ACS chemical biology

Articles

pubs.acs.org/acschemicalbiology

Synthetic Antigens Reveal Dynamics of BCR Endocytosis during Inhibitory Signaling

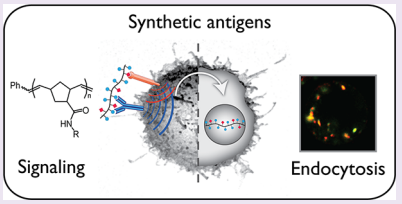
Adam H. Courtney,[†] Nitasha R. Bennett,[‡] Daniel B. Zwick,[†] Jonathan Hudon,[‡] and Laura L. Kiessling*[,†,‡]

[†]Department of Biochemistry, University of Wisconsin—Madison, 433 Babcock Drive, Madison, Wisconsin 53706, United States

[‡]Department of Chemistry, University of Wisconsin—Madison, 1101 University Avenue, Madison, Wisconsin 53706, United States

Supporting Information

ABSTRACT: B cells detect foreign antigens through their B cell antigen receptor (BCR). The BCR, when engaged by antigen, initiates a signaling cascade. Concurrent with signaling is endocytosis of the BCR complex, which acts to downregulate signaling and facilitate uptake of antigen for processing and display on the cell surface. The relationship between signaling and BCR endocytosis is poorly defined. Here, we explore the interplay between BCR endocytosis and antigens that either promote or inhibit B cell activation. Specifically, synthetic antigens were generated that engage the BCR alone or both the BCR and the inhibitory co-receptor CD22. The lectin CD22, a member of the Siglec family, binds sialic acid-containing glycoconjugates found on host tissues, inhibiting BCR signaling to prevent erroneous B cell activation. At low concentrations, antigens that can cocluster the BCR and CD22 promote rapid BCR endocytosis; whereas, slower endocytosis occurs with antigens that bind only the BCR. At higher antigen concentrations, rapid BCR endocytosis occurs upon treatment with either stimulatory or inhibitory antigens. Endocytosis of the BCR, in response to synthetic antigens, results in its entry into early endocytic compartments. Although the CD22-binding antigens fail to activate key regulators of antigen presentation (e.g., Syk), they also promote BCR endocytosis, indicating that inhibitory antigens can be internalized. Together, our observations support a functional role for BCR endocytosis in downregulating BCR signaling. The reduction of cell surface BCR levels in the absence of B cell activation should raise the threshold for BCR subsequent activation. The ability of the activating synthetic antigens to trigger both signaling and entry of the BCR into early endosomes suggests strategies for targeted antigen delivery.



Within the immune system, the B cell antigen receptor (BCR) functions as a detector for foreign antigens. BCR engagement drives the production of antibodies by B cells; dysregulation can result in autoimmunity or immunodeficiency. Essential to these processes is the propagation of signals from the BCR. BCR signaling is initiated by antigen binding the BCR, which triggers local receptor oligomerization and phosphorylation of the BCR associated heterodimer Igα/β. Phosphorylation of Igα/β by Src family kinases (e.g., Lyn) recruits and activates the tyrosine kinase Syk, culminating in the nucleation of a signaling complex.[1,2] The components of this BCR signaling complex include adaptor proteins and cellular effectors that regulate B cell activation. Some of these effectors are generated through the action of the phospholipase PLCγ2, which catalyzes the formation of the secondary messengers inositol trisphosphate (IP_3) and diacylglycerol. These secondary messengers trigger a transient influx of calcium ions into the cytoplasm and B cell activation.[3] Antigen binding to the BCR not only elicits these signals but also leads to endocytosis of the BCR-bound antigen complexes.

The ability of the BCR to engage antigens and mediate their internalization is critical to proper B cell function.[4−6] Endocytosis of the BCR can downregulate and compartmentalize signaling, and it serves as a gateway for uptake of antigen.[7,8] Protein antigens taken up are processed within the endocytic network to afford peptide fragments that are loaded into major histocompatibility class II (MHC II) complexes and presented on the B cell surface.[9] These MHC II complexes are recognized by T cells that coordinate essential B cell functions, such as affinity maturation and isotype switching,[10] that are vital to generating high affinity antibodies against a target pathogen. Thus, BCR endocytosis regulates both BCR signaling and antigen presentation.[7,8,11]

Because BCR signaling and endocytosis are interconnected, the relationship between them is poorly understood. Previous investigations have focused on defining cellular components required for BCR uptake. These studies have implicated several adaptor proteins in BCR endocytosis, including BAM32, LAB, and BLNK; their depletion results in a reduction of endocytosis.[12−15] Furthermore, loss of Vav1/3, which can activate Rac GTPases to carry out actin remodeling, drastically inhibits endocytosis.[13,16,17] Still, many of these proteins also contribute to BCR signaling, obscuring their direct role in endocytosis. For example, BLNK is an essential adaptor, whose deletion results in attenuated BCR signaling.[18] Moreover, there are discrepancies between studies aimed at determining which early BCR signaling events are important for eliciting BCR endocytosis. For instance, it has been reported that BCR

Received: July 15, 2013
Accepted: October 16, 2013
Published: October 16, 2013

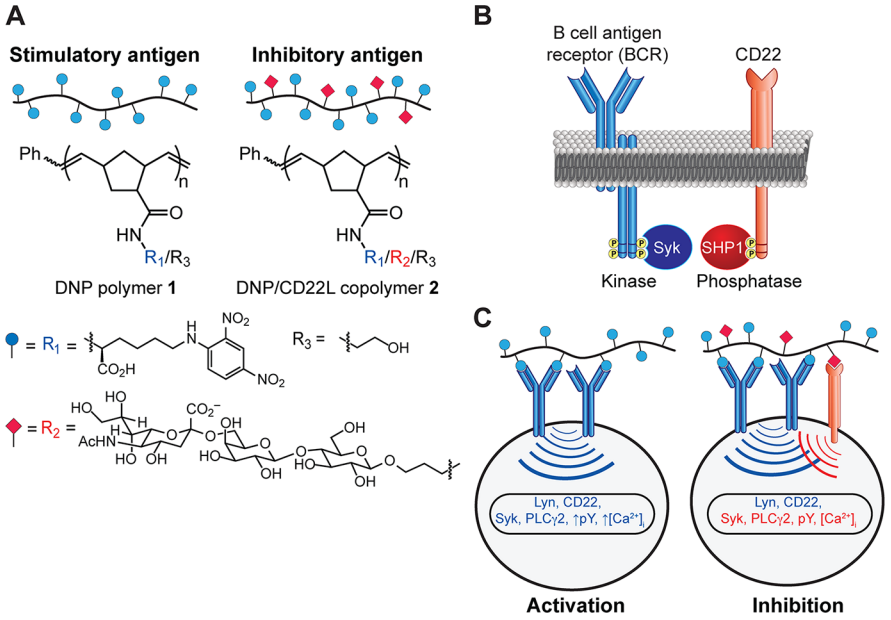


Figure 1. Synthetic antigens used to modulate BCR signaling. (A) Structures of the synthetic antigens that were generated using ROMP include the stimulatory antigen homopolymer **1**, which displays the dinitrophenyl (DNP, R_1, blue) group, and the inhibitory antigen copolymer **2**, which displays both DNP groups and the CD22 ligand Neu5Acα2,6Galβ1,4Glc (CD22L, R_2, red). (B) Targets of the polymers include the DNP-specific BCR and the inhibitory lectin CD22. (C) Engagement of the BCR by polymer **1** results in the activation of multiple signaling components. Activation of many of these components is inhibited in copolymer **2**-treated cells.[21,22,24]

endocytosis is inhibited when early signaling events, such as Lyn kinase activation, are blocked,[11,12] yet other studies report that endocytosis does not depend on this early signaling event.[19] These conflicting results highlight the complexity of dissecting BCR signaling and endocytosis. Loss-of-function experiments are especially difficult to interpret. Despite the importance of both processes to proper B cell function, the relationship between BCR signaling and endocytosis remains largely undefined.

An alternative approach would be to employ antigens capable of modulating BCR signaling. Investigations of BCR endocytosis have predominantly relied on receptor clustering agents, such as anti-BCR antibodies, as antigen mimics. Typically, these reagents are used in combination with strategies to perturb intracellular components (e.g., small molecule inhibitors, protein overexpression, or genetic deletion). How antigen properties influence BCR endocytosis is less explored. In one example, extensive intermolecular cross-linking of antibodies directed against the BCR was shown to slow endocytosis,[20] but the underlying molecular mechanism was unclear. Many antigens can engage co-receptors on the B cell surface. Co-receptors provide context to BCR signaling events by augmenting or inhibiting BCR signaling through the recruitment of intracellular effectors, such as kinases or phosphatases. We therefore reasoned that antigens tailored to bind co-receptors could reveal the relationship between specific steps in signaling and BCR endocytosis. Our test case was to examine the recruitment of the inhibitory co-receptor CD22 to the BCR complex. We postulated two potential outcomes. First, BCR endocytosis could be attenuated upon inhibitory co-receptor recruitment to prevent spurious antigen uptake. Second, endocytosis could occur regardless to downregulate cell surface BCR.

To distinguish between these possibilities, we generated ligands that could bind the BCR and the inhibitory lectin co-receptor CD22. Antigens bearing CD22 ligands, α2,6-linked sialic acid residues, prevent BCR signaling even when the BCR is engaged.[21,22] CD22 attenuates BCR signaling by recruiting a negative regulator of B cell activation, the protein tyrosine phosphatase SHP-1.[21−23] Engagement of CD22 by sialylated antigens blocks early events in BCR signaling, such as phosphorylation of the kinase Syk and the phospholipase PLCγ2.[21] We predicted that the ability of sialylated antigens to engage the BCR, while attenuating BCR signaling events, would provide insight into how BCR endocytosis is regulated. Our data indicate that chemically defined antigens that give rise to disparate signaling events can reveal how differences in signaling influence BCR endocytosis.

RESULTS

Synthetic Antigens As Probes of BCR Endocytosis. Our investigation into the relationship between BCR signaling and receptor endocytosis relies upon antigens capable of eliciting distinct signaling outputs. To investigate how inhibitory signaling affects BCR endocytosis, antigens that either promote or attenuate B cell activation were synthesized (Figure 1A and Supporting Figure S1, Supporting Information).[21,24] Antigen design was guided by the observation that sialylated antigens that can engage CD22 suppress B cell activation (Figure 1B).[21−23] We were especially interested in assessing the roles of B cell signaling components mobilized in the presence of activating antigens (e.g., polymer **1**) but inhibited upon exposure to glycosylated antigens that can bind CD22 (e.g., polymer **2**) (Figure 1C). One signaling component of interest is the tyrosine kinase Syk, which appears to be dispensable for BCR endocytosis yet required for antigen presentation.[25] Syk is essential for BCR signaling, and its loss abrogates proper B cell development.[26,27] Recruitment of the inhibitory co-receptor CD22 to the BCR prevents Syk phosphorylation and dramatically attenuates BCR signaling.[21,22] Because the stimulatory antigen activates Syk but the inhibitor

dx.doi.org/10.1021/cb400532y | ACS Chem. Biol. 2014, 9, 202−210

antigen does not, these compounds can therefore report on the role of regulators like Syk in BCR endocytosis.

Synthetic antigens were generated using the ring-opening metathesis polymerization (ROMP) reaction, because this reaction affords defined polymers.[28−30] Antigens generated using ROMP can engage the BCR and promote B cell activation.[21,24] The polymers employed herein display multiple copies of the 2,4-dinitrophenyl (DNP) hapten, which is recognized by a murine B cell line displaying a DNP-specific BCR (A20.2J HL_{TNP}, denoted A20HL).[31] The succinimidyl ester-substituted polymer was treated with DNP-lysine to afford homopolymer **1**. This polymeric antigen is of sufficient length ($n \approx 215$) to promote B cell activation (Figure 1C). To generate inhibitory antigens, we coupled DNP-lysine as well as the trisaccharide ligand Neu5Acα2,6Galβ1,4Glc (denoted CD22L), because the latter binds the inhibitory co-receptor CD22.[23] The resulting copolymer **2** can bind the BCR and CD22 (Figure 1B) and thereby function as an inhibitory antigen (Figure 1C).[21,22] A common stock of succinimidyl ester-substituted polymer was used to prepare all antigens. As a result, the final polymeric antigens have identical molecular masses and polydispersity,[28−30] so their activities can be compared directly.

Sialylated Antigens Reveal Distinct Kinetics of BCR Endocytosis. We previously reported that copolymer **2** inhibits early signaling events in A20HL cells (a murine B cell line). Data suggest that the mechanism is through recruitment of the protein tyrosine phosphatase SHP-1 to the B cell signaling complex. Copolymer **2** treatment prevents phosphorylation of Syk and PLCγ2 and therefore their activation (Figure 1C);[21,32] consequently, we employed it to probe whether Syk or PLCγ2 activity is required for BCR endocytosis. Guided by our previous studies of ROMP-derived antigens, we added an antigen concentration (5 μM DNP) of homopolymer **1** that induces robust B cell activation, and the same concentration of copolymer **2** was employed.[21,24] After A20HL cells were stimulated with synthetic polymeric antigens **1** or **2** for defined periods of time, cell surface BCR levels were assessed to determine the rate of BCR endocytosis (Supporting Figure S2, Supporting Information).[7,12,25] Homopolymer **1** resulted in rapid BCR uptake, as did copolymer **2**. A comparison of the rates showed little difference (Figure 2A).

We next investigated the influence of antigen concentration. At high antigen concentrations some activation of early signaling components, such as Syk or PLCγ2, might occur even with the inhibitory antigen **2**, so we tested a 10-fold lower antigen concentration. When cells were exposed to polymer **1** (0.5 μM DNP), the rate of BCR endocytosis was slower. Treatment with DNP/CD22L copolymer **2** afforded a rate similar to that obtained with the previous antigen concentration (Figure 2B). To quantify this surprising difference, the remaining BCR on the cell surface in response to polymer treatment at both high and low antigen concentration was fit to an exponential decay, and the half-life ($t_{1/2}$) was determined (Figure 2C). For homopolymer **1**, a striking increase of $t_{1/2}$ from 1.6 to 11.9 min was observed. In contrast, only modest differences in the rate of BCR endocytosis were detected for DNP/CD22L copolymer **2** treated cells. Because copolymer **2** prevents Syk and PLCγ2 activation (Figure 1C),[21] neither Syk nor PLCγ2 activation is responsible for the endocytosis observed.

Upon endocytosis, the BCR enters early endosomes before trafficking to antigen-processing compartments.[33,34] We therefore tested whether the loss of BCR from the cell surface results in its accumulation within the endocytic network. We used fluorophore-labeled transferrin as a marker of early endosomes, because the transferrin receptor constitutively recycles between early endosomes and the cell surface.[35] Treatment with either

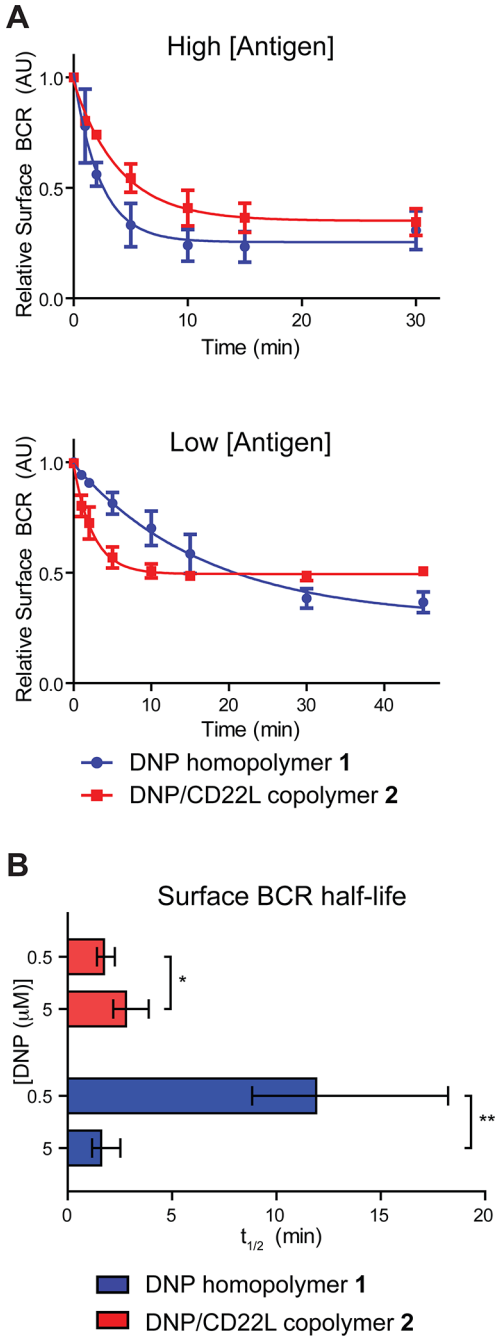


Figure 2. Influence of synthetic antigens on the kinetics of BCR endocytosis. (A) A20HL cells were treated with either DNP homopolymer **1** (blue) or DNP/CD22L copolymer **2** (red). Relative surface BCR was assessed following synthetic antigen stimulation at high (5 μM DNP) or low (0.5 μM DNP) antigen concentration. BCR remaining on the cell surface was determined using fluorophore-labeled anti-BCR antibodies (Fab fragment). Error bars represent ±1 standard deviation from the mean of three independent experiments. (B) Data points were fit to an exponential decay to determine the half-life ($t_{1/2}$) of the BCR on the cell surface in response to polymer stimulation. Error bars represent 95% confidence intervals. *$P < 0.05$; **$P < 0.001$ (Student's t-test).

dx.doi.org/10.1021/cb400532y | ACS Chem. Biol. 2014, 9, 202−210

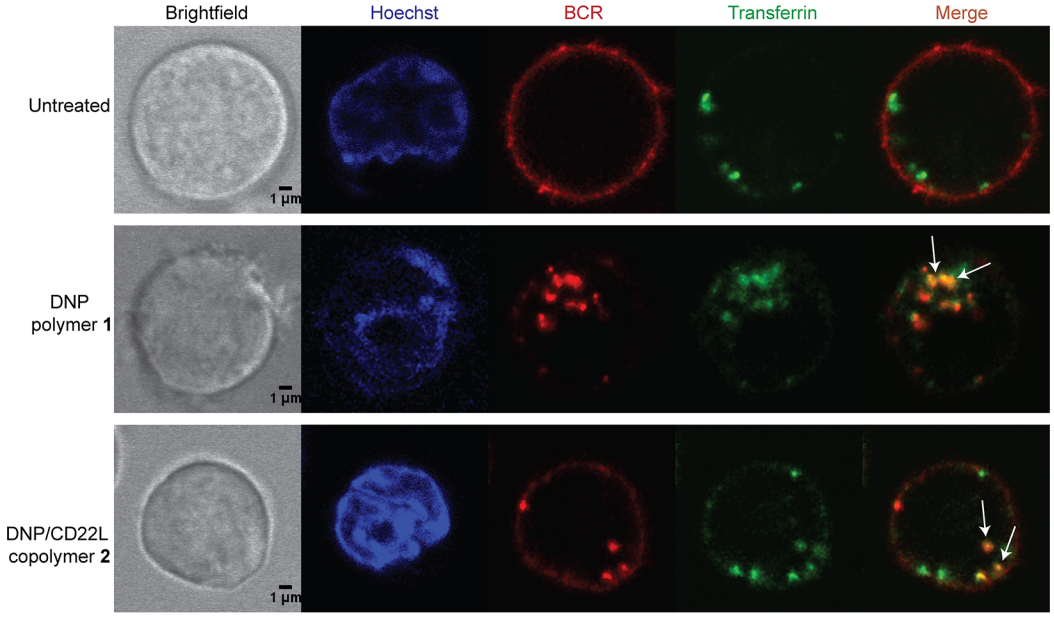


Figure 3. Trafficking of the BCR in response to synthetic antigens. The BCR of A20HL cells was stained prior to stimulation using a DyLight 549-conjugated anti-BCR Fab fragment (red), and early endosomes were labeled with Alexa Fluor-488 conjugated transferrin (green). Following labeling, cells were treated with either DNP polymer **1** or DNP/CD22L copolymer **2** (5 μM DNP) for 15 min, cooled to prevent further endocytosis, and analyzed using confocal microscopy. Nuclei were stained with Hoechst dye (blue). Regions of extensive colocalization between the BCR and transferrin are denoted by arrows. Scale bar, 1 μm.

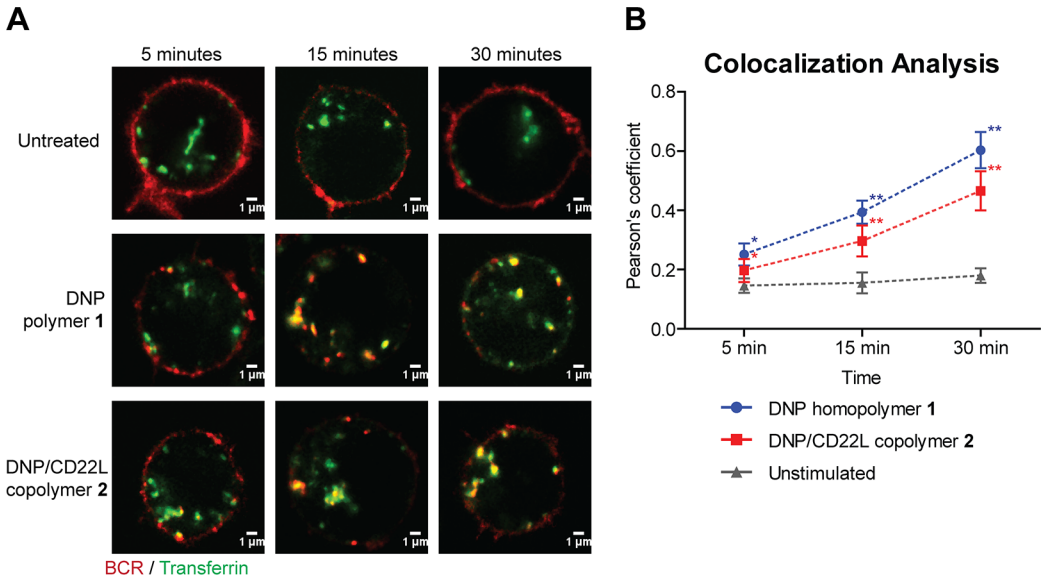


Figure 4. Time course for BCR entry into the endocytic network following synthetic antigen exposure. (A) A20HL cells were stained with anti-BCR fab fragments (red) and transferrin (green). Cells were treated with either DNP polymer **1** or DNP/CD22L copolymer **2** (5 μM DNP), and the results were compared with those from untreated cells. BCR and transferrin colocalization over time was assessed. A representative cell from three independent experiments is shown. (B) The Pearson's coefficient was calculated using at least 30 cells for each condition from three independent experiments. Error bars represent 95% confidence intervals. Scale bar, 1 μm. *P < 0.05 and **P < 0.001 relative to unstimulated control (Mann–Whitney test).

homopolymer **1** or copolymer **2** triggered a rapid loss of cell surface BCR (Figure 3). This loss coincided with the appearance of discrete puncta, which colocalized with transferrin. These observations are consistent with antigen-induced BCR endocytosis and entry of the receptor into early endosomes.

We determined the timeline for the transition of the BCR from the cell surface into early endosomes. The extent of BCR colocalization with transferrin was assessed in either the presence or the absence of antigen (5 μM DNP) at 5, 15, and 30 min (Figure 4A). In the absence of antigen, the BCR resides primarily on the cell surface. Five minutes after exposure to either homopolymer **1** or copolymer **2**, the BCR is found mainly on or near the cell surface; however, some puncta are clearly visible. By 15 minutes, extensive endocytosis has occurred. The presence of the BCR in early endosomal compartments is readily detected at this time point, and its accumulation increases further after 30 minutes. The extent of

dx.doi.org/10.1021/cb400532y | ACS Chem. Biol. 2014, 9, 202–210

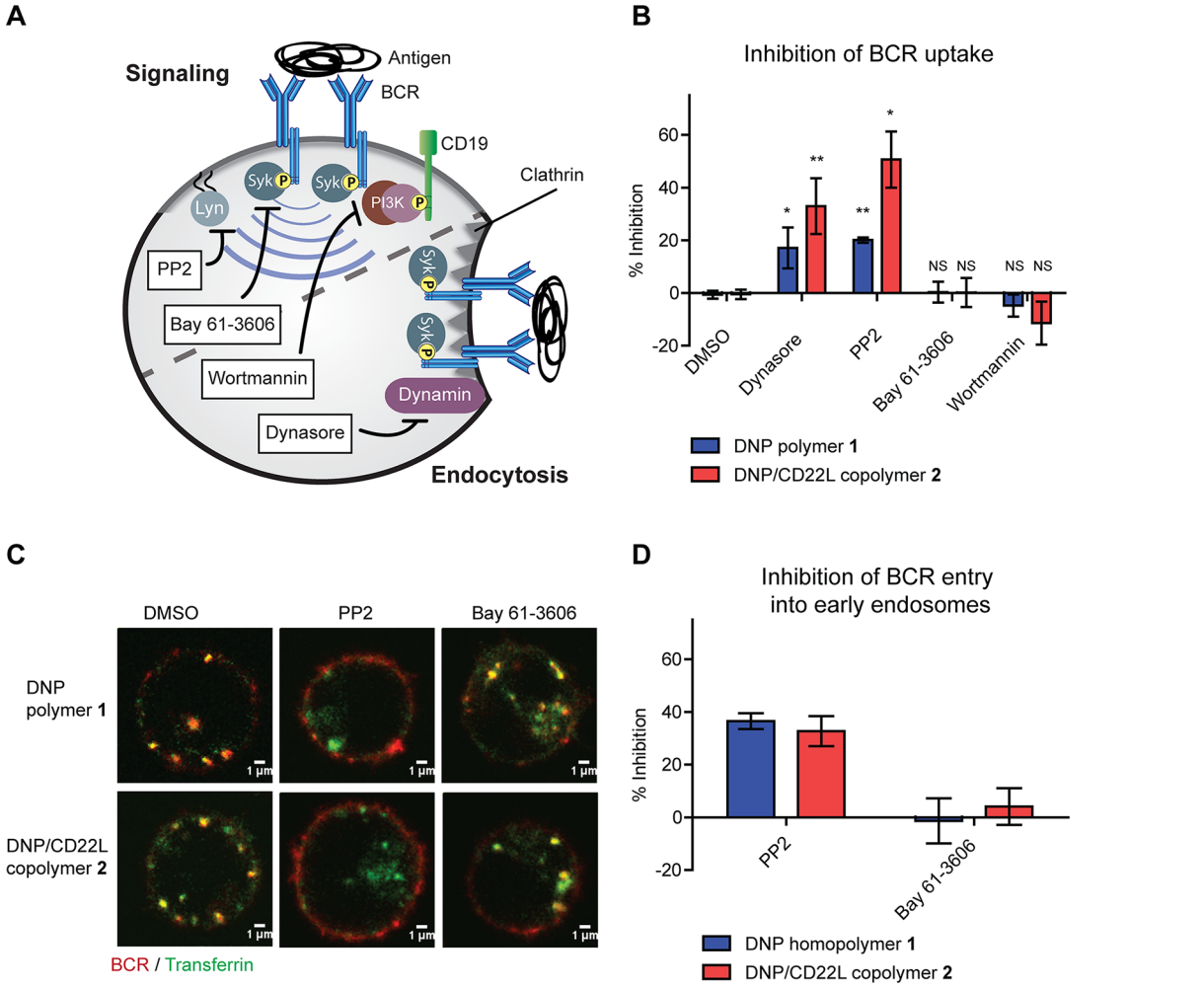


Figure 5. Evaluation of the dual nature of receptor-mediated BCR endocytosis and signaling. (A) Small molecules were used to inhibit proteins implicated in BCR signaling and endocytosis: Src-family kinases, such as Lyn (PP2), Syk kinase (bay 61−3606), PI3K (wortmannin), and dynamin (dynasore). (B) A20HL cells were exposed to small molecule inhibitors for 15 min prior to treatment with DNP homopolymer **1** or DNP/CD22L copolymer **2**: Percent inhibition of BCR uptake after 10 min following antigen stimulation at 5 μM DNP. (C) BCR and early endosome colocalization analysis in inhibitor treated cells. Representive images of inhibitor treated cells following stimulation with **1** or **2**. Cells were stained with anti-BCR Fab fragments (red) and transferrin (green). (D) Percent inhibition of BCR trafficking to early endosomes. All Error bars represent ±1 standard deviation from the mean of three independent experiments. Scale bar, 1 μm. *P < 0.02 and **P < 0.01 relative to DMSO control (Student's t-test). The abbreviation NS denotes a result that is not statistically significant (P > 0.05).

BCR and transferrin colocalization was quantified for individual cells (n > 30) at each time interval (Figure 4B). By 15 minutes, the increase in BCR accumulation within early endosomes in response to synthetic antigens is dramatic. Similar BCR accumulation within early endosomes was observed with treatment of either **1** or **2** at a concentration of 0.5 μM DNP (Supporting Figure S3, Supporting Information). Thus, the rapid loss of BCR from the cell surface following stimulation with either stimulatory or inhibitory antigens results in BCR entry into the endocytic network.

B cells mobilize different signaling components in response to treatment with activating polymer **1** versus inhibitory copolymer **2**. We probed the role of these components using small molecule inhibitors (Figure 5A). One protein that is critical for endocytosis is the GTPase dynamin, which is required for scission of endocytic vesicles during either caveolin- or clathrin-mediated endocytosis. BCR endocytosis has been reported to be predominately mediated by a clathrin-dependent pathway and therefore should be sensitive to dynamin inhibition.[7] We evaluated the ability of dynasore, a dynamin inhibitor, to prevent BCR uptake.[36] When cells were treated with either polymer **1** or copolymer **2**, dynasore inhibited BCR uptake (Figure 5B).

Copolymer **2** prevents Syk phosphorylation yet promotes BCR endocytosis. These data suggest that Syk activity is dispensable for BCR endocytosis.[21] Because of the critical role of Syk in BCR signaling,[26,27] we evaluated BCR endocytosis in the presence of an inhibitor of Syk activity, bay 61-3606.[37] Treatment with bay 61-3606 potently inhibited Syk signaling, abolishing Erk phosphorylation following BCR engagement (Supporting Figure S4A, Supporting Information). The inhibitor had no effect on BCR uptake in antigen-treated B cells (Figure 5B), which is consistent with Syk activity being dispensable. We also assessed the ability of bay 61-3606 to disrupt BCR entry into early endosomes and found that it had no effect (Figures 5C,D). These experiments are consistent with the conclusion that Syk activity is not required for BCR endocytosis.

When Syk is activated, it cooperates with phosphatidylinositol kinase (PI3K),[38] both the tyrosine kinase and the lipid

dx.doi.org/10.1021/cb400532y | ACS Chem. Biol. 2014, 9, 202−210

kinase are components of the B cell signalosome. Downstream of these components is BAM32, an adaptor implicated in BCR endocytosis that translocates in response to BCR engagement. Specifically, when the action of PI3K generates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) the ability of BAM32 to bind this phospholipid recruits the adaptor to the plasma membrane.[39] Although Syk activity appears to be dispensable for endocytosis, inhibition of PI3K should block BAM32 recruitment and therefore limit the ability of BAM32 to facilitate BCR endocytosis.[12] We added the PI3K inhibitor wortmannin[40] to cells treated with either polymer **1** or copolymer **2**. Although wortmannin potently inhibited PI3K signaling (Supporting Figure S4B, Supporting Information), no change in BCR endocytosis was observed. These data indicate that if BAM32 is critical for BCR uptake, it is not through activation of PI3K or Syk (Figure 5B).

The Src family kinase Lyn is activated in both homopolymer **1** and copolymer **2** treated cells.[21] It catalyzes the phosphorylation of the BCR complex as well as a variety of co-receptors including CD22.[41] Lyn activity has been suggested to be a requirement for BCR endocytosis.[11] We therefore tested the effects of the Src family kinase inhibitor PP2[42] on BCR uptake under either stimulatory or inhibitory conditions. The addition of this inhibitor diminished BCR uptake in either polymer **1** or copolymer **2** treated cells (Figure 5B). When we evaluated the ability of the BCR to enter early endosomes, the majority of the BCR was found on the cell surface (Figures 5C,D).

DISCUSSION

Our data indicate that chemically defined antigens that give rise to distinct signaling outputs are powerful probes of the relationship between BCR signaling and endocytosis. Kinetic profiles of BCR uptake obtained using our synthetic antigens reveal rate differences (Figure 2). Antigens that give rise to inhibitory signaling events trigger rapid endocytosis of the BCR independent of antigen concentration. The rapid BCR uptake upon treatment with copolymer **2** suggests that endocytosis of the BCR upon coligation with CD22 promotes rapid internalization, which should downregulate BCR signaling. Compared with antigens that bind only the BCR, antigens that cocluster the stimulatory co-receptor CD21/19 can extend the duration of the BCR on the cell surface.[43,44] Our findings suggest co-receptors may play an important role in regulating BCR endocytosis: Inhibitory antigens trigger rapid BCR internalization. In contrast, stimulatory antigens can extend the duration of the BCR on the cell surface for sustained signaling.

In addition to examining how co-receptor recruitment influences BCR endocytosis, we also examined the role of antigen concentration. The rate of BCR uptake in response to stimulatory antigen **1** depends on its concentration (Figure 2). At high homopolymer **1** concentration BCR uptake is rapid, while it is slower at lower antigen concentration. At this stimulatory antigen concentration, BCR signaling components are recruited rapidly, and receptor uptake also occurs rapidly. In contrast, we postulate that at lower stimulatory antigen concentrations nucleation of a BCR signaling complex is slower. As a result of this bottleneck, the rate of receptor uptake is reduced. In the case of copolymer **2**, which does not activate Syk or PLCγ2, no signaling complex should form; therefore, the rate of BCR uptake is largely independent of antigen concentration.

Our finding that the rate of BCR uptake is sensitive to engagement of inhibitory co-receptors is consistent with a model in which BCR endocytosis is regulated by the composition of the BCR signaling complex (Figures 1 and 2B). This composition is altered when negative effectors such as the phosphatase SHP-1 are recruited by inhibitory co-receptors. Several early BCR signaling events have been reported to be required for endocytosis,[11,12,25] but conflicting evidence also has been presented.[19] Both our stimulatory antigen **1** and inhibitory antigen **2** activate the Src family kinase Lyn[21] and trigger BCR endocytosis (Figures 2 and 3). Accordingly, BCR uptake in cells treated with the Src family kinase inhibitor PP2 was inhibited for both polymers **1** and **2** (Figure 5). Previous studies revealed a requirement for Src family kinase activity for the uptake of stimulatory antigens[11,12,25] and we found this activity is also required for inhibitory antigen uptake (Figure 5). Thus, Src family kinases, including Lyn, not only catalyze the phosphorylation of both positive and negative regulators of BCR signaling,[41] they also are critical for BCR endocytosis under both stimulatory and inhibitory conditions (Figure 5).

Phosphorylation of the BCR complex recruits and activates the tyrosine kinase Syk, an essential mediator of BCR signaling.[1,2] Data have been reported that suggest that Syk kinase activation is not required for endocytosis.[25] Our findings are consistent with this conclusion that Syk activity is not required for initial BCR uptake. Specifically, Syk activation is suppressed in cells treated with copolymer **2**,[21] yet BCR uptake occurs (Figures 2 and 3). Moreover, inhibition of Syk activity using the small molecule inhibitor bay 61-3606 has no apparent effect on BCR entry into early endosomes. Similarly, inhibition of PI3K by wortmannin has no effect on BCR uptake (Figure 5). It has been reported that Syk activation and Syk interaction with the ubiquitin ligase c-Cbl are required for successful antigen trafficking and presentation.[25,45] Together, the available data indicate that the antigen processing is regulated through steps that occur after BCR endocytosis. The evidence suggests that the fate of BCR–antigen complexes is controlled at the level of BCR trafficking.[25,45]

The capacity of both inhibitory and stimulatory antigens to trigger BCR endocytosis highlights the potential impact of BCR uptake in regulating signaling. The ability of inhibitory antigens to reduce the level of cell surface BCR should diminish the B cell's capacity to respond to subsequent antigenic stimuli.[46] In vivo, when naive B cells within a mature polyclonal repertoire encounter self-antigen, they decrease the concentration of cell surface BCR and with it, B cell signaling.[47] There is evidence to suggest that CD22 plays a critical role in controlling B cell reactivity toward self-antigens as deletion of CD22 contributes to spontaneous autoimmunity in mice.[48,49] Additionally, when mice are exposed to inhibitory antigens that display ligands to CD22, they become less responsive to subsequent stimulatory antigen exposure.[22] Our results indicate that antigens that bind the inhibitory co-receptor CD22, and therefore suppress B cell activation,[21] trigger robust BCR uptake. These results suggest that B cells can discriminate antigens through BCR signaling, while exploiting endocytosis to regulate cell surface BCR levels. What emerges is a model where exposure of B cells to stimulatory antigen can increase the duration of the BCR on the cell surface to support sustained signaling, whereas inhibitory antigens rapidly decrease cell surface BCR levels to reduce signaling and increase the threshold for activation to subsequent antigen encounter.

ROMP-derived polymers have been utilized extensively as probes of cell surface receptor clustering and signaling,[50] yet their influence on the endocytosis of these receptors has been largely unexplored. Synthetic antigens generated using ROMP can be used to probe the relationship between receptor engagement and endocytosis. These findings have implications for the design of synthetic vaccines, targeting agents, and strategies to suppress autoimmune responses.

MATERIALS AND METHODS

Cell Culture. A20/2J cells stably transfected with DNP/TNP-specific mIgM[31,51] to generate the A20/2J HL_{TNP} cell line were provided by A. Ochi (University Health Network, Toronto, ON, Canada). A20/2J 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.

Assessing Surface BCR. A20HL cells were suspended in 1% BSA, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ PBS (pH 7.4) at 37 °C at ~2×10^6 cells mL^{-1}. Cells were incubated at 37 °C for 5 min before stimulation with polymeric antigens. Polymers were added followed by gentle mixing with aliquots removed at discrete time points. Aliquots were placed in prechilled tubes on ice to prevent further endocytosis. Aliquoted A20HL cells were labeled on ice with 1.3 μg mL^{-1} anti-IgM μ-chain specific Fab fragment DyLight 649 conjugate (Jackson Immunoresearch) for 45 min. These Fab fragments do not compete for antigen binding and are monovalent, ensuring that BCR clustering does not occur. Cells were then rinsed twice using ice cold 0.1% BSA PBS before analysis by flow cytometry using a FACScaliber (BD Biosciences) to determine the extent of BCR labeling. For each data point, 20000 events were collected. Data analysis was performed using the FlowJo software suite. Relative surface BCR was determined using aliquots of unstimulated cells as a reference and plotted versus time. For screening inhibitors, BSA was reduced to 0.1% to reduce inhibitor sequestration.

Microscopy. A20HL cells were resuspended at 1.5×10^6 cells/mL in 1% BSA RPMI 1640 and incubated for 30 min with 8.8 μg/mL Alexa Fluor 488 conjugated transferrin (Jackson ImmunoResearch) at 37 °C. Cells were pelleted and resuspended on ice using the previous buffer, prechilled. Cell surface BCR was labeled using 15 μg/mL goat anti-mouse IgM μ-chain specific Fab fragments (Jackson ImmunoResearch) for 20 min on ice. Cells were then pelleted and resuspended in PBS, pH 7.4, supplemented with 1% BSA, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ at 37 °C. Cells were then treated with synthetic antigens and samples were placed on ice at desired time intervals. Hoechst 33348 (Life Technologies) was added to label nuclei. Cells were visualized in no. 1.5 borosilicate four-well chambered coverglass (Nunc). Images were collected on a Nikon A1R confocal microscope using a 100× objective. Images were analyzed using ImageJ software (National Institutes of Health, NIH), and the Pearson's coefficient was calculated for individual cells using the colocalization threshold plugin. Images were despeckled to remove noise.

Small Molecule Inhibitors. Inhibitors were obtained from Calbiochem and used at concentrations previously reported to provide robust inhibition according to the manufacturer's instructions. Inhibitors were utilized at the following concentrations: dynasore 80 μM, nystatin 27 μM, PP2 10 μM, bay 61-3606 2 μM, and wortmannin 0.5 μM. Inhibitors were added to cells in PBS, pH 7.4, at 37 °C prior to antigen stimulation. Cells were stimulated in PBS, pH 7.4, supplemented with 1% BSA, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ at 37 °C and analyzed. For flow cytometry analysis, cells were gated using propidium iodide to assess cell viability.

Polymer Synthesis. Antigenic polymers were prepared as previously described.[24] 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 with a solution of 9:1 ether/benzene and centrifuged. The supernatant was decanted, and the remaining polymer was dried to afford an off-white solid.

CD22L Synthesis. The CD22 ligand trisaccharide was synthesized using a modification of the enzymatic protocol reported by Yu et al.[52] Briefly, a two-enzyme system consisting of CMP sialic acid synthetase and α-2,6-sialyltransferase were added to a buffered solution (pH 8.6) of 3-azidopropyl lactoside, sialic acid, and CTP and incubated at RT. After 12 h, ethanol was added to precipitate proteins, and the product was isolated by reverse-phase HPLC on a C18 column with a mobile phase of 0.1% formic acid, 2% acetonitrile, and 97.9% water. The product was further purified by ion-exchange chromatography on a basic resin (Amberlyst, A26(OH)), eluting with water followed by 2% formic acid solution. The azide group was then reduced to an amine and conjugated to the polymer backbone.[23]

Polymer Functionalization. Polymers displaying succinimidyl esters (1 equiv) were functionalized with DNP-lysine (0.4 equiv; Sigma Aldrich) in DMSO containing *N*-methyl morpholine (5 equiv) to generate homopolymers. Synthesis of the aminopropyl linked trisaccharide ligand (CD22L) is described above.[23] The copolymer was prepared by exposing polymer (1 equiv) to DNP-lysine (0.40 equiv) followed by CD22L (0.3 equiv) in the presence of *N*-methyl morpholine (5 equiv) as with homopolymer. The conjugation reactions were quenched with an excess of ethanolamine (5 equiv) to react with any remaining succinimidyl esters. Polymers **1** and **2** were purified by size-exclusion chromatography (PD-10, GE Healthcare). Aqueous polymer was then lyophilized, and functionalization was determined by ^{1}H NMR. Polymer stocks were diluted to 10 mM DNP based on weight of lyophilized solids and confirmed by absorbance of DNP at 355 nm.

ASSOCIATED CONTENT

Supporting Information

A description of the polymers employed, a summary of the BCR endocytosis assay, fluorescence micrographs showing the localization of the BCR and transferrin in presence of different polymers, effect of polymers in the presence of kinase inhibitors on markers of B cell activation, procedures for immunoblots, and a listing of antibodies employed. This material is available free of charge via the Internet at http://pubs/acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: kiessling@chem.wisc.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The A20.2J HL_{TNP} cell line was a generous gift of A. Ochi. We thank R. Sheridan, D. Wesener, and L. Rodenkirch for helpful discussions. We also thank the UWCCC Flow Cytometry facility, Keck Laboratory for Biological Imaging, and the Martin Group for use of equipment and assistance. This work was supported by National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Grant R01-AI055258. N.R.B. acknowledges support from the NIGMS-supported Biotechnology Training Program (Grant T32 GM008349).

REFERENCES

(1) Kurosaki, T., Johnson, S. A., Pao, L., Sada, K., Yamamura, H., and Cambier, J. C. (1995) Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. *J. Exp. Med. 182*, 1815−1823.

(2) Rowley, R. B., Burkhardt, A. L., Chao, H. G., Matsueda, G. R., and Bolen, J. B. (1995) Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Ig alpha/Ig beta immunoreceptor tyrosine

activation motif binding and autophosphorylation. *J. Biol. Chem. 270*, 11590−11594.
(3) Feske, S. (2007) Calcium signalling in lymphocyte activation and disease. *Nat. Rev. Immunol. 7*, 690−702.
(4) Cheng, P. C., Dykstra, M. L., Mitchell, R. N., and Pierce, S. K. (1999) A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. *J. Exp. Med. 190*, 1549−1560.
(5) Cheng, P. C., Steele, C. R., Gu, L., Song, W., and Pierce, S. K. (1999) MHC class II antigen processing in B cells: accelerated intracellular targeting of antigens. *J. Immunol. 162*, 7171−7180.
(6) Aluvihare, V. R., Khamlichi, A. A., Williams, G. T., Adorini, L., and Neuberger, M. S. (1997) Acceleration of intracellular targeting of antigen by the B-cell antigen receptor: Importance depends on the nature of the antigen-antibody interaction. *EMBO J. 16*, 3553−3562.
(7) Stoddart, A., Jackson, A. P., and Brodsky, F. M. (2005) Plasticity of B cell receptor internalization upon conditional depletion of clathrin. *Mol. Biol. Cell 16*, 2339−2348.
(8) Chaturvedi, A., Martz, R., Dorward, D., Waisberg, M., and Pierce, S. K. (2011) Endocytosed BCRs sequentially regulate MAPK and Akt signaling pathways from intracellular compartments. *Nat. Immunol. 12*, 1119−1126.
(9) Vascotto, F., Le Roux, D., Lankar, D., Faure-Andre, G., Vargas, P., Guermonprez, P., and Lennon-Dumenil, A. M. (2007) Antigen presentation by B lymphocytes: How receptor signaling directs membrane trafficking. *Curr. Opin. Immunol. 19*, 93−98.
(10) Mitchison, N. A. (2004) T-cell-B-cell cooperation. *Nat. Rev. Immunol. 4*, 308−312.
(11) Stoddart, A., Dykstra, M. L., Brown, B. K., Song, W., Pierce, S. K., and Brodsky, F. M. (2002) Lipid rafts unite signaling cascades with clathrin to regulate BCR internalization. *Immunity 17*, 451−462.
(12) Niiro, H., Allam, A., Stoddart, A., Brodsky, F. M., Marshall, A. J., and Clark, E. A. (2004) The B lymphocyte adaptor molecule of 32 kDas (Bam32) regulates B cell antigen receptor internalization. *J. Immunol. 173*, 5601−5609.
(13) Malhotra, S., Kovats, S., Zhang, W., and Coggeshall, K. M. (2009) Vav and Rac activation in B cell antigen receptor endocytosis involves Vav recruitment to the adapter protein LAB. *J. Biol. Chem. 284*, 36202−36212.
(14) Siemasko, K., Skaggs, B. J., Kabak, S., Williamson, E., Brown, B. K., Song, W., and Clark, M. R. (2002) Receptor-facilitated antigen presentation requires the recruitment of B cell linker protein to Igalpha. *J. Immunol. 168*, 2127−2138.
(15) Siemasko, K., Eisfelder, B. J., Stebbins, C., Kabak, S., Sant, A. J., Song, W., and Clark, M. R. (1999) Ig alpha and Ig beta are required for efficient trafficking to late endosomes and to enhance antigen presentation. *J. Immunol. 162*, 6518−6525.
(16) Malhotra, S., Kovats, S., Zhang, W., and Coggeshall, K. M. (2009) B cell antigen receptor endocytosis and antigen presentation to T cells require Vav and dynamin. *J. Biol. Chem. 284*, 24088−24097.
(17) Mutch, C. M., Sanyal, R., Unruh, T. L., Grigoriou, L., Zhu, M., Zhang, W., and Deans, J. P. (2007) Activation-induced endocytosis of the raft-associated transmembrane adaptor protein LAB/NTAL in B lymphocytes: evidence for a role in internalization of the B cell receptor. *Int. Immunol. 19*, 19−30.
(18) Ishiai, M., Kurosaki, M., Pappu, R., Okawa, K., Ronko, I., Fu, C., Shibata, M., Iwamatsu, A., Chan, A. C., and Kurosaki, T. (1999) BLNK required for coupling Syk to PLC gamma 2 and Rac1-JNK in B cells. *Immunity 10*, 117−125.
(19) Caballero, A., Katkere, B., Wen, X. Y., Drake, L., Nashar, T. O., and Drake, J. R. (2006) Functional and structural requirements for the internalization of distinct BCR-ligand complexes. *Eur. J. Immunol. 36*, 3131−3145.
(20) Thyagarajan, R., Arunkumar, N., and Song, W. (2003) Polyvalent antigens stabilize B cell antigen receptor surface signaling microdomains. *J. Immunol. 170*, 6099−6106.
(21) Courtney, A. H., Puffer, E. B., Pontrello, J. K., Yang, Z. Q., and Kiessling, L. L. (2009) Sialylated multivalent antigens engage CD22 in trans and inhibit B cell activation. *Proc. Natl. Acad. Sci. U. S. A. 106*, 2500−2505.
(22) Duong, B. H., Tian, H., Ota, T., Completo, G., Han, S., Vela, J. L., Ota, M., Kubitz, M., Bovin, N., Paulson, J., and Nemazee, D. (2010) Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo. *J. Exp. Med. 207* (173−187), S171−174.
(23) Yang, Z. Q., Puffer, E. B., Pontrello, J. K., and Kiessling, L. L. (2002) Synthesis of a multivalent display of a CD22-binding trisaccharide. *Carbohydr. Res. 337*, 1605−1613.
(24) Puffer, E. B., Pontrello, J. K., Hollenbeck, J. J., Kink, J. A., and Kiessling, L. L. (2007) Activating B cell signaling with defined multivalent ligands. *ACS Chem. Biol. 2*, 252−262.
(25) Le Roux, D., Lankar, D., Yuseff, M. I., Vascotto, F., Yokozeki, T., Faure-Andre, G., Mougneau, E., Glaichenhaus, N., Manoury, B., Bonnerot, C., and Lennon-Dumenil, A. M. (2007) Syk-dependent actin dynamics regulate endocytic trafficking and processing of antigens internalized through the B-cell receptor. *Mol. Biol. Cell 18*, 3451−3462.
(26) Cheng, A. M., Rowley, B., Pao, W., Hayday, A., Bolen, J. B., and Pawson, T. (1995) Syk tyrosine kinase required for mouse viability and B-cell development. *Nature 378*, 303−306.
(27) Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L., and Tybulewicz, V. L. (1995) Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature 378*, 298−302.
(28) Bielawski, C. W., and Grubbs, R. H. (2007) Living ring-opening metathesis polymerization. *Prog. Polym. Sci. 32*, 1−29.
(29) Lee, Y., and Sampson, N. S. (2006) Romping the cellular landscape: Linear scaffolds for molecular recognition. *Curr. Opin. Struct. Biol. 16*, 544−550.
(30) Schrock, R. R. (2005) High oxidation state alkylidene and alkylidyne complexes. *Chem. Commun.*, 2773−2777.
(31) Watanabe, M., Wegmann, D. R., Ochi, A., and Hozumi, N. (1986) Antigen presentation by a B-cell line transfected with cloned immunoglobulin heavy- and light-chain genes specific for a defined hapten. *Proc. Natl. Acad. Sci. U. S. A. 83*, 5247−5251.
(32) Doody, G. M., Justement, L. B., Delibrias, C. C., Matthews, R. J., Lin, J., Thomas, M. L., and Fearon, D. T. (1995) A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. *Science 269*, 242−244.
(33) Bryant, P., and Ploegh, H. (2004) Class II MHC peptide loading by the professionals. *Curr. Opin. Immunol. 16*, 96−102.
(34) Siemasko, K., and Clark, M. R. (2001) The control and facilitation of MHC class II antigen processing by the BCR. *Curr. Opin. Immunol. 13*, 32−36.
(35) Maxfield, F. R., and McGraw, T. E. (2004) Endocytic recycling. *Nat. Rev. Mol. Cell Biol. 5*, 121−132.
(36) Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C., and Kirchhausen, T. (2006) Dynasore, a cell-permeable inhibitor of dynamin. *Dev. Cell 10*, 839−850.
(37) Yamamoto, N., Takeshita, K., Shichijo, M., Kokubo, T., Sato, M., Nakashima, K., Ishimori, M., Nagai, H., Li, Y. F., Yura, T., and Bacon, K. B. (2003) The orally available spleen tyrosine kinase inhibitor 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide dihydrochloride (BAY 61-3606) blocks antigen-induced airway inflammation in rodents. *J. Pharmacol. Exp. Ther. 306*, 1174−1181.
(38) Beitz, L. O., Fruman, D. A., Kurosaki, T., Cantley, L. C., and Scharenberg, A. M. (1999) SYK is upstream of phosphoinositide 3-kinase in B cell receptor signaling. *J. Biol. Chem. 274*, 32662−32666.
(39) Marshall, A. J., Niiro, H., Lerner, C. G., Yun, T. J., Thomas, S., Disteche, C. M., and Clark, E. A. (2000) A novel B lymphocyte-associated adaptor protein, Bam32, regulates antigen receptor signaling downstream of phosphatidylinositol 3-kinase. *J. Exp. Med. 191*, 1319−1332.
(40) Powis, G., Bonjouklian, R., Berggren, M. M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W. F., Dodge, J., Grindey, G., and Vlahos, C. J. (1994) Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Res. 54*, 2419−2423.

(41) Cornall, R. J., Cyster, J. G., Hibbs, M. L., Dunn, A. R., Otipoby, K. L., Clark, E. A., and Goodnow, C. C. (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.
(42) Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A., and Connelly, P. A. (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J. Biol. Chem. 271*, 695−701.
(43) Cherukuri, A., Cheng, P. C., and Pierce, S. K. (2001) The role of the CD19/CD21 complex in B cell processing and presentation of complement-tagged antigens. *J. Immunol. 167*, 163−172.
(44) Cherukuri, A., Cheng, P. C., Sohn, H. W., and Pierce, S. K. (2001) The CD19/CD21 complex functions to prolong B cell antigen receptor signaling from lipid rafts. *Immunity 14*, 169−179.
(45) Katkere, B., Rosa, S., and Drake, J. R. (2012) The Syk-binding Ubiquitin Ligase c-Cbl Mediates Signaling-dependent B Cell Receptor Ubiquitination and B Cell Receptor-mediated Antigen Processing and Presentation. *J. Biol. Chem. 287*, 16636−16644.
(46) O'Neill, S. K., Veselits, M. L., Zhang, M., Labno, C., Cao, Y., Finnegan, A., Uccellini, M., Alegre, M. L., Cambier, J. C., and Clark, M. R. (2009) Endocytic sequestration of the B cell antigen receptor and toll-like receptor 9 in anergic cells. *Proc. Natl. Acad. Sci. U. S. A. 106*, 6262−6267.
(47) Zikherman, J., Parameswaran, R., and Weiss, A. (2012) Endogenous antigen tunes the responsiveness of naive B cells but not T cells. *Nature 489*, 160−U185.
(48) Jellusova, J., Wellmann, U., Amann, K., Winkler, T. H., and Nitschke, L. (2010) CD22 x Siglec-G double-deficient mice have massively increased B1 cell numbers and develop systemic autoimmunity. *J. Immunol. 184*, 3618−3627.
(49) O'Keefe, T. L., Williams, G. T., Batista, F. D., and Neuberger, M. S. (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.
(50) Kiessling, L. L., Gestwicki, J. E., and Strong, L. E. (2006) Synthetic multivalent ligands as probes of signal transduction. *Angew. Chem., Int. Ed. 45*, 2348−2368.
(51) Sato, K., and Ochi, A. (1998) Inhibition of B-cell receptor-antigen complex internalization by FcgammaRIIB1 signals. *Immunol. Lett. 61*, 135−143.
(52) Yu, H., Chokhawala, H. A., Huang, S., and Chen, X. (2006) One-pot three-enzyme chemoenzymatic approach to the synthesis of sialosides containing natural and non-natural functionalities. *Nat. Protoc. 1*, 2485−2492.