ACS chemical biology

Articles

pubs.acs.org/acschemicalbiology

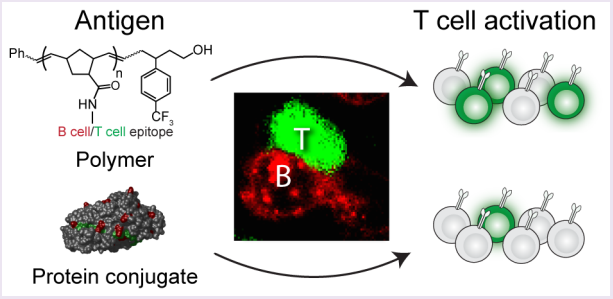
# Multivalent Antigens for Promoting B and T Cell Activation

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

[†]Department of Chemistry, [‡]Department of Biochemistry, University of Wisconsin−Madison, Madison, Wisconsin 53706, United States

Supporting Information

**ABSTRACT:** Efficacious vaccines require antigens that elicit productive immune system activation. Antigens that afford robust antibody production activate both B and T cells. Elucidating the antigen properties that enhance B−T cell communication is difficult with traditional antigens. We therefore used ring-opening metathesis polymerization to access chemically defined, multivalent antigens containing both B and T cell epitopes to explore how antigen structure impacts B cell and T cell activation and communication. The bifunctional antigens were designed so that the backbone substitution level of each antigenic epitope could be quantified using $^{19}$F NMR. The T cell peptide epitope was appended so that it could be liberated in B cells via the action of the endosomal protease cathepsin D, and this design feature was critical for T cell activation. Antigens with high BCR epitope valency induce greater BCR-mediated internalization and T cell activation than did low valency antigens, and these high-valency polymeric antigens were superior to protein antigens. We anticipate that these findings can guide the design of more effective vaccines.






Vaccines are needed to prevent infectious disease caused by HIV, tuberculosis, and other pathogens recalcitrant to traditional strategies. This demand is driving advances in our understanding of the immune system and new approaches to antigen design. Most successful vaccines require production of neutralizing antibodies.[1,2] Robust antibody responses, characterized by high-affinity antibodies and immunological memory, are typically triggered by T cell-dependent antigens, agents that contain both B and T cell epitopes.[3] Such antigens are recognized and processed by antigen-specific B cells to provide peptide epitopes that are presented to CD4$^+$ helper T cells.[4,5] Direct contact with T cells provides signals that promote B cell activation. Accordingly, the structural features of the antigen that promote B−T cell communication must be identified.

The activation of T cells by antigen-presenting B cells involves multiple steps (Figure 1).[6] B cells recognize antigen through the B cell receptor (BCR), a membrane-bound antibody that is complexed to an intracellular signaling domain. Multivalent interactions promote BCR clustering and signaling and facilitate receptor-mediated internalization of antigen. Internalized antigen is processed by endosomal proteases to release peptides that can be loaded onto major histocompatibility complex type II (MHCII) molecules. Peptide−MHCIIs are shuttled to the cell surface, and T cells scan the B cell surface until the T cell receptor (TCR) recognizes a cognate peptide−MHCII complex. Direct B−T cell contact allows bidirectional signaling that promotes B cell proliferation and differentiation.[7−9] For a B cell to effectively recruit T cell help, antigen must engage the BCR and trigger the cascade of events that results in presentation.

Antigen features, such as epitope affinity, valency, or coreceptor recruitment, can impact B or T cell signaling.[10−16] Signaling by B and T lymphocytes is closely linked: the antigen−BCR interactions that trigger B cell signaling and antigen uptake are necessary for downstream T cell signaling. Despite this connection, the influence of antigen on immune signaling is typically examined solely in B cells or solely in T cells but not in tandem. To determine which antigen structural features impact B−T cell communication, antigens are required that can engage the BCR and undergo processing and presentation such that they lead to T cell activation. Protein conjugates are typically employed, but they have limitations: features such as the valency of B and T cell epitopes are difficult to control or modify. Incisive identification of antigen features that enhance presentation and T cell activation requires defined antigens that can be readily manipulated.

The advent of controlled polymerization reactions has opened new opportunities to explore biological processes that benefit from multivalency.[17,18] Immune signaling pathways are excellent testing grounds, as knowledge of how antigen properties influence output responses can guide the design of effective tolerogens or vaccines. As tools to study immune cell responses, we reasoned that epitope-functionalized polymers could overcome the limitations of traditional protein antigens. We showed previously that polymers decorated with B cell epitopes can oligomerize the BCR and induce both signaling and uptake.[13,16,19,20] Using ring-opening metathesis polymer-

**Received:** April 3, 2015
**Accepted:** May 13, 2015
**Published:** May 13, 2015

DOI: 10.1021/acschembio.5b00239
*ACS Chem. Biol.* 2015, 10, 1817−1824

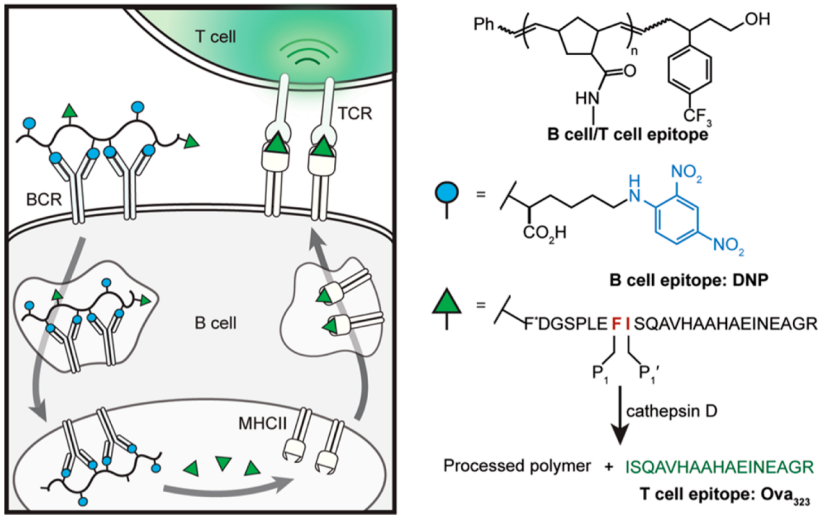



**Figure 1.** General attributes of bifunctional antigens. (Left) Events required for dual activation of B and T cells with a multivalent antigen. The bifunctional antigen must (a) engage and cluster the B cell receptor (BCR) to activate signaling and uptake, (b) undergo endosomal processing to release a T cell epitope for loading and presentation on MHCII, and (c) elicit T cell activation. (Right) General design of polymers generated by ROMP. Polymer backbones were functionalized with B cell epitope (DNP, blue) and a peptide epitope recognized by the T cell receptor ($Ova_{323}$, green). The T cell epitope was appended through a linker that can be cleaved by the endosomal protease cathepsin D. Cathepsin D-mediated cleavage should occur between the residues highlighted in red that occupy the $P_1$ and $P_1'$ sites of the protease. Full structures of the antigens used in this study are depicted in Figure 3.



ization (ROMP) to control key features of polymer structure, such as length and ligand conjugation, we examined how antigen valency influences B cell signaling[16] and how co-clustering of the BCR and the lectin CD22[13,19] attenuates BCR signaling and promotes endocytosis. We postulated that defined polymers could be functionalized with B and T cell epitopes to investigate how antigen properties influence downstream events, including B−T cell communication.

To this end, we synthesized bifunctional antigens equipped with a B cell epitope that binds the BCR and a peptide epitope that can be released from the polymer backbone for presentation to T cells (Figure 1). These antigens cluster the BCR to trigger signaling and receptor-mediated internalization. Upon antigen internalization, the T cell epitope is liberated by the endosomal protease cathepsin D and loaded onto MHCII complexes for display to cognate T cells. B cells treated with the bifunctional antigens promote T cell activation, which could be visualized directly. We used chemical synthesis to optimize antigen features to facilitate their uptake and processing by B cells, which ultimately elicits higher levels of T cell activation. Our results validate a strategy to access modular synthetic antigen capable of activating both B and T cells and provide insight into the parameters that regulate antigen presentation and B−T cell communication.

## RESULTS AND DISCUSSION

**Design and Synthesis of Dual-Activation Polymer.** We showed previously that ROMP can be used to prepare polymers displaying multiple copies of a B cell epitope and that the polymers function as antigens to activate BCR signaling.[13,16] Both short (25mer) and long (500mer) polymers activate signaling, but longer polymers are more potent. The ability to use ROMP to vary polymer length was valuable for elucidating the influence of BCR clustering on B cell signaling. Specifically, ROMP with a functional group-tolerant ruthenium carbene initiator facilitated the synthesis of polymers with a narrow molecular weight distribution and whose lengths were controlled by the monomer-to-initiator ratio (M:I).[21,22] The multivalent antigens were obtained by polymerizing a monomer bearing an *N*-hydroxy succinimidyl ester to generate a polymer backbone to which amine-substituted epitopes were appended.[23] The epitope density was controlled by altering the ratio of amine to activated succinimidyl ester.[23,24] Variations on this general approach have been used to assemble multivalent ligands that can cluster cell-surface receptors.[18,25,26] The modularity and reproducibility of this approach prompted its use to assemble antigens designed to mediate B−T cell communication.

The antigens were designed to exploit B cell and $CD4^+$ T cell lines capable of mutual recognition and synapse formation. The specificity of the antigen receptors present on each cell line dictated the epitopes that were affixed to the polymer backbone. The B cell line (A20.2J $HL_{TNP}$ denoted as A20HL) expresses a BCR that recognizes the 2,4-dinitrophenyl (DNP) hapten.[27,28] Accordingly, polymers bearing $N_\varepsilon$-DNP-L-Lys (DNP-Lys) activate signaling and should undergo receptor-mediated internalization and entry into the endocytic network. We employed the immunogenic peptide $Ova_{323-339}$ (denoted $Ova_{323}$), derived from chicken ovalbumin (Ova), as our T cell epitope. The $Ova_{323}$ peptide has good affinity for the MHCII I-$A^d$ haplotype expressed by A20HL cells,[29,30] and the presence of the $Ova_{323}$/MHCII I-$A^d$ complex can elicit activation of the $CD4^+$ T cell line (DO-11.10).[31,32] This system can reveal whether polymers functionalized with a DNP epitope and $Ova_{323}$ peptide can be used to activate B cells and T cells and thereby probe B−T cell communication.

To generate a B−T cell synapse, B cells must process the T cell epitope and load it into MHCIIs for presentation. As the antigen migrates through the endosomal network, it encounters conditions that are increasingly acidic, reducing, and proteolytic. Reduction-sensitive[33] or pH-sensitive[34] linkers have been used to release peptides within the endolysosomal compartment of dendritic cells. For B cell processing, we employed a linker that is protease-sensitive because we


DOI: 10.1021/acschembio.5b00239
*ACS Chem. Biol.* 2015, 10, 1817−1824

envisioned that it would have a number of advantages: the cleavage site can be readily installed, it should be stable in the absence of the protease, and its sequence can be altered to examine or exploit the roles of specific proteases. The endosomal processing of ovalbumin is primarily mediated by cathepsin D, an aspartyl protease located in the late endosome.[35,36] We therefore designed our polymers to possess a cathepsin D-specific cleavage site between the backbone and the $Ova_{323}$ epitope. The chosen linker sequences were guided by the reported substrate specificity of human cathepsin D.[37] We tested two 25mer peptides with murine cathepsin D. Each contained the $Ova_{323}$ epitope linked either to a sequence hypothesized to be protease-sensitive (**8**, S-$Ova_{323}$) or -insensitive (**9**, IS-$Ova_{323}$) (Supporting Information Figure S1). The products of protease treatment were monitored over time using MALDI-TOF. IS-$Ova_{323}$ peptide **9** was not a substrate, but cleavage of S-$Ova_{323}$ peptide **8** was complete by 15 min to yield the $Ova_{323}$ epitope (Supporting Information Figure S2). The susceptible sequence was cleaved only at the expected position. These results indicate that cathepsin D processes the S-$Ova_{323}$ peptide sequence efficiently and selectively. We therefore had the requisite features to devise polymer probes of B−T cell communication.

To compare B and T cell responses to different polymers, the relative epitope substitution levels were controlled and assessed. Our synthetic approach was designed so that the substitution levels of each pendant functional group could be determined. Specifically, the proportion of DNP-Lys conjugated to polymer was quantified by integration of distinct $^1$H NMR signals that arise from the BCR epitope and the backbone. The level of peptide substitution was difficult to judge because $Ova_{323}$ lacks signals in a nonoverlapping region of the spectrum. We therefore installed fluorinated functional groups on both the peptide and the polymer terminus such that the loading of conjugated $Ova_{323}$ peptide could be assessed by $^{19}$F NMR spectroscopy (Figure 2). We anticipate that this strategy can serve as a general means of determining the level of polymer substitution.

To implement our plan for ascertaining backbone functionalization levels, we needed to generate an appropriate enol ether capping agent to install a trifluoromethyl end group. This capping agent was synthesized from 4-(trifluoromethyl)-phenyl boronic acid and 5,6-dihydro-2*H*-pyran-2-one, which undergo a rhodium/(*S*)-Binap catalyzed reaction to generate $\beta$-aryl lactone **3** (Figure 2).[38] The ester was reduced to incipient aldehyde **4**, which was subjected to a Wittig reaction to afford enol ether **5** as a 1:1 mixture of *E*/*Z* isomers. This agent was used to terminate ROMP reactions of norbornene derivative **6** using ruthenium initiator **7** to afford antigen precursors **2a**−**c** (Figure 2).[21] Succinimidyl ester-substituted polymers in a range of lengths were generated, with the degree of polymerization (DP) controlled by the ratio of monomer to initiator. The resulting succinimidyl ester-substituted polymers **2a**−**c** were exposed to DNP-Lys and $Ova_{323}$ peptide **8** to generate multifunctional polymers bearing B and T cell epitopes (**1a**−**c**, Figure 3). For the T cell epitope, we incorporated a 2-trifluoromethyl-L-phenylalanine (2-$CF_3$-Phe) residue at the $Ova_{323}$ peptide N-terminus to serve as an NMR handle for quantification. Unreacted succinimidyl esters were converted to neutral functionality by treatment with excess ethanolamine. The DNP-substitution level was determined by $^1$H NMR, and $^{19}$F NMR spectroscopy was used to assess the relative amount of $Ova_{323}$ substitution. The epitope loading levels obtained

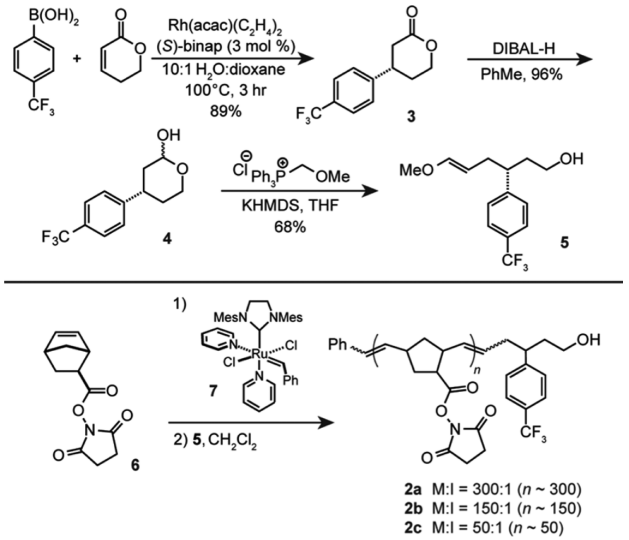



**Figure 2.** Synthetic scheme used to prepare polymeric antigens. (Top) Route employed to generate a capping agent bearing a fluorinated label. (Bottom) ROMP of succinimidyl ester-substituted norbornene **6** yielded polymers whose length was controlled by the ratio of monomer **6** to initiator **7**. The polymerization was terminated with capping agent **5** to install unique functional groups at the polymer terminus.

were predictable and consistent for polymers of different lengths. Specifically, polymers functionalized with 0.4 equiv of DNP-Lys had a substitution level of 37−40%, which is sufficient for eliciting strong BCR signals.[16] A substitution with 4−5% $Ova_{323}$ peptide **8** was selected to allow intracellular delivery of multiple $Ova_{323}$ copies per polymer molecule internalized, while minimizing the possibility that peptide substituents would interfere with epitope−BCR interactions.

**Antigen Valency Modulates BCR-Mediated Internalization.** We tested the ability of DNP/$Ova_{323}$-substituted polymers to activate BCR signaling, a requirement for efficient B cell antigen uptake. Using total tyrosine phosphorylation as a measure of B cell activation,[39,40] we compared BCR signals elicited by the DNP/$Ova_{323}$-substituted 300mer **1a**, 150mer **1b**, and 50mer **1c** to the DNP-substituted 150mer **3b**, which we have shown initiates strong BCR signaling.[16] Polymers functionalized with either DNP epitope alone or DNP and $Ova_{323}$ epitopes triggered an increase in phosphorylation, indicative of B cell activation (Supporting Information Figure S3). We specifically evaluated phosphorylation of PLC$\gamma$2, a critical mediator of B cell activation. Stimulation with either DNP-substituted polymer or DNP/$Ova_{323}$-substituted polymer produced a rapid increase in PLC$\gamma$2 phosphorylation (Supporting Information Figure S3). Together, these results indicate that the DNP/$Ova_{323}$-substituted polymers engage the BCR to activate signal transduction.

We next assessed the ability of DNP/$Ova_{323}$ polymers to induce BCR-mediated uptake. Longer polymer antigens induce greater BCR clustering,[16] suggesting that they would promote more BCR internalization. We compared cell-surface levels of DNP-specific BCR as a function of time after exposure to 300mer **1a**, 150mer **1b**, or 50mer **1c** (5 $\mu$M DNP) (Figure 4). The 300mer **1a** and 150mer **1b** resulted in rapid BCR internalization to similar extents; at 26 min, 65 ± 4% and 56 ± 5% of cell-surface BCR was internalized, respectively. In contrast, the shorter 50mer **1c** induced a much lower level of


DOI: 10.1021/acschembio.5b00239
*ACS Chem. Biol.* 2015, 10, 1817−1824

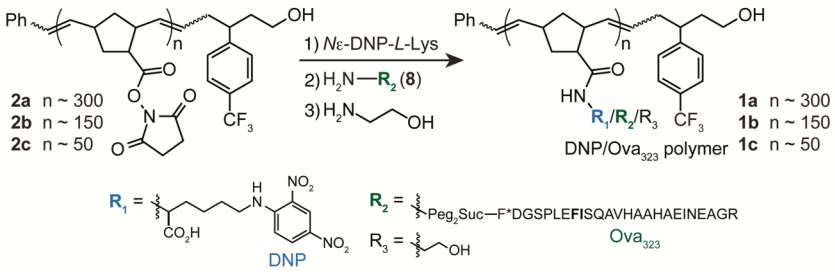



**Figure 3.** Synthetic route to polymers bearing B cell ($R_1$ = DNP, blue) and T cell epitopes ($R_2$ = $Ova_{323}$, green). Polymers of controlled length were functionalized with a DNP derivative (approximately 40% substitution) and $Ova_{323}$ peptide (approximately 4%). The 4% value was determined by comparing the integration of the signals arising from the fluorinated substituents on the polymer backbone and the peptide N-terminus (F* = 2-$CF_3$-Phe).

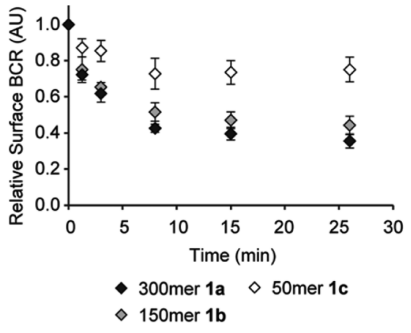



**Figure 4.** Effect of antigen valency on BCR-mediated uptake. A20HL B cells were treated with DNP/$Ova_{323}$ 300mer **1a**, 150mer **1b**, or 50mer **1c** (5 $\mu$M DNP) for distinct time points. Cell surface BCR was labeled with a fluorescent anti-BCR Fab, and fluorescence was measured by flow cytometry. Error bars represent ±1 standard deviation from the mean of three independent experiments.

internalization: after 26 min, only 25 ± 7% of cell-surface BCR was internalized. The concentration of 300mer that afforded maximal BCR uptake was approximately 6-fold lower than that required for the 50mer (0.04 vs 0.25 $\mu$M, respectively), and even at this lower concentration, the 300mer promoted more BCR internalization. These results indicate that BCR-mediated uptake is sensitive to antigen valency, such that high valency antigens promote increased levels of internalization.

**Polymer Delivers Peptide for Presentation and Elicits T Cell Activation.** We assessed the ability of DNP/$Ova_{323}$ polymers to deliver the $Ova_{323}$ epitope for presentation to T cells. When T cells encounter B cells presenting MHCII loaded with antigenic peptide, an immunological synapse can form wherein the TCR engages peptide−MHCII. Ligation of the TCR initiates a T cell signaling cascade that results in an increase in intracellular calcium.[41] Calcium signaling dramatically alters T cell gene expression, which leads to increased expression of cytokines, including IL-2. Accordingly, B cells were treated with bifunctional antigen, fixed, and co-cultured with T cells, and the amount of IL-2 produced was quantified by an enzyme-linked immunosorbent assay (ELISA). The DNP/$Ova_{323}$ 150mer **1b** promoted IL-2 production in a dose-dependent manner (Supporting Information Figure S4). These data suggest that the DNP/$Ova_{323}$ polymer is processed to generate functional $Ova_{323}$−MHCII complexes on the B cell surface. We verified that presentation of $Ova_{323}$−MHCII requires B cell uptake and processing by showing that fixed B cells treated with DNP/$Ova_{323}$-substituted polymer failed to elicit IL-2 production (Supporting Information Figure S5).

We measured IL-2 production in the presence of chloroquine, a small molecule inhibitor that disrupts endocytic function and prevents antigen processing.[42] As expected, chloroquine-treated cells did not secrete IL-2 (Supporting Information Figure S4), indicating blockage of polymer processing. We further tested whether polymer internalization and peptide presentation depend upon the BCR. BCR-mediated endocytosis is the most efficient mode of antigen capture for presentation, but B cells can also present antigen internalized through fluid-phase pinocytosis.[4,43] We therefore used A20 B cells that lack a DNP-specific BCR. In nonspecific B cells, IL-2 production was observed only with very high polymer doses. We therefore conducted subsequent experiments at a dose range that requires BCR-specific antigen uptake for presentation.

To assess the role of cathepsin D in processing of the polymer antigens, we compared IL-2 production elicited by 150mer polymers with peptides linked via sensitive (**1b**) or insensitive (**1d**) linkers (Figure 5). Bifunctional antigens with a cathepsin D-sensitive linkage (**1b**) elicited IL-2 production, whereas those with the insensitive linker (**1d**) did not (Figure 5, bottom left). These data suggest that the accelerated and

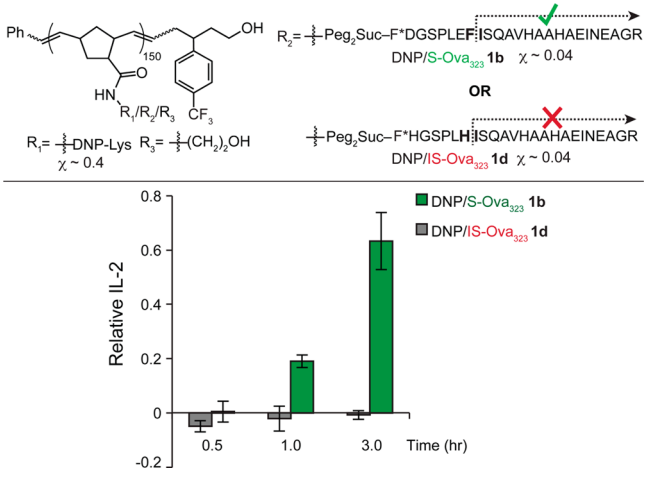



**Figure 5.** Role for the protease-sensitive linker in B cell presentation and T cell activation. (Top) Polymers ($n$ ~ 150) were functionalized with BCR ligand ($R_1$ = DNP) and T cell epitope bearing either a cathepsin D-sensitive linker ($R_2$ = S-$Ova_{323}$, green) or an insensitive linker ($R_2$ = IS-$Ova_{323}$, red). (Bottom) An ELISA was used to measure IL-2 production by DO-11.10 T cells in response to A20HL B cells that had been treated with either DNP/S-$Ova_{323}$ polymer **1b** or DNP/IS-$Ova_{323}$ polymer **1d** (0.4 $\mu$M $Ova_{323}$) for 0.5, 1, or 3 h.




DOI: 10.1021/acschembio.5b00239
ACS Chem. Biol. 2015, 10, 1817−1824

enhanced antigen presentation of the DNP/S-Ova$_{323}$ polymer depends on its cathepsin D-sensitive cleavage site.

**BCR Ligand Valency Modulates IL-2 Production by T Cells.** The increase in BCR internalization with polymer length (Figure 4) suggests that long polymers deliver more Ova$_{323}$ epitope within the cell, resulting in higher levels of peptide−MHCII presentation. Since T cell activation is sensitive to the concentration of peptide−MHCII displayed,[44] we hypothesized that stronger T cell activation would result from longer polymers. We tested this hypothesis by comparing the activities of the DNP/Ova$_{323}$ 300mer **1a**, 150mer **1b**, or 50mer **1c**. The data are consistent with this model: the longer 300mer and 150mer polymers induce more cytokine production than does the 50mer polymer (Figure 6A). This potency of the 300mer over 50mer is not due to differences in Ova$_{323}$ concentration (Figure 6A). These data are consistent with the model in which reduced uptake of the short polymer results in lower levels of intracellular peptide delivery.

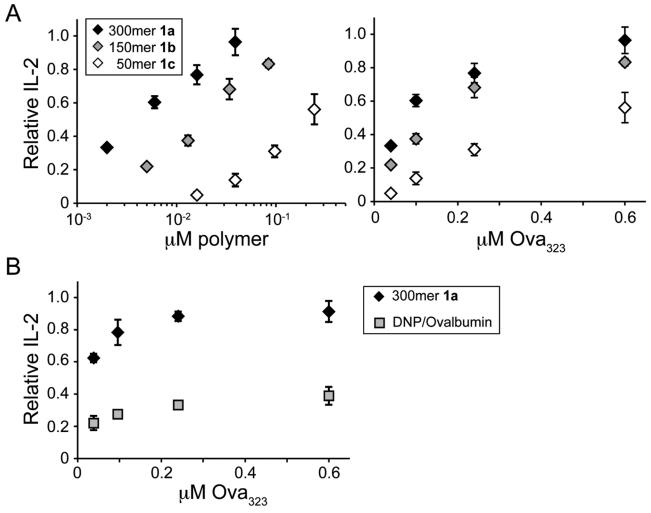



**Figure 6.** IL-2 production by T cells depends on antigen properties. (A) IL-2 produced by DO-11.10 T cells in response to B cells (A20HL) exposed to DNP/Ova$_{323}$-substituted 300mer **1a**, 150mer **1b**, or 50mer **1c** for 3 h. The response is indicated as a function of polymer concentration (left) and Ova$_{323}$ concentration (right). (B) IL-2 produced by T cells in response to B cells treated with either DNP/Ova$_{323}$-substituted 300mer **1a** or the haptenated protein antigen DNP/ovalbumin. Data shown were normalized to IL-2 levels obtained when T cells are exposed to B cells pretreated with peptide **8** (5 $\mu$M Ova$_{323}$). Error bars represent ±1 standard deviation from the mean ($n$ = 3).

Immune responses are stronger against T cell-dependent antigens bearing multivalent B cell epitopes.[45,46] This enhanced immunity is generally attributed to their ability to augment BCR clustering and thereby trigger B cell signaling above an activation threshold. Yet, robust antibody responses also require that antigen-specific B cells compete for T cell help.[47] Our data suggest that high valency antigens also facilitate greater antigen uptake, which then leads to enhanced presentation and T cell activation. Thus, multivalent antigens may result in enhanced immunity through their ability to both amplify signals and enhance B−T cell communication.

We next assessed T cell activation in response to treatment with high valency polymer versus that obtained from ovalbumin functionalized with DNP (DNP/Ova), a protein-conjugate antigen containing epitopes recognized by the B and T cells in our model system. We compared the responses after B cells were exposed to either 300mer **1a** or DNP/Ova protein for 3 h, sufficient time to induce robust IL-2 production (Figure 5). Both compounds promote IL-2 production, but the DNP/Ova$_{323}$ polymer elicited IL-2 levels that were significantly higher than those induced by the DNP/Ova protein conjugate (Figure 6B). The dramatic difference in T cell activation could result from more efficient BCR-mediated internalization of the polymer antigen. When we tested for this possibility, we found that the percentages of BCR internalization triggered by the protein (53 ± 7% at 26 min) and polymer (64 ± 4%) antigens were similar (Supporting Information Figure S6). These findings indicate that uptake alone cannot account for the difference in T cell activation.

**B Cells Exposed to Polymeric Antigen Activate T Cell Signaling.** The increase in IL-2 production elicited by the polymer versus the protein antigen may arise because the former gives rise to higher levels of cell surface peptide−MHCII. When peptide−MHCII display is augmented, signal-active B−T cell contacts occur more frequently.[48] If the polymer antigen affords higher levels of cell surface peptide−MHCII, then we postulated that it should result in more stimulatory B−T cell interactions. Accordingly, we monitored the formation of B−T cell interactions and T cell calcium signaling. T cells were loaded with Fluo-4, a dye whose fluorescence emission intensity increases upon calcium binding.[49] Fluo-4-treated T cells were added to antigen-treated B cells, and changes in Fluo-4 emission intensity were monitored by live single cell imaging to provide a measure of intracellular calcium ion concentration $[Ca^{2+}]_i$. Over the 75 min period for which cells were imaged, multiple aspects of the calcium signal, such as intensity, frequency, and timing, could be resolved for individual responders within the T cell population.

Responses from T cells cultured with either unstimulated or antigen-stimulated B cells were analyzed. We demarcated signaling as an elevation in $[Ca^{2+}]_i$ that reached a peak intensity at least 1.3 times greater than baseline, and this criterion was used to quantify the percentage of T cells undergoing calcium signaling. Unstimulated B cells rarely yielded calcium signaling events (<1% of T cells), and the few cells that seemed to respond afforded only weak signals (Figure 7B). To establish an upper response level, we measured the T cell response to B cells pulsed with free Ova$_{323}$ peptide **8** (5 $\mu$M). We anticipated that this peptide concentration would saturate B cell-surface MHCII molecules and lead to a greater percentage of responding T cells. As predicted, many more T cells in the population (28%) exhibited calcium flux. With these upper and lower limits serving as benchmarks, we monitored calcium signaling for T cells exposed to B cells that had been pretreated for 3 h with DNP/Ova$_{323}$ polymer **1a** (0.5 $\mu$M Ova$_{323}$). Approximately 8% of T cells formed direct contacts with polymer-treated B cells and underwent calcium signaling (Figure 7B). The strength and the duration of the calcium signal could be charted for each responding T cell population (Figure 7A and Supporting Information Figure S7). Compared to the polymer, the frequency of productive B−T cell interactions was lower for the DNP/Ova protein (0.5 $\mu$M Ova$_{323}$). These results show that exposure to polymer antigen allows B cells to more effectively form signal-active synapses with T cells. This likely stems from increased B cell antigen presentation of polymer antigens relative to the protein antigens.




DOI: 10.1021/acschembio.5b00239
ACS Chem. Biol. 2015, 10, 1817−1824

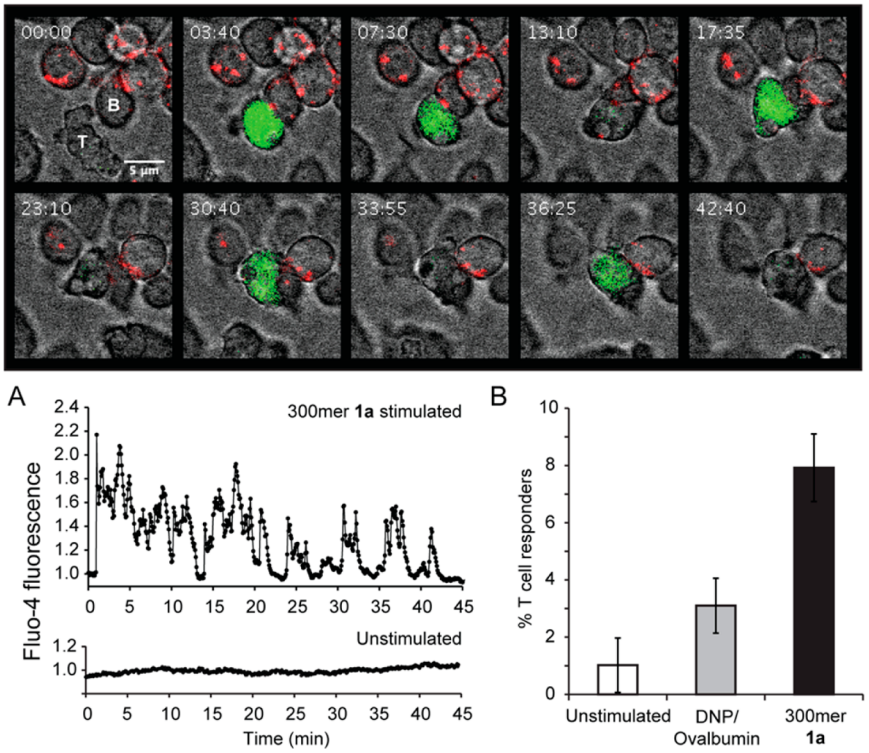



**Figure 7.** B cells stimulated with synthetic antigen form B–T cell synapses that lead to T cell activation. (Top) A20HL B cells labeled with Dylight649-conjugated anti-IgM Fab (red) were treated with DNP/$Ova_{323}$-substituted 300mer **1a** at a concentration of 5 $\mu$M DNP (0.5 $\mu$M $Ova_{323}$) for 3 h. Stimulated live B cells were combined with DO-11.10 T cells loaded with the $Ca^{2+}$-sensitive dye Fluo-4, and samples were imaged by confocal microscopy for approximately 75 min. The Fluo-4 fluorescence emission intensity (green) provides a measure of T cell intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). The frames shown are an overlay of brightfield and fluorescence images and are a single representative T cell signaling event. The movie from which the still images were captured can be found in the Supporting Information. (Bottom) (A) Fluo-4 fluorescence emission as a function of time is shown for a single T cell in response to an unstimulated B cell (bottom) or a B cell stimulated with 300mer **1a** (top). (B) Percentage of T cells demonstrating an increase in $[Ca^{2+}]_i$ upon contact with either unstimulated B cells or B cells treated with DNP/ovalbumin (0.5 $\mu$M $Ova_{323}$) or 300mer **1a** (0.5 $\mu$M $Ova_{323}$).

The stark difference in T cell activation mediated by the polymer versus protein antigen is not likely due to differences in antigen accumulation within B cells, given that both antigens induced BCR internalization to a similar extent. In the case of these two distinct types of antigens, the differences in T cell responses may stem from the relative efficiencies of antigen processing. The polymer antigen requires a single protease cleavage event to release a T cell epitope. Thus, we postulate that the polymer is processed more efficiently than the protein conjugate. The former is readily cleaved by cathepsin D, whereas the latter requires cleavage at multiple sites to release $Ova_{323}$ peptide. Additionally, the polymer antigen bears multiple copies of the $Ova_{323}$ epitope; therefore, it can deliver a higher concentration of antigenic peptide than does the protein antigen, which contains only a single $Ova_{323}$ epitope.

The differences in T cell response to polymer versus protein antigen highlight key features that can be designed into antigens to optimize B and T cell activation and thereby elicit robust immune responses. These parameters include the valency of the B cell epitope, the specificity of the protease-sensitive cleavage site, and loading of the T cell epitope. Protein carriers are static scaffolds that cannot be readily modified to enhance B and T cell activation. In contrast, defined polymers offer the ability to vary antigen structure in a modular fashion. This ability can be exploited to elucidate the effects of B cell ligand–receptor interactions, processing of antigen, and intracellular T cell epitope delivery on antigen presentation and T cell activation.

**Conclusions.** We have developed a modular strategy to synthesize polymers that activate both B and T cells, and it yielded antigens that are more effective than widely used protein conjugates. Using ROMP, we assembled bifunctional antigens that can promote BCR signaling, antigen internalization, antigen processing, presentation of antigenic peptides, and activation of T cells. Multiple features of this powerful polymerization method can be exploited to investigate antigen features that promote immune responses. For example, polymer end capping with a fluorinated capping agent provides the means to control and quantify epitope loading. By exploiting the ability to control polymer length, we determined that polymers with high epitope valency induce greater BCR internalization and T cell activation. Finally, by appending the T cell epitope with a cathepsin D-sensitive linker, we revealed the necessity of endosomal processing to achieve T cell activation. Thus, our ability to promote stronger T cell activation stems from the ability to endow antigens with features that promote increased uptake and processing. While we have exploited the attributes of ROMP to explore and optimize antigen features, our findings can be generalized to other scaffolds.

T cell-independent antigens, such as carbohydrates, offer promising targets for immunotherapies designed to treat cancer and infectious disease.[50,51] However, such antigens are often




DOI: 10.1021/acschembio.5b00239
ACS Chem. Biol. 2015, 10, 1817–1824

conjugated to protein carriers to increase immunogenicity.[52−54] Some disadvantages of these protein carriers have been noted, including challenging conjugation chemistry, a tendency to elicit off-target antibody responses, and instability at high or low temperature.[55,56] Polymer chemistry offers exciting alternatives for synthesizing antigens that can address these drawbacks. A polymer scaffold can be readily functionalized with select B and T cell epitopes to generate an immune response against a pathogen of interest. Moreover, adjuvant ligands, such as those recognized by the toll-like receptors (TLRs),[57,58] can be appended to the scaffold. The ease with which such scaffolds can be generated and manipulated offers a promising strategy to access a new generation of vaccine antigens.

## ■ ASSOCIATED CONTENT

### Supporting Information

(Movie files) Clips of T cell calcium flux events in response to B cells exposed to DNP/$Ova_{323}$ 300mer as well as the full movie from which the still images in Figure 7 were captured. (PDF) I. General procedures and materials. II. Reagent synthesis and characterization. III. Experimental methods. IV. Additional figures. Figure S1: Solid-phase peptide synthesis of $Ova_{323}$ peptides. Figure S2: Cathepsin D-sensitive linker undergoes cleavage to yield a T cell epitope. Figure S3: DNP-substituted polymer bearing $Ova_{323}$ peptide activates BCR signaling. Figure S4: B cell stimulation with DNP/Ova323 polymer results in T cell IL-2 production and requires both cellular processing and BCR-mediated uptake. Figure S5: Antigen presentation of DNP/Ova polymer requires cellular uptake and processing. Figure S6: DNP/Ova protein activates BCR-mediated internalization to a similar extent as that of polymer antigen. Figure S7: B cells stimulated with DNP/$Ova_{323}$ polymer form B−T cell synapses that activate T cell signaling. V. References. VI. Spectral data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00239.

## ■ AUTHOR INFORMATION


### Corresponding Author

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

### Present Address

§(A.H.C.) Department of Medicine, Division of Rheumatology, University of California−San Francisco, San Francisco, California 94143, United States.


### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

This research was supported by the National Institutes of Health (AI055258). N.R.B. was supported by a NIH Biotechnology Training Grant (T32 GM08349) and NSF Graduate Research Fellowship (DGE-1256259). We thank R. Sheridan for helpful discussions. The UW−Madison Chemistry Instrument Center is supported by the NSF (CHE-9208463, CHE-8813550, CHE-9629688). The UW−Madison Soft Materials Laboratory is supported by the NSF UW Nanoscale Science and Engineering Center (NSEC, DMR-0425880) and UW Materials Research Science and Engineering Center (MRSEC, DMR-0520527). The A20.2J $HL_{TNP}$ cell line was a generous gift of A. Ochi (University Health Network, Toronto, ON, Canada), and the DO.11-10 cell line was kindly provided by the Marrack and Kappler Group (National Jewish Health, Denver, CO, USA).

## ■ REFERENCES


(1) Kaufmann, S. H. (2007) The contribution of immunology to the rational design of novel antibacterial vaccines. *Nat. Rev. Microbiol. 5*, 491−504.

(2) Pulendran, B., and Ahmed, R. (2011) Immunological mechanisms of vaccination. *Nat. Immunol. 12*, 509−517.

(3) Takahashi, Y., Dutta, P. R., Cerasoli, D. M., and Kelsoe, G. (1998) In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *J. Exp Med. 187*, 885−895.

(4) Lanzavecchia, A. (1985) Antigen-specific interaction between T-cells and B-cells. *Nature 314*, 537−539.

(5) Lanzavecchia, A. (1990) Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol. 8*, 773−793.

(6) Yuseff, M.-I., Pierobon, P., Reversat, A., and Lennon-Duménil, A.-M. (2013) How B cells capture, process and present antigens: a crucial role for cell polarity. *Nat. Rev. Immunol. 13*, 475−486.

(7) Goodnow, C. C., Vinuesa, C. G., Randall, K. L., Mackay, F., and Brink, R. (2010) Control systems and decision making for antibody production. *Nat. Immunol. 11*, 681−688.

(8) McHeyzer-Williams, M., Okitsu, S., Wang, N., and McHeyzer-Williams, L. (2012) Molecular programming of B cell memory. *Nat. Rev. Immunol. 12*, 24−34.

(9) Tangye, S. G., Deenick, E. K., Palendira, U., and Ma, C. S. (2012) T cell-B cell interactions in primary immunodeficiencies. *Ann. N.Y. Acad. Sci. 1250*, 1−13.

(10) Batista, F. D., Arana, E., Barral, P., Carrasco, Y. R., Depoil, D., Eckl-Dorna, J., Fleire, S., Howe, K., Vehlow, A., Weber, M., and Treanor, B. (2007) The role of integrins and coreceptors in refining thresholds for B-cell responses. *Immunol. Rev. 218*, 197−213.

(11) Morris, G. P., and Allen, P. M. (2012) How the TCR balances sensitivity and specificity for the recognition of self and pathogens. *Nat. Immunol. 13*, 121−128.

(12) 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.

(13) 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.

(14) Fleire, S. J., Goldman, J. P., Carrasco, Y. R., Weber, M., Bray, D., and Batista, F. D. (2006) B cell ligand discrimination through a spreading and contraction response. *Science 312*, 738−741.

(15) Kouskoff, V., Famiglietti, S., Lacaud, G., Lang, P., Rider, J. E., Kay, B. K., Cambier, J. C., and Nemazee, D. (1998) Antigens varying in affinity for the B cell receptor induce differential B lymphocyte responses. *J. Exp. Med. 188*, 1453−1464.

(16) 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.

(17) 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.

(18) Kiessling, L. L., and Grim, J. C. (2013) Glycopolymer probes of signal transduction. *Chem. Soc. Rev. 42*, 4476−4491.

(19) Courtney, A. H., Bennett, N. R., Zwick, D. B., Hudon, J., and Kiessling, L. L. (2014) Synthetic antigens reveal dynamics of BCR endocytosis during inhibitory signaling. *ACS Chem. Biol. 9*, 202−210.

(20) Mangold, S. L., Carpenter, R. T., and Kiessling, L. L. (2008) Synthesis of fluorogenic polymers for visualizing cellular internalization. *Org. Lett. 10*, 2997−3000.

(21) Choi, T. L., and Grubbs, R. H. (2003) Controlled living ring-opening-metathesis polymerization by a fast-initiating ruthenium catalyst. *Angew. Chem., Int. Ed. 42*, 1743−1746.






DOI: 10.1021/acschembio.5b00239
*ACS Chem. Biol.* 2015, 10, 1817−1824

(22) Bielawski, C. W., and Grubbs, R. H. (2007) Living ring-opening metathesis polymerization. *Prog. Polym. Sci. 32*, 1−29.
(23) Strong, L. E., and Kiessling, L. L. (1999) A general synthetic route to defined, biologically active multivalent arrays. *J. Am. Chem. Soc. 121*, 6193−6196.
(24) Kolonko, E. M., Pontrello, J. K., Mangold, S. L., and Kiessling, L. L. (2009) General synthetic route to cell-permeable block copolymers via ROMP. *J. Am. Chem. Soc. 131*, 7327−7333.
(25) Baessler, K. A., Lee, Y., and Sampson, N. S. (2009) $\beta$1 integrin is an adhesion protein for sperm binding to eggs. *ACS Chem. Biol. 4*, 357−366.
(26) Brown, J. M., Xia, J., Zhuang, B., Cho, K.-S., Rogers, C. J., Gama, C. I., Rawat, M., Tully, S. E., Uetani, N., Mason, D. E., Tremblay, M. L., Peters, E. C., Habuchi, O., Chen, D. F., and Hsieh-Wilson, L. C. (2012) A sulfated carbohydrate epitope inhibits axon regeneration after injury. *Proc. Natl. Acad. Sci. U.S.A. 109*, 4768−4773.
(27) Sato, K., and Ochi, A. (1998) Inhibition of B-cell receptor-antigen complex internalization by Fc [gamma] RIIB1 signals. *Immunol. Lett. 61*, 135−143.
(28) 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.
(29) Buus, S., Sette, A., Colon, S. M., Jenis, D. M., and Grey, H. M. (1986) Isolation and characterization of antigen-la complexes involved in T cell recognition. *Cell 47*, 1071−1077.
(30) Buus, S., Sette, A., Colon, S. M., Miles, C., and Grey, H. M. (1987) The relation between major histocompatibility complex (MHC) restriction and the capacity of IA to bind immunogenic peptides. *Science 235*, 1353−1358.
(31) Kappler, J. W., Skidmore, B., White, J., and Marrack, P. (1981) Antigen-inducible, H-2-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J. Exp. Med. 153*, 1198−1214.
(32) Shimonkevitz, R., Kappler, J., Marrack, P., and Grey, H. (1983) Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J. Exp. Med. 158*, 303−316.
(33) Hirosue, S., Kourtis, I. C., van der Vlies, A. J., Hubbell, J. A., and Swartz, M. A. (2010) Antigen delivery to dendritic cells by poly(propylene sulfide) nanoparticles with disulfide conjugated peptides: cross-presentation and T cell activation. *Vaccine 28*, 7897−7906.
(34) Cohen, J. A., Beaudette, T. T., Tseng, W. W., Bachelder, E. M., Mende, I., Engleman, E. G., and Frechet, J. M. (2009) T-cell activation by antigen-loaded pH-sensitive hydrogel particles in vivo: the effect of particle size. *Bioconjugate Chem. 20*, 111−119.
(35) Diment, S. (1990) Different roles for thiol and aspartyl proteases in antigen presentation of ovalbumin. *J. Immunol. 145*, 417−422.
(36) Rodriguez, G. M., and Diment, S. (1992) Role of cathepsin D in antigen presentation of ovalbumin. *J. Immunol. 149*, 2894−2898.
(37) Pimenta, D. C., Oliveira, A., Juliano, M. A., and Juliano, L. (2001) Substrate specificity of human cathepsin D using internally quenched fluorescent peptides derived from reactive site loop of kallistatin. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol. 1544*, 113−122.
(38) Takaya, Y., Senda, T., Kurushima, H., Ogasawara, M., and Hayashi, T. (1999) Rhodium-catalyzed asymmetric 1,4-addition of arylboron reagents to alpha,beta-unsaturated esters. *Tetrahedron: Asymmetry 10*, 4047−4056.
(39) Campbell, M. A., and Sefton, B. M. (1990) Protein tyrosine phosphorylation is induced in murine B lymphocytes in response to stimulation with anti-immunoglobulin. *EMBO J. 9*, 2125−2131.
(40) Gold, M. R., Law, D. A., and DeFranco, A. L. (1990) Stimulation of protein tyrosine phosphorylation by the B-lymphocyte antigen receptor. *Nature 345*, 810−813.
(41) Lewis, R. S. (2001) Calcium signaling mechanisms in T lymphocytes. *Annu. Rev. Immunol. 19*, 497−521.
(42) Ziegler, H. K., and Unanue, E. R. (1982) Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T-cells. *Proc. Natl. Acad. Sci. U.S.A. 79*, 175−178.
(43) 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.
(44) Depoil, D., Zaru, R., Guiraud, M., Chauveau, A., Harriague, J., Bismuth, G., Utzny, C., Muller, S., and Valitutti, S. (2005) Immunological synapses are versatile structures enabling selective T cell polarization. *Immunity 22*, 185−194.
(45) Jardine, J., Julien, J. P., Menis, S., Ota, T., Kalyuzhniy, O., McGuire, A., Sok, D., Huang, P. S., MacPherson, S., Jones, M., Nieusma, T., Mathison, J., Baker, D., Ward, A. B., Burton, D. R., Stamatatos, L., Nemazee, D., Wilson, I. A., and Schief, W. R. (2013) Rational HIV immunogen design to target specific germline B cell receptors. *Science 340*, 711−716.
(46) Kaba, S. A., Brando, C., Guo, Q., Mittelholzer, C., Raman, S., Tropel, D., Aebi, U., Burkhard, P., and Lanar, D. E. (2009) A nonadjuvanted polypeptide nanoparticle vaccine confers long-lasting protection against rodent malaria. *J. Immunol. 183*, 7268−7277.
(47) Schwickert, T. A., Victora, G. D., Fooksman, D. R., Kamphorst, A. O., Mugnier, M. R., Gitlin, A. D., Dustin, M. L., and Nussenzweig, M. C. (2011) A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. *J. Exp. Med. 208*, 1243−1252.
(48) Huang, J., Brameshuber, M., Zeng, X., Xie, J., Li, Q. J., Chien, Y. H., Valitutti, S., and Davis, M. M. (2013) A single peptide−major histocompatibility complex ligand triggers digital cytokine secretion in $CD4^+$ T cells. *Immunity 39*, 846−857.
(49) Minta, A., Kao, J. P., and Tsien, R. Y. (1989) Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem. 264*, 8171−8178.
(50) Astronomo, R. D., and Burton, D. R. (2010) Carbohydrate vaccines: developing sweet solutions to sticky situations? *Nat. Rev. Drug Discovery 9*, 308−324.
(51) Danishefsky, S. J., Shue, Y. K., Chang, M. N., and Wong, C. H. (2015) Development of globo-h cancer vaccine. *Acc. Chem. Res. 48*, 643−652.
(52) Lin, F. Y., Ho, V. A., Khiem, H. B., Trach, D. D., Bay, P. V., Thanh, T. C., Kossaczka, Z., Bryla, D. A., Shiloach, J., Robbins, J. B., Schneerson, R., and Szu, S. C. (2001) The efficacy of a *Salmonella typhi* Vi conjugate vaccine in two-to-five-year-old children. *N. Engl. J. Med. 344*, 1263−1269.
(53) Schneerson, R., Barrera, O., Sutton, A., and Robbins, J. B. (1980) Preparation, characterization, and immunogenicity of Haemophilus influenzae type b polysaccharide−protein conjugates. *J. Exp. Med. 152*, 361−376.
(54) Verez-Bencomo, V., Fernandez-Santana, V., Hardy, E., Toledo, M. E., Rodriguez, M. C., Heynngnezz, L., Rodriguez, A., Baly, A., Herrera, L., Izquierdo, M., Villar, A., Valdes, Y., Cosme, K., Deler, M. L., Montane, M., Garcia, E., Ramos, A., Aguilar, A., Medina, E., Torano, G., Sosa, I., Hernandez, I., Martinez, R., Muzachio, A., Carmenates, A., Costa, L., Cardoso, F., Campa, C., Diaz, M., and Roy, R. (2004) A synthetic conjugate polysaccharide vaccine against *Haemophilus influenzae* type b. *Science 305*, 522−525.
(55) Schutze, M. P., Leclerc, C., Jolivet, M., Audibert, F., and Chedid, L. (1985) Carrier-induced epitopic suppression, a major issue for future synthetic vaccines. *J. Immunol. 135*, 2319−2322.
(56) Buskas, T., Li, Y., and Boons, G. J. (2004) The immunogenicity of the tumor-associated antigen Lewis(y) may be suppressed by a bifunctional cross-linker required for coupling to a carrier protein. *Chemistry 10*, 3517−3524.
(57) Wang, X., Smith, C., and Yin, H. (2013) Targeting Toll-like receptors with small molecule agents. *Chem. Soc. Rev. 42*, 4859−4866.
(58) Mancini, R. J., Stutts, L., Ryu, K. A., Tom, J. K., and Esser-Kahn, A. P. (2014) Directing the immune system with chemical compounds. *ACS Chem. Biol. 9*, 1075−1085.


DOI: 10.1021/acschembio.5b00239
*ACS Chem. Biol.* 2015, 10, 1817−1824