Sialylated multivalent antigens engage CD22 *in trans* and inhibit B cell activation

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CD22 is an inhibitory coreceptor on the surface of B cells that attenuates B cell antigen receptor (BCR) signaling and, therefore, B cell activation. Elucidating the molecular mechanisms underlying the inhibitory activity of CD22 is complicated by the ubiquity of CD22 ligands. Although antigens can display CD22 ligands, the receptor is known to bind to sialylated glycoproteins on the cell surface. The propinquity of CD22 and cell-surface glycoprotein ligands has led to the conclusion that the inhibitory properties of the receptor are due to *cis* interactions. Here, we examine the functional consequences of *trans* interactions by employing sialylated multivalent antigens that can engage both CD22 and the BCR. Exposure of B cells to sialylated antigens results in the inhibition of key steps in BCR signaling. These results reveal that antigens bearing CD22 ligands are powerful suppressors of B cell activation. The ability of sialylated antigens to inhibit BCR signaling through *trans* CD22 interactions reveals a previously unrecognized role for the Siglec-family of receptors as modulators of immune signaling.

B cell antigen receptor | multivalency | sialic acid | siglec | autoimmunity

The initiation of an immune response or the prevention of autoimmunity depends upon the ability of the B cell antigen receptor (BCR) to transmit signals that positively or negatively regulate B lymphocyte survival, proliferation, and differentiation (1). To avoid detrimental autoimmune responses, a means of differentiating between foreign and self-antigens is required; coreceptors that modulate BCR signaling can ensure that these distinctions are made. CD22 is an inhibitory coreceptor that can attenuate BCR signaling (2, 3). CD22 null mice possess hyperresponsive B cells (4), illustrating a role for CD22 in establishing a threshold for B cell activation. Specifically, an increase in intracellular Ca^{2+} ion concentration is a hallmark of B cell activation (5, 6), and B cells isolated from CD22 null mice display increased Ca^{2+} flux in response to antigen (4, 7). Thus, loss of CD22 results in a lowering of the threshold for B cell activation. Other data also support this conclusion: CD22 null mice exhibit increased serum IgM concentrations, decreased surface IgM levels on peripheral B cells, increased induction of apoptosis in response to BCR cross-linking, and increased serum autoantibody titers (8). These observations are consistent with the loss of CD22 leading to increased sensitivity and chronic B cell activation.

The process of B cell activation ensues upon binding of multivalent antigen to the BCR. Antigen-induced clustering elicits phosphorylation of the cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs), which are present in the BCR-associated signaling proteins Igα/β. The phosphorylation reaction is catalyzed by Src-family kinases such as Lyn. Upon phosphorylation of the BCR components, Syk kinase is recruited to the BCR signaling complex (9). Syk is essential for propagating BCR signaling (10, 11). It acts along with other accessory proteins to activate the phospholipase PLCγ2 (12, 13). Once activated PLCγ2, effects lipid hydrolysis to afford the secondary messengers diacylglycerol and inositol trisphosphate. The latter triggers the release of intracellular Ca^{2+} stores, which promotes a transient influx of extracellular Ca^{2+}. CD22 can modulate B cell activation, but the molecular mechanism by which it functions has not been clear.

One domain of CD22 that is critical for its ability to attenuate BCR activation is the intracellular region that possesses immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The kinase Lyn catalyzes the phosphorylation of these ITIMs (14), which facilitates the recruitment of negative regulators of BCR signaling. One such regulator, the phosphatase SHP-1, can promote the hydrolysis of phosphorylated signaling components thereby diminishing BCR activation (15). The ability of CD22 to attract proteins like SHP-1 is consistent with its inhibitory role, yet how CD22 ligands influence these processes is unclear.

CD22 (Siglec-2) is a member of the sialic acid-binding Ig-like lectin (Siglec) family of receptors. Like other members of this family, it interacts with glycoconjugates possessing terminal sialic acid residues. Studies examining the carbohydrate specificity of CD22 have revealed its preference for α2,6-linked sialylated glycans (16). These glycoconjugates are present on some antigens, yet they also are abundant on the B-cell surface (3, 17). *Cis* interactions between CD22 and proximal glycoconjugates can mask the coreceptor to exogenous (*trans*) ligands. Specifically, when CD22-positive cells are exposed to the types of monovalent CD22 ligands that occur naturally, no binding is detected (18–20). Even multivalent presentations of these ligands fail to interact with CD22 (18–20). These observations are consistent with studies indicating that CD22 binds only weakly to naturally occurring carbohydrate ligands (21). Overcoming this masking requires some perturbation of the system—either the degradation or removal of the B cell surface sialic acid residues or the use of nonnatural carbohydrate derivatives that can outcompete the natural ligands for CD22 (22, 23). Thus, data from B cell binding studies emphasize the difficulty of overcoming *cis* interactions.

Complementing the binding studies are investigations indicating a functional role for *cis* CD22 ligands. B cells isolated from mice lacking the sialyltransferase necessary for generating CD22 ligands (*St6gal1*$^{-/-}$) show attenuated Ca^{2+} flux after BCR engagement (24). Thus, the B cell response to antigen is compromised. This finding was rationalized by positing that when B cells lack *cis* ligands, there is increased association of CD22 with the BCR on unstimulated B cells (25). CD22 localizes to clathrin-rich domains before antigen recognition, but the BCR does not. BCR engagement is thought to trigger colocalization of CD22 and the BCR. Accordingly, *cis* interactions of CD22 with cell surface glycoproteins help to sequester it from the BCR before antigen stimulation (24, 25). Mice lacking ST6Gal I also exhibit reduced serum IgM, decreased B cell proliferation after BCR cross-linking, and impaired antibody production in response to antigens. These results suggest that *cis* interactions are critical for CD22 function (24).

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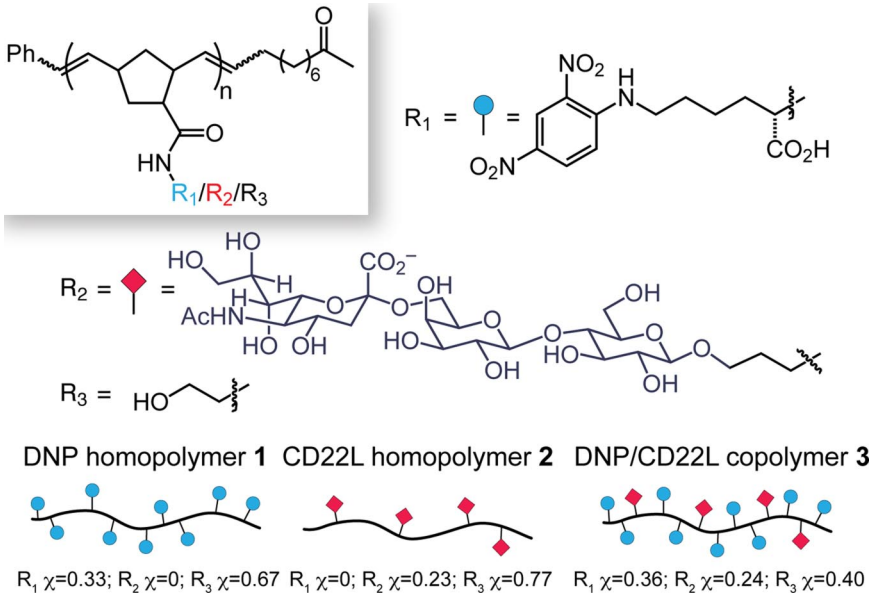

Fig. 1. Synthetic antigens used to investigate CD22 recognition. Polymers with a degree of polymerization of 250 ($n \approx 250$) were used. The substituents include the dinitrophenyl (DNP group, R_1, blue), the CD22 ligand Neu5Acα2,6Galβ1,4Glc (CD22L, R_2, red), or the spacer unit derived from ethanolamine coupling. The level of substitution of each group (mole fraction χ) for compound 1 is R_1 $\chi = 0.33$, R_3 $\chi = 0.67$; for compound 2 is R_2 $\chi = 0.23$, R_3 $\chi = 0.77$; for compound 3 is R_1 $\chi = 0.36$, R_2 $\chi = 0.24$, R_3 $\chi = 0.40$.

In contrast, a functional role for *trans* interactions has been lacking. An understanding of the contribution of *trans* interactions could provide insight into how antigen features influence CD22 function and guide the design of antigens that can exploit it. Several lines of evidence suggest that *trans* interactions may be important. When cells interact, CD22 redistributes to areas of cell–cell contact (17). This redistribution may be due to the aggregate avidity of many glycoconjugates on a cell interacting with many copies of CD22 on another. When cells that fail to present CD22 ligands (ST6Gal I null) were used to display antigen, cognate B cells up-regulated surface markers consistent with activation (26). This observation indicates that, in the absence of *trans* CD22 ligands, B cells can respond more efficiently to antigen on an adjacent cell. It also has been proposed that subpopulations of cells could exist in an unmasked state or that CD22 unmasking can occur after B cell activation (19, 27). We sought to determine whether antigens that bind CD22 elicit responses similar to those that do not.

We hypothesized that multivalent antigens could be used to explore whether *trans* interactions can recruit CD22 function. To this end, we generated a series of synthetic antigens and assessed their influence on BCR signaling. Our results indicate that chemically defined antigens can function as powerful tools for investigating how molecular interactions and receptor localization influence coreceptor function. The data indicate that coengagement of CD22 and the BCR evokes a profound inhibitory effect on B cell activation. Moreover, the synthetic ligands can illuminate key steps in the signal transduction cascade that can be influenced by CD22 engagement.

Results

Synthesis of Antigens for Probing CD22 Function. Our strategy to illuminate CD22 function depends upon the design and synthesis of chemical probes. We and others had shown previously that polymeric antigens can induce B-cell activation (28, 29). We reasoned that polymeric antigens that display a BCR-binding epitope and a CD22 ligand should provide an excellent test for *trans* interactions in CD22 signaling. Specifically, if only *cis* CD22 interactions are relevant, CD22 will be masked, so the antigens will interact solely with the BCR. Their ability to cluster the BCR would elicit B-cell activation. In contrast, if the sialylated antigens can engage both the BCR and CD22 through *trans* interactions, we predicted that they would dampen B-cell activation.

To generate ligands with the necessary attributes, we used the ring-opening metathesis polymerization (ROMP) (Fig. 1). This reaction is useful for antigen synthesis because it affords polymers with defined lengths and narrow molecular mass distributions (30–32). Additionally, compounds generated by ROMP are especially effective at clustering proteins and activating signaling (33, 34). The antigen we incorporated, 2,4-dinitrophenyl (DNP), is convenient because it can be readily attached to the polymer backbone and a murine B cell line displaying a DNP-specific BCR is available (A20.2J HL_{TNP} denoted A20HL) (35, 36). The design of the CD22 ligands was guided by our previous studies indicating that a polymer bearing a sialic acid-terminated trisaccharide can engage CD22 on the cell-surface (20). Using these components and precedents, we synthesized polymers that can bind to cells through the BCR alone (**1**), CD22 alone (**2**), or both (**3**). The target antigens were assembled from succinimidyl ester-substituted polymers, which were elaborated with DNP-lysine and/or an amine-substituted trisaccharide that functions as ligand for CD22 (Neu5Acα2,6Galβ1,4Glc denoted as CD22L) to generate a DNP-substituted homopolymer, a trisaccharide-substituted homopolymer, and a copolymer that displays both recognition elements. After coupling of the recognition elements, any remaining succinimidyl esters were blocked with ethanolamine (Fig. 1). The use of a single succinimidyl ester-substituted polymer stock ensures that antigen characteristics, such as valency and polydispersity, are preserved (37). Other design features of the DNP-substituted antigens are the density of the antigenic epitopes they present and their valency. Both parameters were chosen to elicit robust BCR signaling. Specifically, the hapten density for both homopolymers and copolymers was 33–36%, and we had shown previously that this level of substitution results in BCR binding and signaling (29). The level of substitution of CD22L was 25%, which we anticipated would be sufficient to allow for CD22 binding. The degree of polymerization for all of the synthetic antigens was ≈250 (Fig. 1), and polymers of this length (valency) can activate BCR signaling (see below).

If CD22 is masked on the surface by interactions with B cell surface glycoconjugates, ligands displaying sialylated oligosaccharides should fail to interact [supporting information (SI) Fig. S1] (18–20). To test for *cis* interactions, we treated A20HL cells with fluorophore-labeled polymer **2** that presents a trisaccharide CD22 ligand (Fig. 1) (20). Analysis by flow cytometry revealed that binding of the polymer was blocked (Fig. S1). Thus, even though compound **2** is multivalent, CD22 is not accessible to it. To test

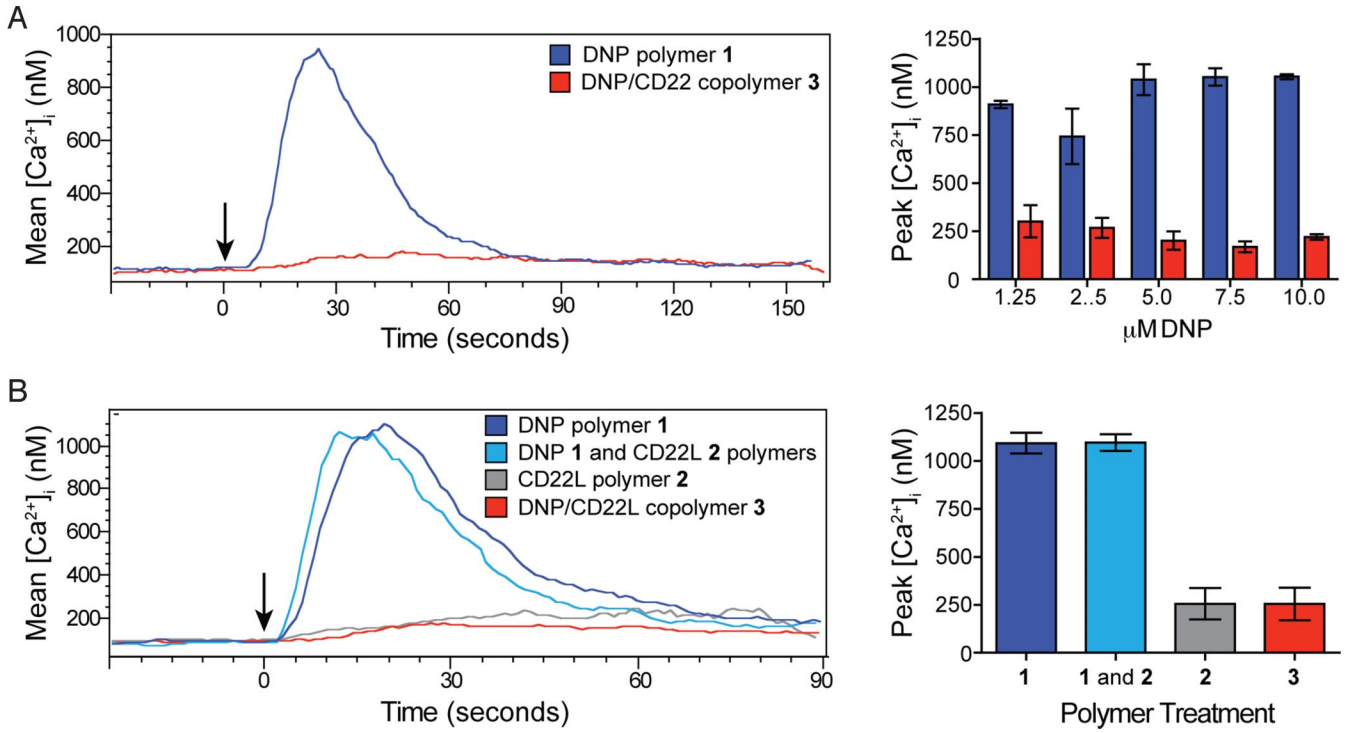

Fig. 2. Antigenic polymers possessing CD22 ligands inhibit calcium flux. (*A*) Calcium flux was assayed by using the ratiometric dye indo-1 and flow cytometry (*Left*). After establishing the base line, cells were treated with polymers (DNP concentration of 5 μM; CD22L concentration of 3.5 μM) at $t = 0$. Peak Ca^{2+} flux from A20HL cells treated (*Right*) with both DNP homopolymer and DNP/CD22L copolymers at 1.25–10 μM DNP. (*B*) Cells also were treated with the CD22L homopolymer alone or CD22L homopolymer followed by DNP homopolymer (*Left*) and peak Ca^{2+} flux (*Right*) determined. All error bars represent 1 standard deviation from the mean of 3 independent experiments.

whether binding can occur when *cis* interactions with cell surface glycoproteins are alleviated, we treated cells with periodate. This reagent effects the oxidative cleavage of surface sialic acid residues, thereby liberating CD22 (18). After this treatment, the polymer probe bearing the CD22L binds. These data indicate that the polymer can complex CD22 in the absence of *cis* interactions, but it cannot compete effectively with cell surface glycoconjugates for the lectin. These results are consistent with previous observations indicating that if CD22 is to function *in trans*, *cis* interactions must be overcome (18–20, 22, 23).

Sialylated Multivalent Ligands Inhibit B Cell Activation. Binding studies indicate that CD22 engages in *cis* interactions. If CD22 functions solely through *cis* interactions, polymers bearing a CD22-binding group and a BCR-specific antigen should elicit B cell activation. To test for this response, we monitored a critical indicator: the transient influx of extracellular Ca^{2+} into the cytosol. Intracellular calcium ion concentration ($[Ca^{2+}]_i$) can be monitored in real-time in live cells with flow cytometry using the ratiometric Ca^{2+}-chelating dye indo-1 (38). We compared the Ca^{2+} flux elicited by DNP-substituted polymer **1**, CD22 ligand (CD22L)-substituted polymer **2**, and DNP/CD22L-substituted copolymer **3** (Fig. 2). Consistent with our previous results, we observed that stimulation with polymers substituted only with the DNP antigen causes a rapid increase in $[Ca^{2+}]_i$ (Fig. 2*A*). In contrast, the addition of CD22L homopolymer **2** had no effect, as would be expected from its binding data. Intriguingly, the DNP/CD22L copolymer **3** did not promote the increase in $[Ca^{2+}]_i$ that is indicative of B cell activation; it elicited little or no change in $[Ca^{2+}]_i$. We tested polymers **1** and **3** over several concentrations, but only the DNP-substituted polymer **1** elicited robust Ca^{2+} flux. Treatment with DNP/CD22L copolymer **3** consistently squelched Ca^{2+} flux (Fig. 2*A* and Fig. S2). To test whether antigen binding results in CD22 unmasking, we added both the CD22L-bearing homopolymer and the DNP-substituted homopolymer. This combination resulted in an increase in $[Ca^{2+}]_i$, which is a response similar to that obtained when cells are treated with the DNP homopolymer **1** alone (Fig. 2*B*). These results suggest that coclustering of CD22 and the BCR block B cell activation. Consistent with this model, antigenic polymers with lower levels of the CD22 ligand were weak activators of Ca^{2+} flux (Fig. S3*A*). In contrast, a copolymer displaying a saccharide that does not bind CD22, Galβ1,4Glc, initiated robust Ca^{2+} flux (Fig. S3*B*). These findings suggest that *trans* interactions with CD22 dampen signaling. To explore further the influence of the sialylated antigen, we examined other signatures of B cell activation.

A feature of BCR signaling is that it results in the phosphorylation of a wide array of signaling components (39, 40) (Fig. S4). Thus, we assessed the influence of the polymeric antigens on the level of protein tyrosine phosphorylation. A20HL cells were treated with the different polymers for various intervals and then lysed; the resulting samples were subjected to immunoblotting to detect tyrosine phosphorylation (Fig. 3). In cells exposed to polymer **2**, which is substituted only with the CD22L, no change in phosphorylation was observed. In contrast, samples treated with DNP-substituted polymer **1** exhibited the high levels of increased tyrosine phosphorylation indicative of activation. These results are consistent with the influence of antigens **1** and **2** on $[Ca^{2+}]_i$. Compared with the responses due to antigenic polymer **1**, treatment with the DNP/CD22L copolymer **3** resulted in levels of phosphotyrosine that were reduced dramatically. This reduction was detectable even at very early time points (30 s). Together, the data suggest that CD22 recruitment attenuates BCR signaling through *trans* ligand recognition.

Copolymer Treatment Promotes the Phosphorylation of CD22 and Lyn but Not Syk or PLCγ2. Although the profiles of phosphotyrosine-containing proteins for untreated and DNP/CD22L copolymer

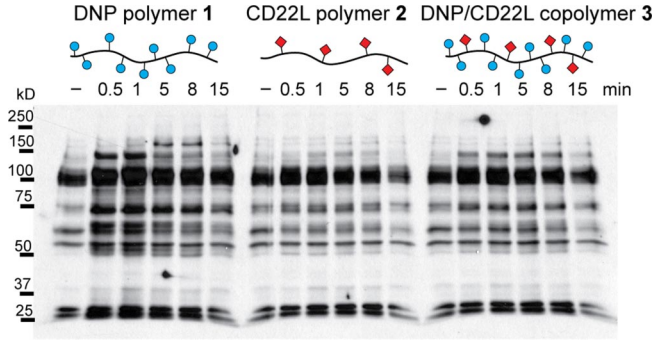

Fig. 3. Antigenic polymers possessing CD22 ligands attenuate tyrosine phosphorylation. Stimulation of A20HL cells with DNP homopolymer results in an increase in cellular protein tyrosine phosphorylation as assessed by immunoblot of cellular lysates using anti-phosphotyrosine antibody. In contrast, DNP/CD22L copolymer-treated cells show little change in tyrosine phosphorylation after stimulation, whereas CD22L homopolymer-treated cells remain unchanged. Cells were stimulated by using a final concentration of 5 μM DNP and/or 3.5 μM CD22L.

3-treated cells were similar, they were not identical. Analysis of the data suggests that the DNP/CD22L antigen **3** does promote some signaling. We therefore compared the ability of the potent activator **1** and the bifunctional **3** to promote the phosphorylation of key proteins that propagate BCR signals.

Neither CD22 nor the BCR signaling components Igα/β possess intrinsic kinase activity; therefore, accessory nonreceptor tyrosine kinases are required to promote their phosphorylation (Fig. S4). This process presumably occurs when CD22 and the BCR are recruited to domains enriched in Src-family kinases, such as Lyn (41). Because Lyn can catalyze the phosphorylation of both the BCR and CD22 (14), we tested the effects of antigens **1** and **3** on Lyn activation. The kinase activity of Lyn is unleashed when its activation loop is phosphorylated; therefore, we probed for this modification (42). Exposure of B cells to either DNP homopolymer **1** or the DNP/CD22L copolymer **3** resulted in rapid and transient phosphorylation of Lyn (Fig. 4*A*). These data indicate that both types of antigens can engage and cluster the BCR and thereby initiate the earliest events in BCR signaling.

If Lyn is activated, it should effect the phosphorylation of CD22 (Fig. S4). Upon BCR engagement, tyrosine residues within the CD22 ITIMs can be modified (14, 43). Because antigenic polymers **1** and **3** can bind the BCR, each should elicit CD22 phosphorylation. As expected, treatment of cells with CD22L homopolymer **2** failed to promote phosphorylation of CD22 (Fig. S5*A*). In contrast, DNP homopolymer **1** promoted CD22 phosphorylation, as did copolymer **3**. These data are consistent with our expectations that BCR engagement should result in CD22 phosphorylation (44). Although both antigens **1** and **3** elicited this modification, there were differences. Comparable levels of CD22 phosphorylation were observed at early time points (30 s–1 min), but at later time points (5–15 min), samples from cells exposed to copolymer **3** had higher levels of CD22 phosphorylation (Fig. 4*B* and Fig. S5*B*). The finding that **3** elicits sustained phosphorylation is notable, because it indicates that responses to antigen are altered dramatically by *trans* CD22 interactions.

To probe whether downstream steps are perturbed, we investigated the status of relevant effectors. An increase in $[Ca^{2+}]_i$ results when the secondary messenger IP_3 is generated through the action of PLCγ2 (Fig. S4). The activity of PLCγ2 is triggered by phosphorylation, and antigen stimulation results in the modification of several different tyrosine residues. To determine whether different polymers vary in their abilities to promote PLCγ2 phosphorylation, we used a phosphospecific antibody against PLCγ2 Y1217 (45). As expected from our previous data, stimulation with the DNP-

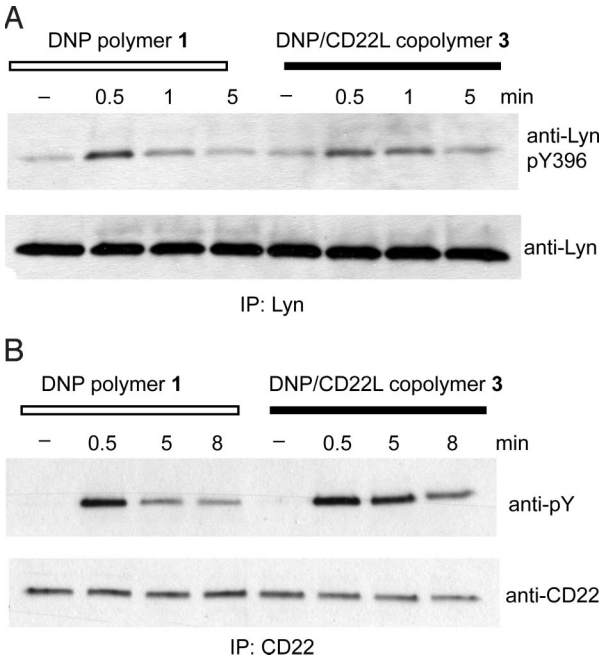

Fig. 4. Polymer stimulation results in Lyn and CD22 phosphorylation. (*A*) A20HL cells were stimulated with polymers and lysed, and the resulting samples were subjected to immunoprecipitation using anti-Lyn. Immunoblot analysis with a phosphospecific antibody was performed. Immunoprecipitation efficiency was monitored by probing with anti-Lyn. Treatment with either the DNP homopolymer or the DNP/CD22L copolymer resulted in Lyn phosphorylation (Y396). (*B*) Similarly CD22 was immunoprecipitated with anti-CD22. Both the DNP homopolymer and the DNP/CD22L copolymer stimulation resulted in CD22 phosphorylation; however, increased phospho-CD22 is observed at later time points with copolymer. Cells were stimulated by using a final concentration of 5 μM DNP (3.5 μM CD22L). See Fig. S6.

substituted homopolymer **1** induced rapid phosphorylation. In contrast, the DNP/CD22L copolymer **3** had no influence (Fig. 5*A*). These observations are consistent with the Ca^{2+} flux data, in which the DNP-substituted antigen **1** affords large increases in $[Ca^{2+}]_i$, but the bifunctional antigen **3** does not.

In addition to testing for PLCγ2, we monitored the activation state of the kinase Syk. This enzyme, which is obligatory for B cell development (10, 11), is critical for propagating BCR signaling (9). Because Syk appears to act downstream of Lyn, we were interested in whether antigens **1** and **3** would influence Syk activation. We found that cells stimulated with DNP homopolymer **1** resulted in an increase in the phosphorylation of tyrosine residues (Y519/520) located within the activation loop of Syk kinase (46). In cells treated with the DNP/CD22L copolymer **3,** however, Syk phosphorylation was almost completely abolished (Fig. 5*B*). These results reveal that antigens that can interact with both the BCR and CD22 give rise to signals that differ dramatically from those elicited by antigens that bind only to the BCR.

Discussion

A variety of studies indicate that *cis* ligands are important for CD22 function, but whether glycosylated antigens can bind CD22 *in trans* and influence signaling was ambiguous. This ambiguity stems from inadequacy of standard approaches for addressing this question. Specifically, CD22 ligands are ubiquitous, so the contributions of *cis* and *trans* interactions are difficult to separate. Additionally, only nonnatural high-affinity ligands have been shown to bind CD22 *in trans*, and because these do not engage the BCR, they would not be predicted to influence signaling (22, 23). Bifunctional antigens provide a means to directly assess the functional roles of cell-surface (*cis*) and

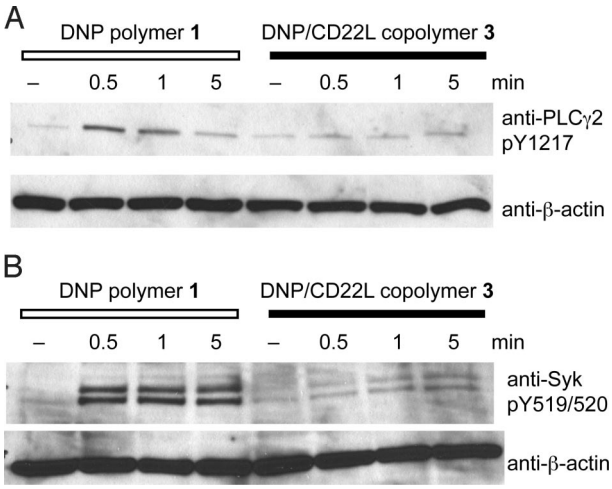

Fig. 5. Antigen sialylation prevents Syk and PLCγ2 activation. (*A*) A20HL cells were stimulated with polymers and lysed, and the resulting samples were subjected to immunoprecipitation using anti-Syk. Immunoblot analysis with a phosphospecific antibody (Y519/520) was performed. Two isoforms are visible. (B) PLCγ2 activation was assessed by immunoblot analysis of lysate from polymer-treated cells using a phosphospecific antibody (Y1217). As with Syk, the DNP homopolymer treatment resulted in PLCγ2 activation as assessed by phosphorylation (Y1217). Cells were stimulated by using a final concentration of 5 μM DNP (3.5 μM CD22L). See Fig. S6.

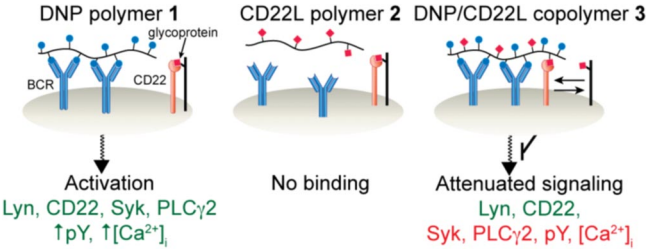

Fig. 6. Antigenic polymers displaying hapten (DNP) initiate activation of early B cell signaling events. Copolymers displaying DNP and a ligand for the coreceptor CD22 result in the attenuation of B cell activation.

antigen-presented (*trans*) CD22 ligands on B cell activation. Thus, we designed compound **3**.

We reasoned that a sialylated antigen like **3** could bind CD22 because interactions between CD22 and its *cis* ligands are weak. Indeed, the monovalent dissociation constants are in the range of 100–200 μM (21). With their low affinities, *cis* interactions with CD22 should be kinetically labile. Given that antigen binding to the B cell receptor results in its localization on the cell surface, antigens bearing even a low-affinity CD22-binding group might compete effectively with cell-surface glycoconjugates. We postulated that antigens that can bind in this way could induce responses distinct from those promoted by antigens that do not bind CD22. If *cis* interactions predominate, compound **3** should activate B cells; if *trans* interactions are important, it should suppress activation. A comparison of B cell signaling in response to antigen **1** versus bifunctional antigen **3** reveals key differences. The most striking is that antigen **1** activates B cells but the bifunctional antigen **3** does not. This conclusion is supported by monitoring 2 hallmarks of B cell activation: an increase in intracellular calcium ion concentration and an increase in phosphotyrosine levels. One explanation for the inability of compound **3** to activate BCR signaling is that the addition of saccharide groups to the polymer backbone prevents DNP binding. To test for this possibility, we synthesized an antigen bearing DNP groups and a saccharide (Galβ1,4Glc) that does not interact with CD22. In contrast to antigen **3**, the copolymer displaying DNP and Galβ1,4Glc groups elicits robust B cell activation (Fig. S3). These findings indicate that the suppression of B cell activation observed with compound **3** arises from its ability to interact with both the BCR and CD22. Together, these results indicate *trans* interactions with CD22 suppress antigen-initiated B cell activation.

The differences in B cell activation elicited by antigen **1** and bifunctional antigen **3** prompted us to examine specific steps in the signal transduction cascade. Both antigen types promote an early step in B cell activation—the phosphorylation of the Src-family kinase Lyn. Both types of antigens also cause CD22 phosphorylation. Still, the duration of CD22 phosphorylation was longer when cells were treated with sialylated antigen **3**. This observation suggests that the proximity of CD22 and the BCR influence the level of CD22 phosphorylation. We also found that in cells treated with antigen **3** key BCR signaling effectors PLCγ2 and Syk were not phosphorylated and therefore not activated (Fig. 5). This response (i.e., phosphorylation of Lyn but not of Syk) is similar to that observed in the presence of the inhibitory coreceptors CD72 and PIR-B, which recruit the phosphatase SHP-1 to the BCR signaling complex (47, 48). Together, the data indicate that antigen **3**, which can bind the BCR and CD22 *in trans*, elicits some initial BCR signaling events but suppresses the activation of downstream effectors.

The influence of the DNP/CD22L-substituted antigen **3** on BCR signaling and the inactivity of CD22L homopolymer **2** provides evidence that recruitment of CD22 to the BCR is critical for signal attenuation. A role for CD22 localization is supported by studies using B cells from ST6 Gal I null mice. CD22 localization on resting B cells is sensitive to α2,6-Sia concentrations on the cell surface (25), a finding that suggests that *cis* interactions sequester CD22 from the BCR before antigen engagement (24). In this model, antigen engagement results in colocalization of CD22 and the BCR, which suppresses activation. Our results provide independent support that when CD22 and the BCR are proximal, BCR signaling is diminished.

The available data indicate that the negative regulatory role of CD22 can be recruited via both *cis* and *trans* interactions. We postulate that both modes are important for modulating B cell activation. *Cis* ligand interactions can sequester CD22 from the BCR, so that B cells can be activated, and upon activation, they help dampen BCR signaling. When antigens that can engage in *trans* interactions are present, CD22 recruitment to the BCR attenuates signaling dramatically. Thus, *cis* and *trans* interactions are not mutually exclusive but rather are different binding modes that are critical for controlling the level of CD22 suppression of B cell activation (Fig. 6).

The ability of CD22 to function *in trans* provides evidence that antigen glycosylation can serve as an innate form of self-recognition (26). Our data indicate that the level of CD22 ligand substitution on an antigen can influence its ability to activate B cells (Fig. S2). Specifically, those antigens displaying no CD22 ligands were potent activators, those with modest levels of CD22 ligand substitution were weak activators, and those with high levels were extremely effective suppressors. These findings are consistent with observations that tolerogenic or immature cells display α2–6 sialic acid moieties at higher levels than immunogenic or activated cells (49). Moreover, our results indicate that changes in carbohydrate expression and recognition can have dramatic effects on immune reactivity and tolerance. By manipulating levels of sialylated glycoconjugates, it may be possible to alter the immunogenicity of an antigen or cell. Additionally, our findings indicate that ligands that can coopt inhibitory receptors such as CD22 may offer a fundamentally different approach to blocking deleterious autoimmune responses.

Materials and Methods

Cell Culture. A20/2J cells stably transfected with DNP/TNP-specific mIgM (35, 36) to generate the A20/2J HL_{TNP} cell line were provided by A. Ochi (University Health Network, Toronto, ON, Canada). A20/2J HL HL_{TNP} cells were cultured in RPMI medium 1640 supplemented with 2 mM L-glutamine, 10% FBS, 50 μM 2-mercaptoethanol, 100 U/mL^{-1} penicillin and 100 U/mL^{-1} streptomycin.

Calcium Flux. Cells were resuspended in DPBS (pH 7.4) at 2×10^6 cells per mL^{-1} and loaded 6 μg mL^{-1} indo-1 a.m. (Invitrogen) for 40 min at 37 °C. Cells were then washed and resuspended at 1×10^6 cells mL^{-1} in DPBS supplemented with 1% BSA and 1 mM $CaCl_2$. Cells were analyzed by using BD FACSVantage SE and LSR II flow cytometers. The indo-1 emission ratio (R) was monitored as F_2/F_1, where F_2 corresponds to Ca^{2+}-bound (405 nm) and F_1 Ca^{2+}-free (490 nm). $[Ca^{2+}]_i$ was determined by using the formula $[Ca^{2+}]_i = K_d B[(R - R_{min})/(R_{max} - R)]$, where Kd is the dissociation constant for indo-1, and Ca^{2+} and $B = F_{1,min}/F_{1,max}$ (38). Minimum and maximum values for R and F_1 were determined from analysis of samples lacking Ca^{2+} (2 mM EDTA) or with saturating levels of Ca^{2+} (4 μM ionomycin). Kinetic analysis of $[Ca^{2+}]_i$ was performed by using the FlowJo software package.

Antibodies. All secondary Ab were obtained from Jackson ImmunoResearch. Anti-CD22 mAB (2D6) was obtained from Southern Biotech. Anti-phosphotyrosine-HRP mAB (PY20) was obtained from Santa Cruz Biotechnology. Phosphospecific and corresponding pABs against PLCγ2, Syk and Src-family kinases were obtained from Cell Signaling Technology.

Immunoblots. Cells were prepared as for Ca^{2+} flux experiments and resuspended at 1–2 $\times$ 10^6 cells mL^{-1} in DPBS supplemented with 1 mM $CaCl_2$ (and 1% BSA for immunoprecipitation). Cells were incubated at 37 °C for 10 min before stimulation. Aliquots were lysed on ice at a final concentration of 1% Triton-X 100, 1 mM EDTA, 2 mM Na_3VO_4, 2 mM Na_2MoO_4, 2 mM NaF and 1:100 HALT protease inhibitor mixture (Pierce). Samples were resuspended in SDS/PAGE sample buffer and run on 10% Tris·HCl gels. For immunoprecipitations samples were pelleted to remove debris (15,000 $\times$ g,10 min, 4 °C) and precleared by using appropriate resin (Pierce) at 4 °C. For immunoblot analysis, samples were transferred to a 0.45-μm PVDF membrane (Millipore) in transfer buffer (14.4 g of glycine, 3.03 g of Tris base, and 10% methanol to 1 L). Membranes were blocked for 1 h at 4 °C using blocking buffer (5% BSA TBS-T) before incubating with primary antibody overnight. Blots were rinsed for 3 times for 10 min before incubating with secondary for 1 h at room temperature. Blots were then rinsed as before and developed with chemiluminescent substrate (ECL; Pierce) and X-ray film (Pierce).

Polymer Synthesis. Antigenic polymers were prepared as previously described (29). Briefly, a succinimidyl ester-substituted norbornene monomer was dissolved in a deoxygenated solution of dichloromethane to which a ruthenium carbene initiator was added. The reaction was terminated with an excess of an enol ether. The polymer was precipitated by using a solution of 9:1 ether/benzene and centrifuged. The supernatant was decanted and the remaining polymer dried to afford an off-white solid.

Further experimental details and figures are found in the *SI Text*

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