydrate Galβ1-4GlcNAc (NP–PA), compared with the conjugates NP–PA–NeuGc or NP–PA–bNeuGc. (B and C) Mice were immunized 7 d previously with 20 μg of the indicated conjugates in PBS. Unless otherwise stated, the antigen dose given in all experiments of this study was 20 μg in PBS. Results shown are representative of at least three independent experiments. All statistical tests given used the two-tailed Student's *t* test. Means + SD are shown. *, P < 0.05; ***, P < 0.005. (B) Antibody response to NP–PA–bNeuGc (+) versus NP–PA (−; n = 4/group). (C) Antibody response to NP–PA–NeuGc (+) versus NP–PA (−; n = 7/group).

it rescued a subsequent response to NP–PA. These data show that NP-specific B cells protected from apoptosis neither responded to nor were tolerized by sialylated conjugate, indicating that B cell tolerance involved both apoptotic and nonapoptotic mechanisms.

### In vitro responses to TI–2 conjugates with or without siglec ligands

To further probe the effects of antigen sialylation on B cell responses, NP–PA, NP–PA–bNeuGc, and PA–bNeuGc were compared for their abilities to bind and to activate NP-specific B cells. Both NP and bNeuGc made important independent contributions to overall binding, which was enhanced with conjugates carrying both ligands (Fig. 8 A). In response to NP–PA challenge in vitro, NP-reactive splenic B cells mobilized $Ca^{2+}$ (Fig. 8 B) and proliferated (Fig. 8 C), whereas they responded poorly to NP–PA–bNeuGc (Fig. 8, B and C). PA–bNeuGc had no effect on B cell proliferation in vitro (unpublished data). Thus, despite improving binding to B cells, sialylated antigen suppressed NP-specific B cell responsiveness in vitro.

Tyrosine phosphorylation analysis of NP-specific B cells stimulated with tolerogenic compared with immunogenic NP conjugates revealed that sialylation altered both overall and CD22-specific phosphorylation (Fig. 8, D–G). NP–PA stimulated robust tyrosine phosphorylation of many substrates (Fig. 8 D, lane 2), whereas NP–PA–bNeuGc triggered a comparatively reduced overall tyrosine phosphorylation (lane 4), with the notable exception of CD22, which was more strongly phosphorylated (Fig. 8 E, lane 4 vs. 2). Control con-

jugate without NP failed to induce phosphorylation, except perhaps in CD22 (lanes 6 and 7), but as shown in Fig. S1, this had no effect on subsequent responses to NP. CD22 immunoprecipitates revealed, as expected, that upon activation with conjugate a fraction of SHP-1 was bound (Fig. 8 E, lanes 2 and 4). However, the relative levels of associated SHP-1 were similar in both NP–PA– and NP–PA–bNeuGc–stimulated cells and were also significant in PA–bNeuGc–stimulated cells, despite the differences in overall tyrosine phosphorylation. Phosphorylation of Y531 on CD19 is known to recruit PI3K activity leading to Akt activation and attendant S473 phosphorylation (Tuveson et al., 1993; Otero et al., 2001; Wang et al., 2002). At 5 min after NP–PA stimulation, CD19 (Y531) and Akt (S473) phosphorylation were elevated (Fig. 8, F and G, lane 2), whereas in NP–PA–bNeuGc–challenged cells no increased phosphorylation was seen (lane 4). This indicated that the most striking effect of BCR/Siglec coligation was suppression of CD19 phosphorylation and downstream PI3K/Akt activation rather than SHP-1 recruitment alone. Thus, sialylated antigen induced more CD22 phosphorylation, suppressed early activation signals, and directly suppressed antigen-specific B cell proliferation in vitro.

### DISCUSSION

With their high molecular weights and multimeric arrays of identical epitopes, most host cell surfaces and fragments thereof are potential TI-2 antigens, but rather than inducing a response, host cell-surface antigens are strongly tolerogenic for mature B cells (Russell et al., 1991; Murakami et al., 1992). An important difference from immunogenic TI-2 antigens
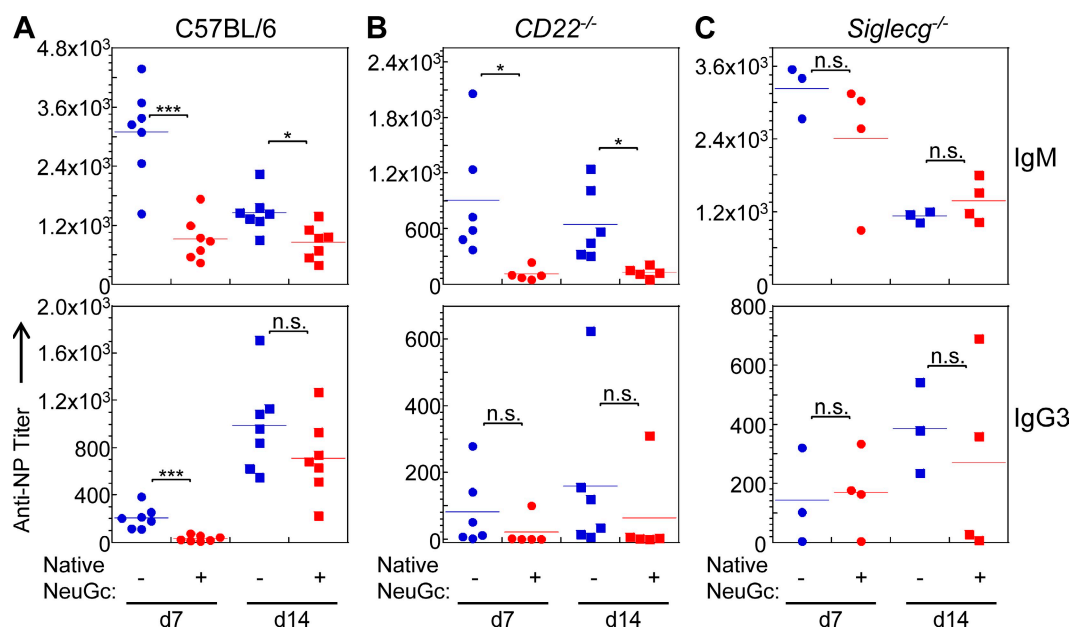


**Figure 3.    Serum anti-NP responses to unsialylated (−; NP–PA) or natively sialylated (+; NP–PA–NeuGc) compounds.** (A–C) Responses of (A) wild-type, (B) *CD22*⁻/⁻, and (C) *Siglecg*⁻/⁻ mice. Anti-NP titers were assessed on days 7 and 14 as indicated. (top) IgM anti-NP titers; (bottom) IgG3 anti-NP titers. Each point represents the response of an individual mouse of the indicated genotype. Results shown are representative of at least two independent experiments. Horizontal bars represent means. *, P < 0.05; and ***, P < 0.005 using the two-tailed Student's *t* test. n.s., not significant.
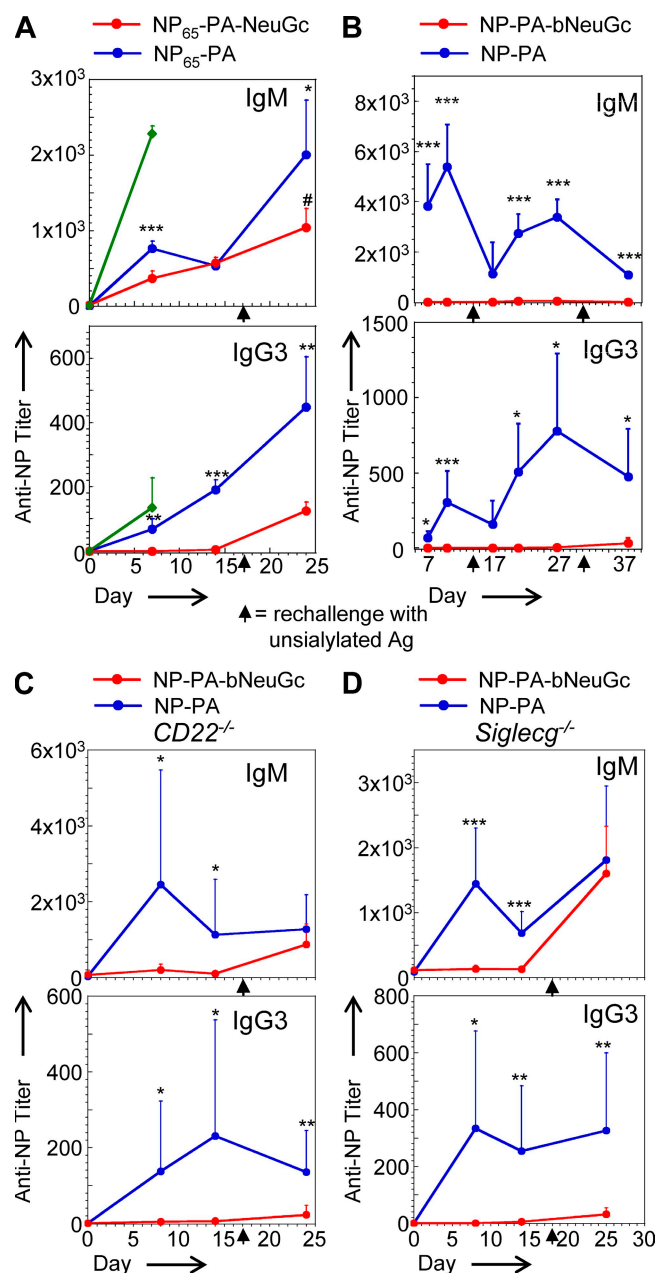
**Figure 4. Analysis of tolerance induction by the sialylated antigens NP–PA–NeuGc and NP–PA–bNeuGc, and responses in siglec-deficient mice.** (A–D) Functional assay of in vivo tolerance induction. Results shown are representative of at least two independent experiments. (A) Mice ($n = 4$/group) were challenged with $NP_{65}$–PA–NeuGc on day 0 and rechallenged with $NP_{200}$–PA on day 17. IgM and IgG3 antibody levels were assessed at the indicated time points. Control mice received either $NP_{65}$–PA on day 0 and were rechallenged with $NP_{200}$–PA as in the experimental group, or received just $NP_{200}$–PA without pretreatment (green lines). #, a significant reduction in $NP_{65}$–PA–NeuGc–pretreated mice at 7 d after challenge with $NP_{200}$–PA ($P = 8.84 \times 10^{-5}$) compared to nonpretreated animals at 7 d after $NP_{200}$–PA challenge (green line). (B) Mice ($n = 4$/group) were challenged with NP–PA–bNeuGc on day 0 and rechallenged with NP–PA on days 14 and 31. Control mice received NP–Ficoll on day 0 and were rechallenged with NP–PA as in the experimental group. (C and D) Tolerance induction in siglec-deficient mice. Serum IgM and IgG3 anti-

may be that host tissues are sialylated and can engage siglecs. Indeed, sialic acids have been estimated to be present on cell surfaces at concentrations of >10 mM (Collins et al., 2004). In an in vitro model of B cell reactivity to cell-surface antigen, Lanoue et al. (2002) demonstrated that expression of CD22 ligands on cells could suppress induction of B cell co-stimulatory molecules through a CD22-dependent pathway, presumed to result from redistribution of CD22 to the site of cell contact by trans ligands on the antigen-presenting cell (Collins et al., 2004). We find that when TI-2 antigens are sialylated it renders them tolerogenic or nonimmunogenic in vivo, and present genetic evidence that both CD22 and Siglec-G contribute to both suppression of immune responses and tolerance induction. Previous studies have shown that coengagement of CD22 and the BCR can suppress early B cell signal transduction and blunt activation (Lanoue et al., 2002; Tedder et al., 2005; Courtney et al., 2009), but experiments designed to directly assess B cell tolerance in the presence and absence of CD22 or its downstream signaling components failed to reveal a defect in tolerance induction (Cyster and Goodnow, 1995; Cornall et al., 1998; Ferry et al., 2005). The primary difference in our studies is the analysis of responses to TI-2 antigens conjugated to siglec ligands to introduce missing self. To our knowledge, the present studies are the first to clearly demonstrate that siglecs facilitate B cell tolerance, as opposed to simply blunting antigen recognition, and suggest a general paradigm for the role of B cell siglecs in regulating BCR response to antigens in the context of distinguishing self from nonself.

In this study we have focused on siglecs and sialic acid–containing glycans as self-markers. Although CD22 is highly specific for α2-6 sialosides (Crocker et al., 2007), it is notable that Siglec-G recognizes both α2-3– and α2-6–linked sialosides (Fig. 1). Thus, the specificities of these two siglecs are complementary and together cover the most common sequences found on cell-surface glycans, providing a broad basis for self-recognition with two siglecs. Although siglecs are a rapidly evolving family (Crocker et al., 2007), CD22 expression is conserved in mammals, and human B cells express Siglec-10, an orthologue of Siglec-G, as well as Siglec-5 (Crocker et al., 2007; Yamanaka et al., 2009). The specificity of these three siglecs combined cover all the major sialoside sequences expressed on human cells (Crocker et al., 2007). Thus, although the ligands recognized by the specific human and mouse B cell siglecs have evolved, the ligand specificities cover the major sialoside sequences expressed as self-markers on cells.

NP responses of mice that were primed on day 0 with either NP–PA–bNeuGc or NP–PA and boosted on days 17 or 18 with NP–PA. (C) Responses in $CD22^{-/-}$ mice ($n = 8$ NP–PA; $n = 9$ NP–PA–bNeuGc). Secondary challenge was done on day 17. Because of the known weak TI-2 responses in $CD22^{-/-}$ mice, all conjugate injections were performed with a double dose of antigen (40 µg/mouse). (D) Responses in $Siglecg^{-/-}$ mice ($n = 8$ NP–PA; $n = 9$ NP–PA–bNeuGc). Secondary challenge was done on day 18. Shown are means + SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$ using the two-tailed Student's $t$ test.

Although tolerance in the present study was induced by the native ligand with NeuGc, it is most efficiently induced using antigen conjugates carrying bNeuGc, which binds to both CD22 and Siglec-G with higher affinity. We attribute this to more efficient competition with cis ligands and recruitment of siglecs to the site of antigen engagement with the BCR. As evident in the studies with B cells from the QM mice, the polymer containing both antigen and siglec ligands binds to B cells better than polymers containing either one alone, demonstrating synergy in binding. In this regard, Courtney et al. (2009) recently reported that a mouse 2,4-dinitrophenol–specific B cell line suppressed early triggering responses to 2,4-dinitrophenol when displayed on a polymer backbone with NeuAcα2-6Galβ1-4Glc, despite the fact that this glycan has low affinity for mouse CD22 (<10% that of the preferred NeuGc ligand; Fig. 1 A; Blixt et al., 2003; Collins et al., 2006a). Thus, siglec ligands can produce graded responses of B cells depending on the affinity and degree of recruitment of siglec to the site of antigen engagement. Our finding that copolymers of antigens with high affinity siglec ligands both reduce an immune response and facilitate induction of tolerance suggests possible uses in clinical settings in which induction of antigen-specific B cell tolerance might be beneficial. Further studies are required to define the optimal parameters of conjugates for efficacy in therapeutic tolerance induction.

The polymeric presentation of antigen and siglec ligands in this study is intended to mimic their simultaneous presentation in a more biological context, such as a cell surface. Given the multivalency required for TI–2 responses, the observations made in this study might be highly relevant to B cell tolerance and response to high molecular weight self-antigens in addition to cell surfaces. Siglec ligands may be especially important in restraining these responses because of their ubiquity and association with secreted and cell-surface proteins. Cell surfaces should be particularly tolerogenic owing to the high number of self-moieties able to engage inhibitory receptors on B cells, such as siglecs, PirB, CD72, FcγRIIb, BTLA, PD-1, and CD31, which recognize, respectively, sialic acids, MHC I, CD100, IgG-Fc, HVEM, PD-L1/2, and
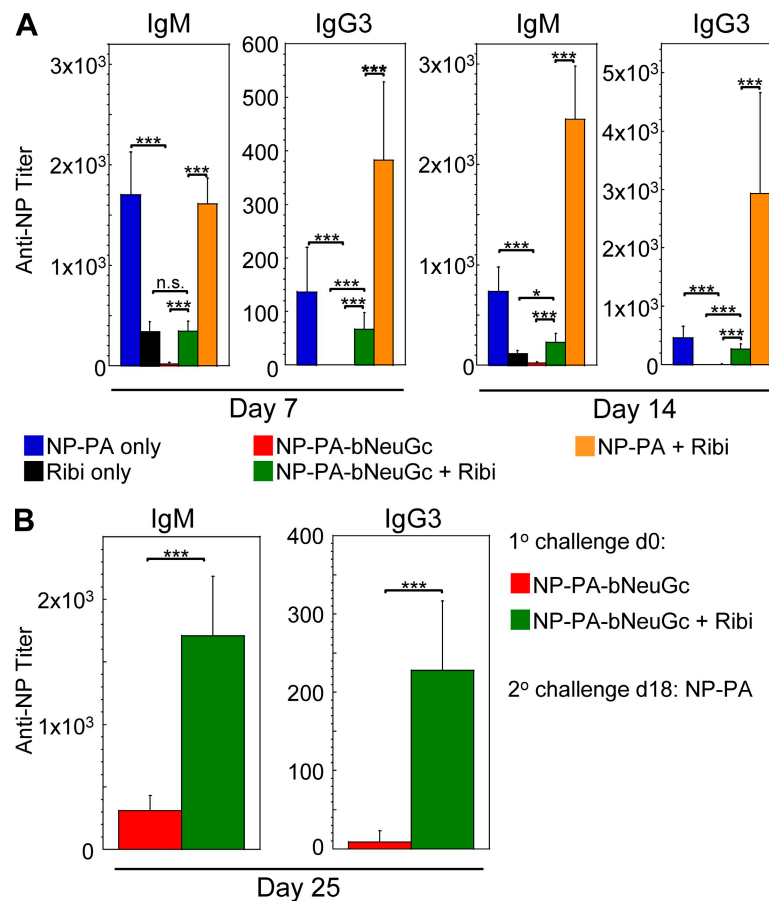


**Figure 5. Effect of Ribi coadministration on tolerance induction and response to sialylated and unsialylated antigens.** (A) Mice received the indicated conjugates in the presence or absence of Ribi; serum IgM and IgG3 anti-NP levels were measured on days 7 and 14. (B) Mice were treated with the tolerogenic compound NP–PA–bNeuGc with or without Ribi on day 0, and were rechallenged on day 18 with NP–PA. Shown are serum anti-NP titers monitored on day 25. Results are representative of three experiments. For all experimental groups, eight mice were analyzed. Shown are means + SD. All statistical tests given used the two-tailed Student's t test. *, P < 0.05; ***, P < 0.005. n.s., not significant.

Missing self–recognition by B cells | Duong et al.

heparin (Long, 2008). If tissues defend themselves through expression of self-markers, then cell type–specific heterogeneity or defects in that expression are predicted to contribute to tissue-specific autoimmune disease.

In the present study, ligands carrying bNeuGc moieties were not only nonimmunogenic in all contexts analyzed but usually promoted long-lived tolerance, preventing B cell responses to rechallenge with immunogenic conjugate. Mice deficient in CD22, Siglec-G, or both failed to respond. Tolerance developed slowly over several days and was associ-

ated with B cell proliferation, a block in plasma cell differentiation, and lack of CD93 expression (unpublished data), features that distinguish tolerized B cells from conventional anergic cells (Adams et al., 1990; Merrell et al., 2006). The time required for tolerance induction might in part be a function of a slow rate of antigen trafficking from the site of injection, but we noted splenic responses to peritoneal injection by 7 d. Tolerance induction by NP–PA–bNeuGc was inhibitable by Bcl2 expression in B cells, which did not permit direct response to the ligand but instead simply rescued
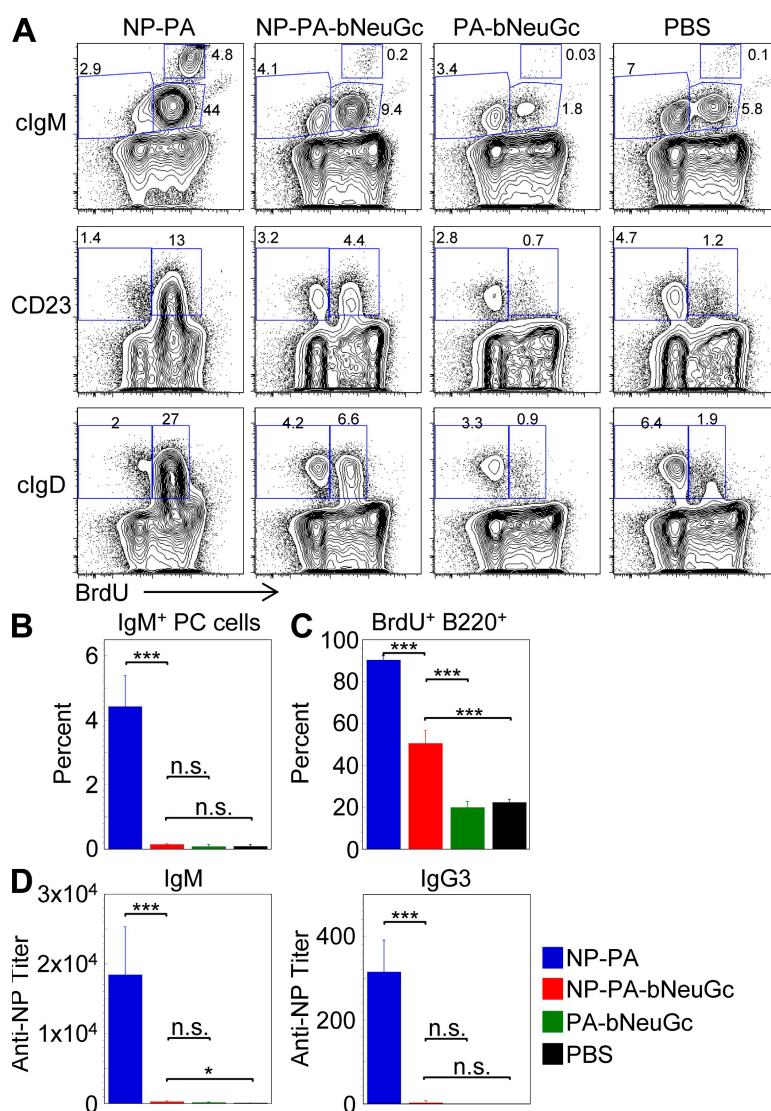


**Figure 6. Flow cytometry and serum antibody analysis of in vivo responses of NP–specific B cells to sialylated and unsialylated conjugates.** *Rag1$^{-/-}$* mice that received 10$^7$ isolated splenic QM transgenic B cells were challenged with 40 µg of the indicated conjugates 2 h later. From the time of reconstitution they were labeled with BrdU as indicated in Materials and methods. Similar results were obtained in two independent experiments similar in design but using CFSE-prelabeled B cells rather than BrdU to measure cell division (e.g., Fig. S4). (A) At day 7 after reconstitution, spleen cells were analyzed for BrdU uptake and B cell marker expression. A lymphocyte gate was used for analysis. Plots shown were representative of mice receiving NP–PA–bNeuGc (*n* = 5), NP–PA (*n* = 5), PA–bNeuGc (*n* = 3), and PBS (*n* = 3). Percentages are shown. (B) Quantitation of percentages of IgM$^+$ plasma cells as defined by high levels of cytoplasmic IgM. (C) Percentages of B220$^+$ cells scoring positive for BrdU uptake. (D) Serum anti-NP IgM and IgG3 antibody titers of the indicated recipients obtained at day 7 after reconstitution/challenge. Shown are means + SD. *, P < 0.05; and ***, P < 0.005 using the two-tailed Student's *t* test. n.s., not significant.

the ability to respond to rechallenge by unsialylated compound. Thus, the mode of tolerance observed is complex, regulated both by differentiative arrest preventing plasma cell formation and apoptosis. It is unlikely that the impaired tolerance induction observed in the *Bcl2 Tg* mice could be explained by their elevated total NP-specific B cell numbers, because tolerance was readily inducible in mice receiving QM B cells, which had an NP-specific precursor frequency many orders of magnitude over that of non-BCR–transgenic mice. The significant delay and antigen-specific B cell proliferation associated with tolerance induction might serve to prevent premature elimination of foreign antigen-specific B cells, permitting their rescue by antigen-linked second signals, such as TLR ligands or T cell help, should they become available late in the response. Perhaps more importantly, unsialylated TI-2 antigens promote rapid antibody responses that can precede the development of second signals or even occur in their absence. In the context of a microbial infection, such responses would provide early adaptive
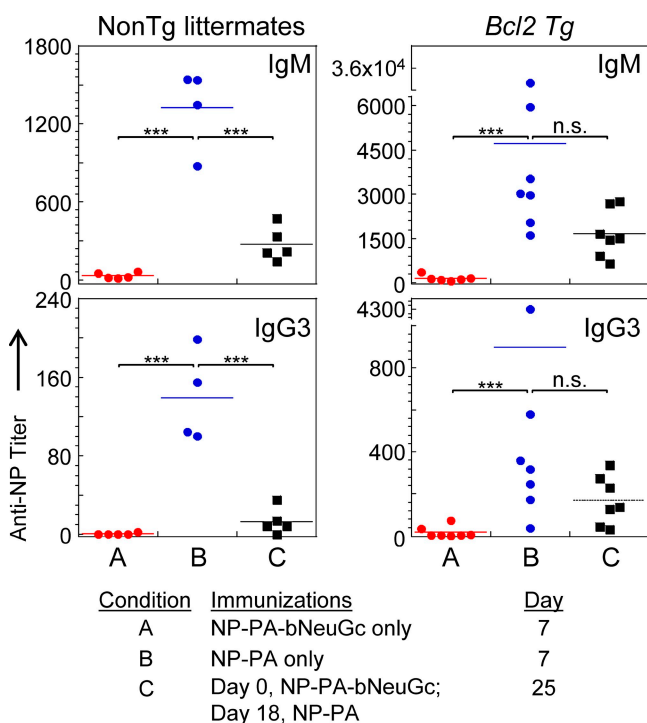
immunity to repetitive ligands when second signals are still inadequate.

Though they failed to respond directly to NP–PA–bNeuGc, mice lacking CD22, Siglec-G, or both showed impaired tolerance induction, as revealed by rechallenge with NP–PA. In these mutants, IgG3 responses failed to recover but IgM responses were restored, indicating that interaction with these siglecs contributed to the tolerance induction in vivo. Why was only IgM and not IgG3 antibody restored by knockout of CD22 or Siglec-G? We presume that there is preferential rescue in the knockouts of those B cell clones with lower average affinity for NP, which might score well in the IgM assay but not in the IgG3 assay, or might fail to switch owing to this lower affinity. Because NP–PA–bNeuGc was not itself immunogenic even in $CD22^{-/-};Siglecg^{-/-}$ mice, presumably its bNeuGc moiety interacts with other molecules besides CD22 and Siglec-G to effect tolerance and to suppress direct anti-NP responses. One possible candidate receptor is Siglec-E, which is expressed in marginal zone B cells (Zhang et al., 2004) and might be responsible for the residual binding of PA–bNeuGc to $CD22^{-/-};Siglecg^{-/-}$ B cells.

Although we cannot exclude that immunogenic conjugates somehow activate accessory cells in promoting TI-2 antibody responses, we believe that our data show that sialylated conjugates work directly on B cells for the following reasons. Had the effect not been direct, but instead through antigen-nonspecific accessory cells, treatment of mice with sialylated conjugate lacking hapten would have reduced subsequent responses to the haptenated conjugate (Fig. S1 A). Moreover, direct signaling and proliferation effects were seen in cultures of purified B cells. Finally, given its structure, it is difficult to imagine that a B cell could effectively contact NP–PA–bNeuGc conjugate without coengaging siglecs.

We suspect that there are deep evolutionary origins to the siglec receptor function we have investigated. Siglecs have been suggested to play a role in self-recognition in the context of myeloid cell recognition, where they negatively regulate phagocytosis and activation (Varki and Angata, 2006; Carlin et al., 2009b; Chen et al., 2009). Siglec-G was recently proposed to play a role in regulating the innate immune responses of dendritic cells to liver injury through the recognition of the carbohydrate-rich molecule CD24 (Chen et al., 2009). Moreover, Siglec 7 is expressed on NK cells, where it appears to carry out classical inhibitory functions (Nicoll et al., 2003). These observations, combined with ours in this study, support the notion that missing self–recognition is a common ancestral mechanism of self-/nonself-discrimination in leukocytes.

A feature predicted to be common to missing self–signaling is the potential of cis ligands to dampen recognition by inhibitory receptors of self-markers in trans. Indeed, in NK cells, B cells, and likely many other leukocytes, loss of expression of putative self-markers, such as MHC I and sialic acids, leads to hyperreactivity as a consequence of a lack of tonic inhibitory signaling (for review see Held and Mariuzza, 2008). The ability



| Condition | Immunizations | Day |
|---|---|---|
| A | NP-PA-bNeuGc only | 7 |
| B | NP-PA only | 7 |
| C | Day 0, NP-PA-bNeuGc; Day 18, NP-PA | 25 |

**Figure 7. In vivo analysis of the effect of B cell–enforced Bcl2 expression on tolerance induction with NP–PA–bNeuGc.** *Bcl2 Tg* or nontransgenic littermates were challenged with NP–PA–bNeuGc on day 0, followed by rechallenge with NP–PA on day 17. Shown are IgM and IgG3 antibody titers of sera obtained at day 7 after primary or secondary challenge. Transgenic and nontransgenic littermates were immunized and sera were taken in three independent, noncontemporaneous immunization experiments; these sera were analyzed together by ELISA. Note that one *Bcl2 Tg* mouse had an extremely high titer that is given on a different scale than the other data points. No statistically significant differences were seen between *Bcl2 Tg* and nontransgenic littermate responses to NP–PA only (Fig. S5). ***, P < 0.005 using the two-tailed Student's *t* test. n.s., not significant.

Missing self–recognition by B cells | Duong et al.

to more efficiently overcome competition with cis ligands probably explains the greater effectiveness, compared with natively sialylated compounds, of bNeuGc-containing conjugates in suppressing antibody responses and in the induction of tolerance.

Our results have implications for host defense and microbial evasion. T-independent antibody responses can be rapid and elicit antibodies with complement-fixing ability important in resistance to bacteria (Martin et al., 2001; Alugupalli et al., 2004). The ability to produce sialic acid–containing glycosylations is a specialization of pathogenic microbes that has been explained as a means to evade complement-mediated destruction through recruitment of the complement inhibitor factor H (Tomlinson et al., 1994; Ngampasutadol et al., 2008) or to evade recognition by myeloid cells (Varki and Angata, 2006). Our results suggest an additional possibility, namely to dampen the TI-2 antibody response to microbial surface antigens. Selection against these microbial countermeasures

likely explains the rapid coevolution of siglec ligands and their receptors.

Our studies help to explain an uncomfortable exception to the two-signal model with respect to B cell tolerance. For antigens with many epitopes, like TI-2 antigens, the model does not hold unless they are associated with ligands for inhibitory receptors. Nor are our results consistent with the notion that antigen organization alone regulates the TI-2 response (Dintzis et al., 1976; Bachmann et al., 1993). Rather, we see immune recognition of mature B cells in the context of an evolutionarily ancient leukocyte recognition system in which self-markers engaging inhibitory receptors prevent self-reactivity, and in their absence a cell-autonomous, but perhaps self-limited, response is possible.

## MATERIALS AND METHODS
**Synthesis of sialosides, PA–sialoside conjugates, and NP–PA–sialoside conjugates.** bNeuGc was prepared as previously described (Collins et al., 2006a). NeuGc and other glycans (Galβ1-4GlcNAcβ–O-ethylazide,
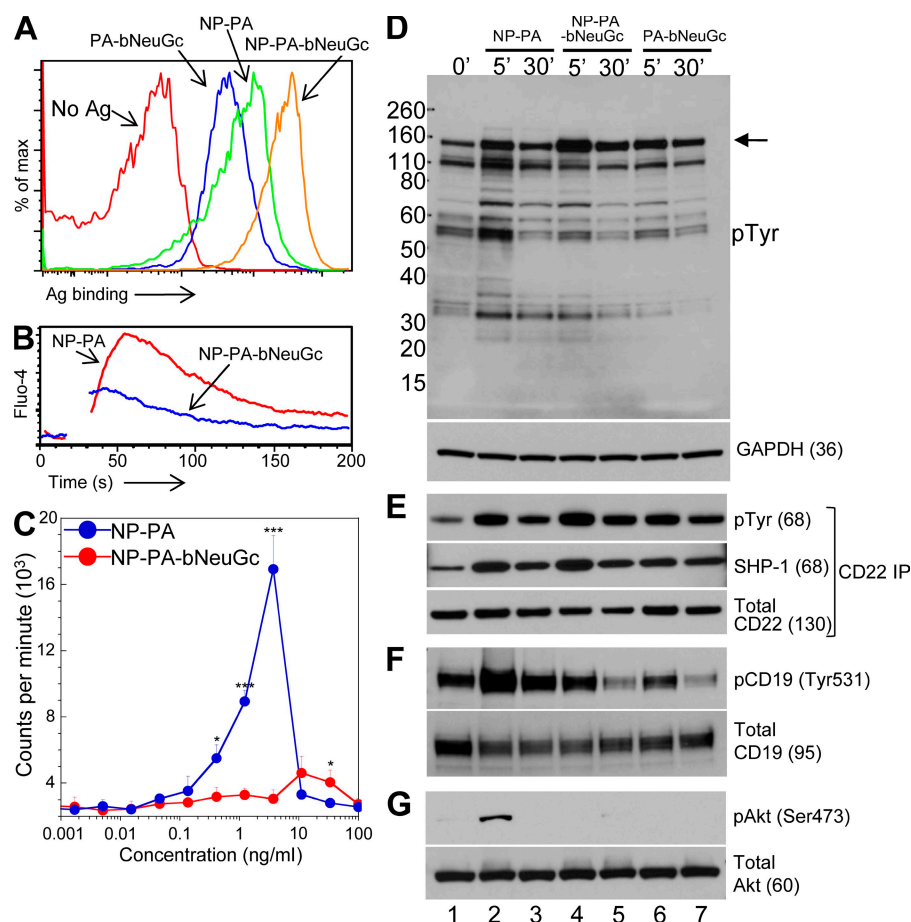


Figure 8. **In vitro analysis of conjugate binding and response by NP–specific B cells.** (A) Flow cytometry analysis of conjugate binding to isolated NP-specific B cells from QM transgenic mice. PA ligands used in this assay carried biotin tags along with the indicated carbohydrate and hapten moieties. (B) Ca²⁺ mobilization response monitored by Fluo-4 dye fluorescence. (C) Analysis of proliferation induced by the indicated concentrations of antigens by ³[H]thymidine uptake. Cells were harvested at 39 h of culture after a 15-h labeling period. Shown are means + SD. *, P < 0.05; and ***, P < 0.005 using the two-tailed Student's t test. (D–G) Western blot analysis of protein phosphorylation induced by the indicated conjugates. The arrow indicates the expected molecular weight of CD22 on the pTyr blot. The number of independent replicates of the experiments shown in the indicated sections were as follows: A, one; B, two; C, three; D, three; and E–G, one.

183

NeuAcα2-6Galβ1-4GlcNAcβ–O-ethylazide, and NeuGcα2-3Galβ1-4GlcNAcβ–O-ethylazide) were prepared as previously described (Blixt et al., 2003). Three PA–sialoside conjugates containing 20 mol% sialoside and 2.5% biotin (PA–NeuGc, PA–NeuGcα2-3Galβ1-4GlcNAcβ, and PA–NeuAcα2-3Galβ1-4GlcNAcβ) were provided by the Consortium for Functional Glycomics (http://www.functionalglycomics.org). Other PA conjugates were prepared as previously described (Bovin et al., 1993) by reducing the corresponding sialoside-azides to the respective sialoside-amines and coupling to PA (1,000 kD), resulting in a conjugate with 20 mol% sialoside and 2.5 mol% biotin. Similarly, NP–PA and NP–PA–sialoside immunogen PA conjugates (1,000 kD) were prepared with 10 mol% NP (NP-hexyl-amine) with 10 mol% NP and 20 mol% of the corresponding ethyl-amines of either Galβ1-4GlcNAc, the bNeuGc trisaccharide, or the NeuGc trisaccharide.

**Mice and TI-2 immunizations.** C57BL/6J mice were obtained from either the Jackson Laboratory or the Scripps Research Institute in-house breeding program. $CD22^{-/-}$ (provided by E. Clark [University of Washington, Seattle, WA] and L. Nitschke [University of Erlangen, Erlangen, Germany]; Otipoby et al., 1996), $Siglecg^{-/-}$ (provided by Y. Liu, University of Michigan, Ann Arbor, MI; Ding et al., 2007), QM (provided by M. Cascalho, University of Michigan, Ann Arbor, MI; Cascalho et al., 1996), $Bcl2\ Tg$ (provided by A. Strasser and A. Harris, Walter and Eliza Hall Institute of Medical Research, Parkville, Australia; Strasser et al., 1991), $Rag1^{-/-}$, and B6Ly5a (B6.SJL-$Ptprc^a$ $Pep3^b$/BoyJ; The Jackson Laboratory) mice have been previously described. All genetically modified mice were on a C57BL/6J background. $CD22^{-/-};Siglecg^{-/-}$ mice were obtained by crossing the $CD22^{-/-}$ and $Siglecg^{-/-}$ mice and PCR screening for recombination of the two null alleles. All mice used in this study were between 6–16 wk of age and housed in a specific pathogen-free facility. Animal protocols were approved by the Institutional Care and Use Committee of the Scripps Research Institute. Unless otherwise stated, all immunizations were administered i.p. at 20 μg of TI-2 antigen in 200 μl PBS per mouse. Ribi, sold as the Sigma Adjuvant System (Sigma-Aldrich), was used according to the manufacturer's protocol, with each mouse receiving 20 μg of the respective TI-2 antigen in 200 μl of reconstituted adjuvant.

**ELISA.** Maxisorp plates (Thermo Fisher Scientific) were coated with 10 μg/ml $NP_{4-7}$-BSA in PBS (Biosearch Technologies), blocked with 1% BSA in Tris-buffered saline (TBS) containing 0.1% Tween 20 (ELISA buffer), and incubated with sera serially diluted in ELISA buffer. For detection, biotinylated rat anti–mouse IgM (clone M41) and IgG3 (BD) antibodies were used at 0.25 μg/ml, followed by streptavidin–horseradish peroxidase (HRP; BD), diluted in ELISA buffer. Signals were developed using 1-Step Ultra-TMB substrate (Thermo Fisher Scientific), stopped with 2 M $H_2SO_4$, and recorded at 450 nm using a microplate reader (VersaMax; MDS Analytical Technologies). In all experiments, standard curves were generated using pooled day 8 sera collected from 16 C57BL/6 mice immunized i.p. with 50 μg/mouse $NP_{41}$-AECM-Ficoll (Biosearch Technologies). Subsequent anti-NP titer calculations were normalized using these standard curves.

**In vitro proliferation.** QM B cells purified by CD4/CD43 negative selection (Miltenyi Biotec) were seeded in 96-well plates at $2 \times 10^5$ cells/well in DMEM containing 10% FBS, 55 μM β-mercaptoethanol, L-glutamine, and penicillin-streptomycin. Cells were stimulated with threefold serial dilutions of the respective TI-2 antigen, starting at 100 ng/ml in a total volume of 200 μl/well. Approximately 24 h later, 1 μCi $^3$[H]thymidine (GE Healthcare) was added into each well. Cells were harvested for scintillation counts 15 h later. All treatments were done in triplicate.

**In vivo QM B cell transfer.** To quantify in vivo proliferative responses of NP-specific B cells to TI-2 antigens, B cells were purified from QM mouse spleens by CD4/CD43 negative selection (Miltenyi Biotec) and injected i.v. into nonirradiated $Rag1^{-/-}$ hosts ($10^7$ cells/mouse). 2 h later, all mice received (i.p.) 40 μg of the respective TI-2 antigen plus 1 mg BrdU in PBS and were placed on drinking water containing 1 mg/ml BrdU for 7 d. In a sec-

ond experiment, QM B cells purified by CD4/CD43 negative selection (Miltenyi Biotec) were first resuspended at $1–2 \times 10^7$ cells/ml in 0.1% BSA in PBS, labeled with 1 μl of 5 mM CFSE per milliliter of cell suspension for 10 min at 37°C, washed two times with DMEM containing 10% FBS, and injected i.v. into B6Ly5a mice at $10^7$ cells/mouse. As before, all mouse recipients subsequently received (i.p.) 40 μg of the respective TI-2 antigen or PBS alone 2 h later. On day 7, total spleen cells were harvested, and fates of QM cells marked by the Ly5b allele were analyzed by flow cytometry.

**Flow cytometric analyses of surface markers, antigen binding, $Ca^{2+}$ flux, and BrdU uptake.** Cell-surface marker stains for FACS analyses were performed using standard protocol. All of the following antibodies were used at 1:200 dilution in FACS buffer (HBSS containing 1% BSA, 2 mM EDTA, and 0.1% $NaN_3$), with $2 \times 10^6$ cells/stain: FITC anti-IgM$^a$ (BD), PE anti-IgD (eBioscience), PE-Cy7 anti-CD23 (eBioscience), Alexa Fluor 647 anti-IgM (clone M41), Pacific blue anti-CD21 (BioLegend), allophycocyanin-Cy7 anti-B220 (BD), and PerCP-Cy5.5–conjugated antibodies used in dump channel (CD4, CD8, F4/80, and Gr-1; BioLegend). To quantitate TI-2 antigen binding to QM cells, $10^6$ spleen cells were incubated with 1 μg of the biotinylated PA antigen in a total volume of 200 μl of FACS buffer on ice for 30 min. After a wash with 2 ml of FACS buffer at 4°C, cells were stained on ice with cocktail containing 0.25 μg streptavidin-PE (eBioscience) along with (1:200 dilution each) Alexa Fluor 647 anti-B220, PerCP-Cy5.5 anti-CD4, and PerCP-Cy5.5 anti-CD8 (all from BioLegend) in a final volume of 200 μl. Similarly, binding of primary C57BL/6 spleen cells to PA probes containing various siglec ligands was assessed as described but by using 0.125 μg of probe per $10^6$ cells with 2 h of incubation on ice. To measure intracellular $Ca^{2+}$ responses to TI-2 antigens, $8 \times 10^6$ QM spleen cells were preincubated with 500 μl Fluo-4 reagent (Invitrogen), 5 μg Fc Block (clone 2.4G2), and 1 μg each of PerCP-Cy5.5–labeled CD4 and CD8 (BioLegend). Aliquots of $10^6$ cells were stimulated with 50 μg/ml of TI-2 antigen at room temperature. $Ca^{2+}$ signals were recorded for 200 s, gating on CD4/CD8 double-negative cells. Flow cytometric analysis of BrdU incorporation was performed using the FITC BrdU Flow Kit (BD) according to the manufacturer's instructions. All flow cytometric data were acquired on a FACSCalibur or LSR II (BD) and were analyzed using the FlowJo program (Tree Star, Inc.).

**Western blotting and immunoprecipitation.** B cells were purified from QM mouse spleens by CD4/CD43 negative selection (Miltenyi Biotec). Aliquots of $10^7$ cells in 1 ml HBSS buffer, prewarmed to 37°C in a water bath, were stimulated with 25 μg of the respective TI-2 antigen for 5 or 30 min. Pelleted cells were lysed in 100 μl of lysis buffer consisting of TBS containing 1% NP-40, Complete EDTA-Free Protease Inhibitor Cocktail (Roche), 5 mM EDTA, 10 mM NaF, and 1 mM $Na_3VO_4$. For straight Western blotting analyses, 10 μl of each lysate after removing insoluble debris by centrifugation was run in 4–12% NuPAGE gels (Invitrogen). For CD22 immunoprecipitation, each lysate of $10^7$ cells/ml was precleared with 30 μl of streptavidin agarose resin (Thermo Fisher Scientific) for 1 h at 4°C, and was then incubated with 5 μg of biotinylated Cy34.1 antibody (BD) overnight at 4°C. On the next day, 30 μl of the streptavidin agarose resin was added into each lysate, washed three times with lysis buffer, and incubated at 70°C for 10 min in 2× NuPAGE loading buffer containing 100 mM dithiothreitol before SDS-PAGE. 0.2-μm nitrocellulose membranes (Bio-Rad Laboratories) were used for all Western blotting analyses. Antiphosphotyrosine (4G10) and anti-GAPDH (6C5) antibodies, used at 1:1,000 and 1:10,000, respectively, diluted in TBS/T containing 5% milk, were purchased from Millipore. Polyclonal antibodies against SHP-1, phospho-CD19 (Tyr531), phospho-Akt (Ser473), and total Akt were purchased from Cell Signaling Technology and used according to the manufacturer's recommendations. Goat anti–mouse CD22 was used at 1:100 dilution in 5% milk in TBS/T (M-20; Santa Cruz Biotechnology, Inc.). All HRP-conjugated secondary polyclonal antibodies, purchased from Jackson ImmunoResearch Laboratories, Inc., were used at 1:30,000 diluted in 5% milk in TBS/T. Signals were developed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). To strip

blots for reprobing, membranes were incubated in TBS buffer, adjusted to pH 2.3, at 65°C with gentle agitation for 15–20 min.

**Online supplemental material.** Fig. S1 shows antigen specificity and time course of tolerance induction. Fig. S2 shows the effects of altering NP hapten density on responses to unsialylated compounds. Fig. S3 shows an analysis of $CD22^{-/-};Siglecg^{-/-}$ mice for tolerance induction by NP–PA–bNeuGc and responses to NP–PA. Fig. S4 shows an analysis of QM B cell proliferation in vivo using the CFSE dilution technique. Fig. S5 shows an alternative display of the data shown in Fig. 7. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091873/DC1.

## REFERENCES

Adams, E., A. Basten, and C.C. Goodnow. 1990. Intrinsic B-cell hyporesponsiveness accounts for self-tolerance in lysozyme/anti-lysozyme double-transgenic mice. *Proc. Natl. Acad. Sci. USA.* 87:5687–5691. doi:10.1073/pnas.87.15.5687

Alugupalli, K.R., J.M. Leong, R.T. Woodland, M. Muramatsu, T. Honjo, and R.M. Gerstein. 2004. B1b lymphocytes confer T cell–independent long-lasting immunity. *Immunity.* 21:379–390. doi:10.1016/j.immuni.2004.06.019

Bachmann, M.F., U.H. Rohrer, T.M. Kündig, K. Bürki, H. Hengartner, and R.M. Zinkernagel. 1993. The influence of antigen organization on B cell responsiveness. *Science.* 262:1448–1451. doi:10.1126/science.8248784

Blixt, O., B.E. Collins, I.M. van den Nieuwenhof, P.R. Crocker, and J.C. Paulson. 2003. Sialoside specificity of the siglec family assessed using novel multivalent probes: identification of potent inhibitors of myelin-associated glycoprotein. *J. Biol. Chem.* 278:31007–31019. doi:10.1074/jbc.M304331200

Bovin, N.V., E.Yu. Korchagina, T.V. Zemlyanukhina, N.E. Byramova, O.E. Galanina, A.E. Zemlyakov, A.E. Ivanov, V.P. Zubov, and L.V. Mochalova. 1993. Synthesis of polymeric neoglycoconjugates based on N-substituted polyacrylamides. *Glycoconj. J.* 10:142–151. doi:10.1007/BF00737711

Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. *Science.* 169:1042–1049. doi:10.1126/science.169.3950.1042

Burnet, F.M., and F. Fenner. 1949. The Production of Antibodies. Second edition. Macmillan, New York. 142 pp.

Campbell, M.A., and N.R. Klinman. 1995. Phosphotyrosine-dependent association between CD22 and protein tyrosine phosphatase 1C. *Eur. J. Immunol.* 25:1573–1579. doi:10.1002/eji.1830250616

Cariappa, A., H. Takematsu, H. Liu, S. Diaz, K. Haider, C. Boboila, G. Kalloo, M. Connole, H.N. Shi, N. Varki, et al. 2009. B cell antigen receptor signal strength and peripheral B cell development are regulated by a 9-O-acetyl sialic acid esterase. *J. Exp. Med.* 206:125–138. doi:10.1084/jem.20081399

Carlin, A.F., Y.C. Chang, T. Areschoug, G. Lindahl, N. Hurtado-Ziola, C.C. King, A. Varki, and V. Nizet. 2009a. Group B *Streptococcus* suppression of phagocyte functions by protein-mediated engagement of human Siglec-5. *J. Exp. Med.* 206:1691–1699. doi:10.1084/jem.20090691

Carlin, A.F., S. Uchiyama, Y.C. Chang, A.L. Lewis, V. Nizet, and A. Varki. 2009b. Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response. *Blood.* 113:3333–3336. doi:10.1182/blood-2008-11-187302

Cascalho, M., A. Ma, S. Lee, L. Masat, and M. Wabl. 1996. A quasi-monoclonal mouse. *Science.* 272:1649–1652. doi:10.1126/science.272.5268.1649

Chen, G.Y., J. Tang, P. Zheng, and Y. Liu. 2009. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. *Science.* 323:1722–1725. doi:10.1126/science.1168988

Collins, B.E., O. Blixt, N.V. Bovin, C.P. Danzer, D. Chui, J.D. Marth, L. Nitschke, and J.C. Paulson. 2002. Constitutively unmasked CD22 on B cells of ST6Gal I knockout mice: novel sialoside probe for murine CD22. *Glycobiology.* 12:563–571. doi:10.1093/glycob/cwf067

Collins, B.E., O. Blixt, A.R. DeSieno, N. Bovin, J.D. Marth, and J.C. Paulson. 2004. Masking of CD22 by cis ligands does not prevent redistribution of CD22 to sites of cell contact. *Proc. Natl. Acad. Sci. USA.* 101:6104–6109. doi:10.1073/pnas.0400851101

Collins, B.E., O. Blixt, S. Han, B. Duong, H. Li, J.K. Nathan, N. Bovin, and J.C. Paulson. 2006a. High-affinity ligand probes of CD22 overcome the threshold set by cis ligands to allow for binding, endocytosis, and killing of B cells. *J. Immunol.* 177:2994–3003.

Collins, B.E., B.A. Smith, P. Bengtson, and J.C. Paulson. 2006b. Ablation of CD22 in ligand-deficient mice restores B cell receptor signaling. *Nat. Immunol.* 7:199–206. doi:10.1038/ni1283

Cornall, R.J., J.G. Cyster, M.L. Hibbs, A.R. Dunn, K.L. Otipoby, E.A. Clark, and C.C. Goodnow. 1998. Polygenic autoimmune traits: Lyn, CD22, and SHP-1 are limiting elements of a biochemical pathway regulating BCR signaling and selection. *Immunity.* 8:497–508. doi:10.1016/S1074-7613(00)80554-3

Courtney, A.H., E.B. Puffer, J.K. Pontrello, Z.Q. Yang, and L.L. Kiessling. 2009. Sialylated multivalent antigens engage CD22 in trans and inhibit B cell activation. *Proc. Natl. Acad. Sci. USA.* 106:2500–2505. doi:10.1073/pnas.0807207106

Coutinho, A., E. Gronowicz, W.W. Bullock, and G. Möller. 1974. Mechanism of thymus-independent immunocyte triggering. Mitogenic activation of B cells results in specific immune responses. *J. Exp. Med.* 139:74–92. doi:10.1084/jem.139.1.74

Crocker, P.R., J.C. Paulson, and A. Varki. 2007. Siglecs and their roles in the immune system. *Nat. Rev. Immunol.* 7:255–266. doi:10.1038/nri2056

Cyster, J.G., and C.C. Goodnow. 1995. Protein tyrosine phosphatase 1C negatively regulates antigen receptor signaling in B lymphocytes and determines thresholds for negative selection. *Immunity.* 2:13–24. doi:10.1016/1074-7613(95)90075-6

Ding, C., Y. Liu, Y. Wang, B.K. Park, C.Y. Wang, P. Zheng, and Y. Liu. 2007. Siglecg limits the size of B1a B cell lineage by down-regulating NFkappaB activation. *PLoS One.* 2:e997. doi:10.1371/journal.pone.0000997

Dintzis, H.M., R.Z. Dintzis, and B. Vogelstein. 1976. Molecular determinants of immunogenicity: the immunon model of immune response. *Proc. Natl. Acad. Sci. USA.* 73:3671–3675. doi:10.1073/pnas.73.10.3671

Doody, G.M., L.B. Justement, C.C. Delibrias, R.J. Matthews, J. Lin, M.L. Thomas, and D.T. Fearon. 1995. A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. *Science.* 269:242–244. doi:10.1126/science.7618087

Ferry, H., T.L. Crockford, K. Silver, N. Rust, C.C. Goodnow, and R.J. Cornall. 2005. Analysis of Lyn/CD22 double-deficient B cells in vivo demonstrates Lyn- and CD22-independent pathways affecting BCR regulation and B cell survival. *Eur. J. Immunol.* 35:3655–3663. doi:10.1002/eji.200535247

Gavin, A.L., K. Hoebe, B. Duong, T. Ota, C. Martin, B. Beutler, and D. Nemazee. 2006. Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. *Science.* 314:1936–1938. doi:10.1126/science.1135299

Ghosh, S., C. Bandulet, and L. Nitschke. 2006. Regulation of B cell development and B cell signalling by CD22 and its ligands alpha2,6-linked sialic acids. *Int. Immunol.* 18:603–611. doi:10.1093/intimm/dxh402

Goodnow, C.C., J. Sprent, B. Fazekas de St Groth, and C.G. Vinuesa. 2005. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature.* 435:590–597. doi:10.1038/nature03724

Grewal, P.K., M. Boton, K. Ramirez, B.E. Collins, A. Saito, R.S. Green, K. Ohtsubo, D. Chui, and J.D. Marth. 2006. ST6Gal-I restrains CD22-dependent antigen receptor endocytosis and Shp-1 recruitment in normal and pathogenic immune signaling. *Mol. Cell. Biol.* 26:4970–4981. doi:10.1128/MCB.00308-06

Haas, K.M., S. Sen, I.G. Sanford, A.S. Miller, J.C. Poe, and T.F. Tedder. 2006. CD22 ligand binding regulates normal and malignant B lymphocyte survival in vivo. *J. Immunol.* 177:3063–3073.

Han, S., B.E. Collins, P. Bengtson, and J.C. Paulson. 2005. Homomultimeric complexes of CD22 in B cells revealed by protein-glycan cross-linking. *Nat. Chem. Biol.* 1:93–97. doi:10.1038/nchembio713

Hanasaki, K., A. Varki, and L.D. Powell. 1995. CD22-mediated cell adhesion to cytokine-activated human endothelial cells. Positive and negative regulation by alpha 2-6-sialylation of cellular glycoproteins. *J. Biol. Chem.* 270:7533–7542. doi:10.1074/jbc.270.13.7533

Held, W., and R.A. Mariuzza. 2008. Cis interactions of immunoreceptors with MHC and non-MHC ligands. *Nat. Rev. Immunol.* 8:269–278. doi:10.1038/nri2278

Hennet, T., D. Chui, J.C. Paulson, and J.D. Marth. 1998. Immune regulation by the ST6Gal sialyltransferase. *Proc. Natl. Acad. Sci. USA.* 95:4504–4509. doi:10.1073/pnas.95.8.4504

Hinton, H.J., A. Jegerlehner, and M.F. Bachmann. 2008. Pattern recognition by B cells: the role of antigen repetitiveness versus Toll-like receptors. *Curr. Top. Microbiol. Immunol.* 319:1–15. doi:10.1007/978-3-540-73900-5_1

Hoffmann, A., S. Kerr, J. Jellusova, J. Zhang, F. Weisel, U. Wellmann, T.H. Winkler, B. Kneitz, P.R. Crocker, and L. Nitschke. 2007. Siglec-G is a B1 cell-inhibitory receptor that controls expansion and calcium signaling of the B1 cell population. *Nat. Immunol.* 8:695–704. doi:10.1038/ni1480

Jin, L., P.A. McLean, B.G. Neel, and H.H. Wortis. 2002. Sialic acid binding domains of CD22 are required for negative regulation of B cell receptor signaling. *J. Exp. Med.* 195:1199–1205. doi:10.1084/jem.20011796

Kärre, K., H.G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature.* 319:675–678. doi:10.1038/319675a0

Lang, J., B. Arnold, G. Hammerling, A.W. Harris, S. Korsmeyer, D. Russell, A. Strasser, and D. Nemazee. 1997. Enforced Bcl-2 expression inhibits antigen-mediated clonal elimination of peripheral B cells in an antigen dose–dependent manner and promotes receptor editing in autoreactive, immature B cells. *J. Exp. Med.* 186:1513–1522. doi:10.1084/jem.186.9.1513

Lanier, L.L. 2008. Up on the tightrope: natural killer cell activation and inhibition. *Nat. Immunol.* 9:495–502. doi:10.1038/ni1581

Lanoue, A., F.D. Batista, M. Stewart, and M.S. Neuberger. 2002. Interaction of CD22 with alpha2,6-linked sialoglycoconjugates: innate recognition of self to dampen B cell autoreactivity? *Eur. J. Immunol.* 32: 348–355. doi:10.1002/1521-4141(200202)32:2<348::AID-IMMU348>3.0.CO;2-5

Long, E.O. 2008. Negative signaling by inhibitory receptors: the NK cell paradigm. *Immunol. Rev.* 224:70–84. doi:10.1111/j.1600-065X.2008.00660.x

Martin, F., A.M. Oliver, and J.F. Kearney. 2001. Marginal zone and B1 B cells unite in the early response against T–independent blood-borne particulate antigens. *Immunity.* 14:617–629. doi:10.1016/S1074-7613(01)00129-7

Mary, C., C. Laporte, D. Parzy, M.L. Santiago, F. Stefani, F. Lajaunias, R.M. Parkhouse, T.L. O'Keefe, M.S. Neuberger, S. Izui, and L. Reininger. 2000. Dysregulated expression of the Cd22 gene as a result of a short interspersed nucleotide element insertion in Cd22a lupus-prone mice. *J. Immunol.* 165:2987–2996.

Merrell, K.T., R.J. Benschop, S.B. Gauld, K. Aviszus, D. Decote-Ricardo, L.J. Wysocki, and J.C. Cambier. 2006. Identification of anergic B cells within a wild-type repertoire. *Immunity.* 25:953–962. doi:10.1016/j.immuni.2006.10.017

Mond, J.J., A. Lees, and C.M. Snapper. 1995. T cell-independent antigens type 2. *Annu. Rev. Immunol.* 13:655–692. doi:10.1146/annurev.iy.13.040195.003255

Murakami, M., T. Tsubata, M. Okamoto, A. Shimizu, S. Kumagai, H. Imura, and T. Honjo. 1992. Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. *Nature.* 357:77–80. doi:10.1038/357077a0

Naito, Y., H. Takematsu, S. Koyama, S. Miyake, H. Yamamoto, R. Fujinawa, M. Sugai, Y. Okuno, G. Tsujimoto, T. Yamaji, et al. 2007. Germinal center marker GL7 probes activation-dependent repression of N-glycolylneuraminic acid, a sialic acid species involved in the negative modulation of B–cell activation. *Mol. Cell. Biol.* 27:3008–3022. doi:10.1128/MCB.02047-06

Nemazee, D., and A. Gavin. 2003. Do B cells take advantage of 'missing self' recognition? *Curr. Dir. Autoimmun.* 6:245–264. doi:10.1159/000066852

Ngampasutadol, J., S. Ram, S. Gulati, S. Agarwal, C. Li, A. Visintin, B. Monks, G. Madico, and P.A. Rice. 2008. Human factor H interacts selectively with *Neisseria gonorrhoeae* and results in species-specific complement evasion. *J. Immunol.* 180:3426–3435.

Nicoll, G., T. Avril, K. Lock, K. Furukawa, N. Bovin, and P.R. Crocker. 2003. Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and –independent mechanisms. *Eur. J. Immunol.* 33:1642–1648. doi:10.1002/eji.200323693

Nishimura, H., N. Minato, T. Nakano, and T. Honjo. 1998. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. *Int. Immunol.* 10:1563–1572. doi:10.1093/intimm/10.10.1563

Nitschke, L., R. Carsetti, B. Ocker, G. Köhler, and M.C. Lamers. 1997. CD22 is a negative regulator of B-cell receptor signalling. *Curr. Biol.* 7:133–143. doi:10.1016/S0960-9822(06)00057-1

Nossal, G.J., and B.L. Pike. 1984. A reappraisal of "T-independent" antigens. II. Studies on single, hapten-specific B cells from neonatal CBA/H or CBA/N mice fail to support classification into TI-1 and TI-2 categories. *J. Immunol.* 132:1696–1701.

O'Keefe, T.L., G.T. Williams, S.L. Davies, and M.S. Neuberger. 1996. Hyperresponsive B cells in CD22-deficient mice. *Science.* 274:798–801. doi:10.1126/science.274.5288.798

O'Keefe, T.L., G.T. Williams, F.D. Batista, and M.S. Neuberger. 1999. Deficiency in CD22, a B cell–specific inhibitory receptor, is sufficient to predispose to development of high affinity autoantibodies. *J. Exp. Med.* 189:1307–1313. doi:10.1084/jem.189.8.1307

Onodera, T., J.C. Poe, T.F. Tedder, and T. Tsubata. 2008. CD22 regulates time course of both B cell division and antibody response. *J. Immunol.* 180:907–913.

Otero, D.C., S.A. Omori, and R.C. Rickert. 2001. Cd19-dependent activation of Akt kinase in B-lymphocytes. *J. Biol. Chem.* 276:1474–1478. doi:10.1074/jbc.M003918200

Otipoby, K.L., K.B. Andersson, K.E. Draves, S.J. Klaus, A.G. Farr, J.D. Kerner, R.M. Perlmutter, C.L. Law, and E.A. Clark. 1996. CD22 regulates thymus–independent responses and the lifespan of B cells. *Nature.* 384:634–637. doi:10.1038/384634a0

Pan, C., N. Baumgarth, and J.R. Parnes. 1999. CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. *Immunity.* 11:495–506. doi:10.1016/S1074-7613(00)80124-7

Parish, C.R. 1996. Signal minus 1: a key factor in immunological tolerance to tissue-specific self antigens? *Immunol. Cell Biol.* 74:278–285. doi:10.1038/icb.1996.49

Pezzutto, A., B. Dörken, G. Moldenhauer, and E.A. Clark. 1987. Amplification of human B cell activation by a monoclonal antibody to the B cell-specific antigen CD22, Bp 130/140. *J. Immunol.* 138:98–103.

Poe, J.C., Y. Fujimoto, M. Hasegawa, K.M. Haas, A.S. Miller, I.G. Sanford, C.B. Bock, M. Fujimoto, and T.F. Tedder. 2004. CD22 regulates B lymphocyte function in vivo through both ligand-dependent and ligand-independent mechanisms. *Nat. Immunol.* 5:1078–1087. doi:10.1038/ni1121

Ravetch, J.V., and L.L. Lanier. 2000. Immune inhibitory receptors. *Science.* 290:84–89. doi:10.1126/science.290.5489.84

Razi, N., and A. Varki. 1998. Masking and unmasking of the sialic acid-binding lectin activity of CD22 (Siglec-2) on B lymphocytes. *Proc. Natl. Acad. Sci. USA.* 95:7469–7474. doi:10.1073/pnas.95.13.7469

Razi, N., and A. Varki. 1999. Cryptic sialic acid binding lectins on human blood leukocytes can be unmasked by sialidase treatment or cellular activation. *Glycobiology.* 9:1225–1234. doi:10.1093/glycob/9.11.1225

Russell, D.M., Z. Dembic, G. Morahan, J.F. Miller, K. Bürki, and D. Nemazee. 1991. Peripheral deletion of self-reactive B cells. *Nature.* 354:308–311. doi:10.1038/354308a0

Sato, S., A.S. Miller, M. Inaoki, C.B. Bock, P.J. Jansen, M.L. Tang, and T.F. Tedder. 1996. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity.* 5:551–562. doi:10.1016/S1074-7613(00)80270-8

Shultz, L.D., P.A. Schweitzer, T.V. Rajan, T. Yi, J.N. Ihle, R.J. Matthews, M.L. Thomas, and D.R. Beier. 1993. Mutations at the

murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene. *Cell.* 73:1445–1454. doi:10.1016/0092-8674(93)90369-2

Strasser, A., S. Whittingham, D.L. Vaux, M.L. Bath, J.M. Adams, S. Cory, and A.W. Harris. 1991. Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease. *Proc. Natl. Acad. Sci. USA.* 88:8661–8665. doi:10.1073/pnas.88.19.8661

Tedder, T.F., J.C. Poe, and K.M. Haas. 2005. CD22: a multifunctional receptor that regulates B lymphocyte survival and signal transduction. *Adv. Immunol.* 88:1–50. doi:10.1016/S0065-2776(05)88001-0

Tomlinson, S., L.C. Pontes de Carvalho, F. Vandekerckhove, and V. Nussenzweig. 1994. Role of sialic acid in the resistance of *Trypanosoma cruzi* trypomastigotes to complement. *J. Immunol.* 153:3141–3147.

Tuveson, D.A., R.H. Carter, S.P. Soltoff, and D.T. Fearon. 1993. CD19 of B cells as a surrogate kinase insert region to bind phosphatidylinositol 3-kinase. *Science.* 260:986–989. doi:10.1126/science.7684160

Varki, A., and T. Angata. 2006. Siglecs—the major subfamily of I-type lectins. *Glycobiology.* 16:1R–27R. doi:10.1093/glycob/cwj008

Vos, Q., A. Lees, Z.Q. Wu, C.M. Snapper, and J.J. Mond. 2000. B-cell activation by T-cell–independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. *Immunol. Rev.* 176:154–170. doi:10.1034/j.1600-065X.2000.00607.x

Walker, J.A., and K.G. Smith. 2008. CD22: an inhibitory enigma. *Immunology.* 123:314–325. doi:10.1111/j.1365-2567.2007.02752.x

Wang, Y., S.R. Brooks, X. Li, A.N. Anzelon, R.C. Rickert, and R.H. Carter. 2002. The physiologic role of CD19 cytoplasmic tyrosines. *Immunity.* 17:501–514. doi:10.1016/S1074-7613(02)00426-0

Yamanaka, M., Y. Kato, T. Angata, and H. Narimatsu. 2009. Deletion polymorphism of SIGLEC14 and its functional implications. *Glycobiology.* 19:841–846. doi:10.1093/glycob/cwp052

Zhang, J.Q., B. Biedermann, L. Nitschke, and P.R. Crocker. 2004. The murine inhibitory receptor mSiglec-E is expressed broadly on cells of the innate immune system whereas mSiglec-F is restricted to eosinophils. *Eur. J. Immunol.* 34:1175–1184. doi:10.1002/eji.200324723