Multimeric Insulin Desensitizes Insulin-Specific B Cells
Stephanie N. Johnson,* J. Daniel Griffin,* Chrys Hulbert, Brandon J. DeKosky, James W. Thomas, and Cory J. Berkland

ABSTRACT: Adaptive immunity plays a central role in the pathogenesis of type 1 diabetes. Among past treatment approaches, B cell ablation has yielded unmistakable therapeutic potency; however, global immunosuppression imposes unacceptable risks to a patient population consisting of children. Multivalent antigen arrays represent a compelling strategy for targeted immunosuppression by selectively engaging and inactivating autoreactive B cells. Here, we report the design and characterization of 4-arm polyethylene glycol-insulin (PEG-Ins) conjugates as multivalent arrays for autoreactive B cell engagement. First, we selectively modified human insulin at the B29 residue to retain antigenicity. Next, we conjugated the modified proteins to 20 kDa, 4-arm polyethylene glycol backbones to produce multivalent PEG-Ins constructs. Mass spectrometry, circular dichroism, and dynamic light scattering indicated that the structure of insulin was maintained in the much larger, multivalent construct. PEG-Ins conjugates demonstrated an ex vivo immunological effect in splenocytes harboring an anti-insulin B cell receptor (VH125SD) by inactivating B cells and promoting an anergic phenotype that was downregulated in B cell receptor expression (CD79b), and PEG-Ins conjugates did not mobilize calcium upon B cell receptor stimulation. These data support the further study of PEG-Ins conjugates in animal models of type 1 diabetes.

KEYWORDS: antigen-specific, insulin, type 1 diabetes, immunotherapy, multivalent, B cell, anergy

INTRODUCTION
Multivalency as a molecular design strategy has enabled a broad range of efforts in drug delivery,4−6 vaccine development,3,4,5,7 and immunotherapies for both autoimmunity8 and cancer.9,10 The association of multiple ligands within the same macromolecule can be advantageous in these applications by increasing the receptor binding avidity. The Dintzis laboratory was the first to describe the effects of valency, particularly in the context of immunon-specific immunity, beginning in the 1970s.11−14 Dintzis’ group demonstrated that immunologic outcomes that can be tuned between tolerance and immunogenicity using antigen valency and spatial density.12 Ultimately, Dintzis’ “rules” codified the immunologic impact of multivalent nanoparticles. These foundational guidelines showed that molecules with fewer than 20 immunons (or antigens) and a molecular weight of less than 100 kDa promoted tolerance, while, conversely, entities larger than 100 kDa and presenting greater than 20 autoantigens were tuned for immunogenicity.15 Laura Kiessling’s group later defined mechanisms of multivalency and its impact on the immune system. Their work illustrated that multivalent antigen-presenting polymers could engage in avid binding with B cell receptors (BCRs) to induce receptor clustering within membrane microdomains, activating cells in a valency-dependent manner.15,16 For low valency, tolerogenic materials, Kiessel substantiated Dintzis’ seminal hypothesis that multivalent presentation of typically agonistic antigens may induce incomplete or unproductive configurations of B cell receptors. Building on these prior efforts, our group previously established the Soluble Antigen Array (SAgA) platform by applying the lessons of Dintzis and Kiessling to manifest a multivalent, antigen-specific polymeric format embodying tolerogenic characteristics for autoimmune disease applications.17−28 SAgAs were designed as small (<100 kDa), water-soluble arrays of low-valency autoantigens (~10 ligands per molecule).29 The autoantigen arrays making up SAgAs have historically been grafted upon 16 kDa linear hyaluronic acid (HA) backbones that confer water solubility and functional chemical handles for antigen conjugation.30 SAgAs were highly efficacious against models of multiple sclerosis,17−19,21,23−26 and type 1 diabetes (T1D)27,28 by selectively targeting and

Received: June 25, 2020
Accepted: July 27, 2020
Published: July 27, 2020

https://dx.doi.org/10.1021/acsabm.0c00782
ACS Appl. Bio Mater. 2020, 3, 6319−6330
disarming B cells. These multivalent platforms represent compelling prospective solutions for antigen-specific auto-immune disease treatments.

While linear, HA-based SAgAs have exhibited therapeutic potential, HA polymers are heterogeneous in size and therefore in the number of functional chemical handles. The heterogeneity of HA polymers imposes difficulty in obtaining monodisperse populations of SAgAs conjugated with discrete numbers of autoantigens. In this work, we envisioned that four-arm polyethylene glycol (PEG) polymers could serve as well-characterizable backbones for the presentation of multivalent autoantigens. We previously determined that low-valency SAgAs displaying ∼4–7 antigens on hyaluronic acid provided optimal B cell inhibition,26 so we hypothesized that the four discrete arms of PEG tetramers with distal functional handles would enable facile production of low valency SAgAs. Furthermore, the iteration of the SAgA structure from a linear backbone molecule to a two-dimensional branched backbone molecule could confer an enhanced ability to avidly engage BCRs.30

Here, we generated 4-arm PEG-insulin (PEG-Ins) conjugates as B cell targeted antigen specific immunotherapies against TID. Human insulin represents a more complex autoantigen than has been previously explored in peptide epitope-induced models of autoimmunity.18–20,26 and it was important to generate a construct where antigenicity could be retained. We employed a VH125SD mouse model of TID for the ex vivo evaluation of our approach, as these mice possess a knocked-in heavy chain BCR sequence that binds to insulin.31–33 Up to 5% of the B cell repertoire in VH125 mice is insulin-specific, providing a robust model for ex vivo analyses of antigen-specific immunomodulation by PEG-Ins conjugates.

Materials and Methods

Materials. Recombinant human insulin was purchased from Millipore Sigma (Burlington, MA). Propargyl N-hydroxysuccinimide (NHS) ester was purchased from Click Chemistry Tools (Scottsdale, AZ). 1,1,3,3-Tetramethylethylendiurea was purchased from Alfa Aesar (Haverhill, MA), and 20 kDa 4-arm PEG-azide was purchased from JenKem Technology USA (Beijing, China). Tris-(3-hydroxypropylpyrrolidinyl)methine (THPTA) and sodium ascorbate (NaAsc) were purchased from Sigma-Aldrich (St. Louis, MO). Copper(II) sulfate pentahydrate (CuSO4·5H2O) was purchased from Acros Organics (Geel, Belgium). All reagents were used as received without further purification.

For in vitro cell assays, red blood cell lysis buffer was purchased from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charles, MO). Complete Roswell Park Memorial Institute (cRPMI) media were prepared by combining from Sigma-Aldrich (St. Charlie
Far-UV circular dichroism (CD) spectroscopy was performed using an Applied Photophysics Chirascan equipped with a 6-cell holder (Applied Photophysics, Leatherhead, UK). Proteins were at concentrations of 0.2 mg/mL on a protein basis, in a 50 mM phosphate buffer, pH 7.4, in a 1 mm quartz cell. CD spectroscopy was conducted from 190−280 nm, using a 1 nm step size with a two second integration time at each step. A sample containing the buffer was subtracted from each sample.

Animals. The anti-insulin VH125SD H chain transgenic mice used in this study were maintained by the J.W. Thomas Laboratory and harbor a randomly integrated H chain sequence on the B6 or nonobese diabetic (NOD) background, as described previously.31,33 Animals used for these studies were aged between 5 and 19 weeks and were backcrossed for >20 generations to B6 or NOD mice. All mice were housed under sterile conditions, and all studies were approved by the Institutional Animal Care and Use Committee of Vanderbilt University, which is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Splenocyte Isolation. NOD and B6 VH125 spleen samples were received as unlysed splenocytes from the J.W. Thomas laboratory after overnight shipping. Unlysed splenocytes were centrifuged at 300 g for 5 min and resuspended with 1 mL of red blood cell lysis buffer (Sigma-Aldrich, St. Charles, MO). Samples were incubated for 7 min at room temperature before 9 mL of cRPMI media was added to stop the lysis. Splenocytes were spun down once more and resuspended in 10 mL of fresh cRPMI media. Gently tapping the resuspended samples on the cell culture hood surface, suspensions were transferred to new tubes to leave behind aggregated connective tissue. The isolated cell suspensions were counted and concentrated for further assays.

Figure 1. (a) Reaction scheme for the selective modification of human insulin with an alkyne handle. (b) 2D HSQC NMR data (x-axis shows the $^1$H spectrum and y-axis shows the $^{13}$C spectrum) of unmodified human insulin enable the visualization of the (c) unique resonance signal for the proton on the alkyne group on the same 2D HSQC spectrum after modification. (d) MS data of Ins-Alk and its fragments confirm the location of the alkyne modification to the B29 lysine residue.
Fluorescent Detection of IBCs. B6 VH125 splenocytes were received and isolated. Aliquots of $1.5 \times 10^6$ splenocytes were washed one additional time in 1× DPBS and resuspended in 50 μL of a Zombie Aqua live/dead cell indicator prepared at a 1:200 dilution (Biolegend, San Diego, CA) in FACS buffer. Samples were incubated for 20 min at room temperature, covered from light. After the incubation, fluorescent antibodies and 10 μg of fluorescent PEG-Ins(2) with 2 fluorescein isothiocyanate (FITC) molecules per backbone were added, and samples were incubated for an additional 30 min at 4 °C (covered from light). Samples were washed twice and resuspended in 200 μL of FACS buffer before analysis on a BD FACSaria Fusion cytometer.

Calcium Mobilization. Calcium mobilization assays were conducted as previously reported. Briefly, NOD or B6 VH125 splenocytes were plated at a concentration of $3 \times 10^6$ cells/mL in 500 μL of cRPMI media with 5 μg of the treatment compound for 5 h. After the incubation, cells were washed and loaded in 500 μL of a 5 μM Fluo-4 calcium indicator (Invitrogen, Carlsbad, CA) for 30 min at room temperature. Cells were simultaneously labeled for CD79b and B220. Labeled samples were washed in DPBS and resuspended in Hanks balanced salt solution media containing calcium and magnesium. At the cytometer, cells were warmed to 37 °C for 2−3 min before a baseline Fluo-4 measurement was taken for 3 s. Then, 20 μg/mL (10 μg) of anti-immunoglobulin M (IgM) F(ab')2 (Invitrogen, Carlsbad, CA) was added, and a stimulated measurement was collected for 2 min and 30 s. Finally, 1 μg of ionomycin was introduced to collect a maximal reading over an additional 30 s.

Receptor Expression by Flow Cytometry. B6 splenocytes were plated at $3 \times 10^6$ cells/mL for 24 or 72 h in cRPMI media containing 5 μg of the treatment compound. Cells were collected and washed in FACS buffer before being incubated for 20 min with a Zombie Aqua live/dead stain (Biolegend, San Diego, CA) at room temperature. Next, fluorescent antibodies were added for 30 min, and samples were incubated for an additional 30 min at 4 °C. Labeled cells were washed twice and resuspended at 200 μL of flow buffer before analysis on a BD FACSaria Fusion cytometer.

Resazurin Cell Metabolism. B6 splenocytes were incubated for 72 h with treatments. After the incubation, plates were spun down to remove the culture supernatant. Removed media were replaced with a 75 μM resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) solution, and the resuspended cells were incubated for 3 h at 37 °C plus 5% CO2. The metabolic reductive capacity was measured by assessing changes in fluorescence measurements at excitation/emission of 560/590 on a Spectramax M5 instrument from Molecular Devices (San Jose, CA). The background fluorescence was subtracted out by equilibrating to cRPMI media alone.

Cytokine Measurement. Supernatants from the 72 h resazurin assay were collected for cytokine analysis. Markers (granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin (IL)-2, IL-21, IL-6, IL-10, IL-17, and IL-23) were detected using a U-Plex kit according to manufacturer standards (Meso Scale Discovery, Rockville, MD). Each assay plate was read using the Quickplex multiplex plate reader (Meso Scale Discovery, Rockville, MD).

Statistical Analysis. Statistical analyses for biological assays were performed using one-way ANOVA with paired data replicates and a
RESULTS

Selective Modification of Insulin at the B29 Residue.
Selective modification of the B29 lysine residue of human insulin was an attractive target, as previous studies have shown that modification at this location does not alter receptor binding.\(^{35}\) Specifically, several insulin analogues currently on the market, such as insulin lispro, aspart, glulisine, and detemir possess modifications at this location.\(^{36}\) The functionalization of human insulin with an alkyne handle was achieved by NHS chemistry under basic conditions, as shown in Figure 1a.\(^{34}\) NMR spectroscopy was utilized to qualitatively confirm the homopropargyl linker on insulin. By comparing the \(\text{\textsuperscript{1}}\text{H}/\text{\textsuperscript{13}}\text{C}\) heteronuclear single quantum coherence (HSQC) spectra of unmodified human insulin (Figure 1b) to that of Ins-Alk (Figure 1c), the unique resonance signal corresponding to the alkyne group (\(\delta(\text{\textsuperscript{1}}\text{H}) \approx 2.6\) ppm and \(\delta(\text{\textsuperscript{13}}\text{C}) \approx 70\) ppm) was evident. Additionally, LC-MS verified both the product identification as well as the location of the modification (Figure 1d). Regarding the modification of human insulin with an alkyne handle, there were three possible substitution locations, including the lysine residue at the B29 position or the N-termini of both the A and B chains.

To determine the location of the modification, 100 \(\mu\text{L}\) of a 1 mg/mL solution of Ins-Alk was incubated with 100 \(\mu\text{L}\) of 2 M \(\text{NH}_4\text{acetoxy (OAc)}\) and 2.5 mg of dithiothreitol for 3 h at room temperature. A LC-MS analysis was then performed, and data were collected in MS E mode for fragmentation. Two major fractions resulting from reduction with dithiothreitol corresponded to masses for the unmodified \(\text{A}\) chain (2384.0 Da) and the \(\text{B}\) chain plus the addition of the alkyne handle (3539.8 Da) (Figure 1d and Figure S1). Collision-induced dissociation (CID) then allowed for further fragmentation of the resulting ions. Examination of the \(\gamma\) ions resulting from fragmentation on the N-terminal or C-terminal side of the proline residue at the B28 location (556.3 and 455.2 Da, respectively) confirmed that the modification of human insulin was selective for the B29 lysine residue (Figure 1d and Figure S1).

Selectively Modified Insulin Enables the Synthesis of Multivalent PEG-Ins Conjugates.
CuAAC chemistry was next applied to conjugate Ins-Alk to 20 kDa 4-arm PEG-N\(_3\) backbones to produce multivalent insulin products (Figure 2a and Figure S2). To qualitatively confirm conjugation, HSQC was again utilized. The spectra of 20 kDa 4-arm PEG-N\(_3\) showed one major resonance corresponding to the PEG backbone (Figure 2b), while the spectra of the insulin-conjugated 4-arm PEG-Ins reveals an overlay of the signatures for both the PEG backbone and Ins-Alk, albeit with the absence of a signal for the homopropargyl group on Ins-Alk (Figure 2c). These data qualitatively confirmed that the final product contained both the polymer and conjugated protein.

Next, it was critical to assess the biophysical properties of PEG-Ins conjugates to characterize the suitability of modified insulin conjugates for biological applications. Dynamic light scattering, Matrix-assisted laser desorption/ionization-time of flight MS, and circular dichroism were each applied toward these ends. DLS was incorporated to characterize the size of conjugates as a function of the number of insulins grafted (1, 2, 3, or 4) and to monitor changes in protein hydrodynamic properties in solution (i.e., aggregation). Notably, human insulin is susceptible to form into higher-order structures consisting of dimers or hexamers; however, insulin is only active in its monomeric form.\(^{37}\) The reported radius of monomeric human insulin is approximately 2 nm.\(^{38}\) DLS measurements yielded hydrodynamic radii that were proportional for all samples (Figure 3). Interestingly, the radius for Ins-Alk was smaller than that of human insulin, a phenomenon potentially arising from increased intramolecular hydrophobic interactions due to the addition of the alkyne handle. Overall, these results suggested that insulin remained in its monomeric form and that aggregation did not occur. MALDI-TOF MS supplemented the DLS findings with measurements for the average molecular weights among the constructs. Results showed that the average molecular weight of 20 kDa 4-arm PEG-N\(_3\) was 20104 Da and that each of the constructs increased by approximately 6 kDa for each conjugated insulin (corresponding to the installment of each molecule of Ins-Alk (Figure 3 and Figure S3). Far-UV spectra of insulin have been reported and demonstrated minima at 209 and 223 nm, which correspond to the known \(\alpha\)-helical structure of insulin.\(^{37,39}\) Therefore, a CD analysis was performed in order to confirm that modified human insulin and each of the conjugated products retained the \(\alpha\)-helical structure. The data showed conserved waveforms among all PEG-Ins conjugates, suggesting that each of the insulin-containing samples retained a native-like \(\alpha\)-helical secondary structure (Figure 3 and Figure S4). This observation was further confirmed by the analysis of the percentage of the \(\alpha\)-helical structure of each molecule, quantified through the BeStSel web server.\(^{40,41}\) As shown in Figure 3, the percentage of the \(\alpha\)-helical structure is consistent for each of the insulin-containing samples.

---

**Table 1:** Biophysical characterization data for each of the PEG-Ins conjugates and their components. DLS and MALDI-TOF analyses were applied to ascertain the relative sizes of the constructs, and a CD analysis was applied to assess the retained \(\alpha\)-helical secondary structure for all insulin-containing species.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Size by DLS (nm)</th>
<th>Size by MALDI-TOF (Da)</th>
<th>CD Waveform</th>
<th>% (\alpha)-helical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>1.7 ± 0.10</td>
<td>N/A</td>
<td>74.6 ± 3.32</td>
<td></td>
</tr>
<tr>
<td>Insulin-Alkynie</td>
<td>1.0 ± 0.10</td>
<td>N/A</td>
<td>74.2 ± 2.63</td>
<td></td>
</tr>
<tr>
<td>20 kDa 4-arm PEG-N(_3)</td>
<td>1.8 ± 0.39</td>
<td>20,104</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>4-arm PEG-Ins(1)</td>
<td>3.0 ± 0.06</td>
<td>26,434</td>
<td>72.8 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>4-arm PEG-Ins(2)</td>
<td>3.7 ± 0.39</td>
<td>32,106</td>
<td>72.8 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>4-arm PEG-Ins(3)</td>
<td>4.2 ± 0.33</td>
<td>38,282</td>
<td>72.8 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>4-arm PEG-Ins(4)</td>
<td>4.8 ± 0.44</td>
<td>44,266</td>
<td>72.8 ± 0.27</td>
<td></td>
</tr>
</tbody>
</table>
Constructs Promote a Calcium Immobile B Cell Phenotype in a Valency-Dependent Fashion. Multivalency is well-known to increase the binding between antigen and cognate receptors through enhancing the avidity of macromolecule−ligand interactions. We designed multiarm PEG-Ins conjugates to harness the benefits of multivalency to engage insulin-specific B cell receptors (BCRs) such as those found in transgenic VH125 cells and authentic T1D alike. We produced fluorescent PEG-Ins(2) (fPEG-Ins) and found that it was able to identify a positive subset of CD19+ insulin-specific B cells (IBCs) among splenocytes from VH125 B6 mice (Figure 4a).

With data supporting the engagement of IBCs by multivalent PEG-Ins, we endeavored to assess functional biological effects ex vivo using splenocytes from VH125 mice. We first conducted calcium mobilization assays to investigate the changes to B cell activation after incubation with constructs. Immediately, from our initial experiments, two populations were evident in preliminary scatter gating (Figure 4b). A “main” population, typical of healthy splenocytes, encompassed B cells that were able to mobilize calcium. An “alternate” population showed no changes in intracellular calcium levels after stimulation with anti-IgM or ionomycin. Interestingly, two distinct CD19+ insulin-specific B cells (IBCs) among splenocytes from VH125 B6 mice (Figure 4a).

PEG-Ins Conjugates Downregulate BCRs and Skew B Cells toward an Anergic Phenotype after 24 h of Incubation. We next isolated splenocytes from VH125 B6 mice and plated them with 5 μg of the treatment compound

![Figure 4](https://dx.doi.org/10.1021/acsabm.0c00782)
(10 μg/mL) to assess the influence of the constructs on cell receptor expression after 24 h (Figure 5, Figure S5, and Figure S6). While PEG-Ins treatment only slightly influenced the total proportion of B cells in culture, PEG-Ins(3) and PEG-Ins(4) skewed the balance between reactive and anergic B cells toward the anergic phenotype (Figure 5a). PEG-Ins(3) and PEG-Ins(4) each significantly increased the proportions of CD19lo+ (anergic) B cells in culture compared to insulin treatment alone. Interestingly, PEG-Ins(1) skewed these proportions toward the reactive CD19hi+ phenotype. The magnitude of fluctuations that were evident in these populations was roughly equivalent to the ∼5% of total cells that constitutes the IBC population (Figure 5b).

PEG-Ins constructs also displayed an apparent reduction of BCR expression among the B cell subsets (Figure 5c); however, the statistical significance was not consistently achieved. CD79b expression was employed as a surrogate marker for BCRs in these observations, as this marker is linked to the receptor complex. PEG-Ins(4) did significantly reduce the total MFI of CD79b among all B cells. In the CD19lo+ cluster, PEG-Ins(1) and PEG-Ins(4) showed a lowered expression. The suppressive performance of PEG-Ins(4) across multiple markers suggested that this higher-valency construct may be most effective for the deactivation of IBCs. The collective trends were highly conserved when the same assays were conducted in VH125 NOD counterpart splenocytes (Figure S7 and Figure S8). Particularly, similar reductions in the BCR expression (CD79b) (Figure S7b and Figure S8b) were evident; however, the skew from CD19hi+ to CD19lo+ B cells was not as pronounced (Figure S7a). This difference was likely accountable to a lower prevalence of insulin-specific B cells in the NOD model.

**Antigen-Presenting B Cells Are Disarmed after 72 h.**

Next, we incubated PEG-Ins conjugates with VH125 B6 splenocytes for 72 h to assess downstream effects (Figure 6). CD19+CD11c+ B cells are a potent antigen-presenting cell (APC) subset that is implicated in autoimmune responses. We probed the CD19+CD11c+ B cell population and its
activation state to investigate insulin-specific immune responses by this potent APC population. PEG-Ins conjugates generally suppressed the proportions of CD19+CD11c+ B cells after 72 h (Figure 6a), with PEG-Ins(4) eliciting statistically significant reductions. CD79b expression was slightly reduced by PEG-Ins conjugates, but only PEG-Ins(1) showed statistically significant changes (Figure 6b). To further link changes in BCRs with B cell activation states, we probed cells for IgD expression as a marker of retained anergy (Figure 6c).44 PEG-Ins(4) treatment yielded the greatest proportion of BCRs having IgD among CD19+CD11c+ B cells; however, statistical significance was not achieved.

To assess the broader immunity among splenocytes, we performed a resazurin cell metabolism assay after 72 h of incubation (Figure 6d). The metabolic activity did not statistically differ from insulin treatment among conjugates, but PEG-Ins(3) and PEG-Ins(4) apparently trended up from lower-valency counterparts. Cytokine analysis also suggested a B cell response. (Figure 6e and Figure S9). IL-2, IL-17, and IL-10 were not significantly influenced by PEG-Ins conjugates; however PEG-Ins(4) significantly increased IL-6 levels over those of the insulin group.

**DISCUSSION**

Self-reactive B cells have been found to contribute to the pathology of T1D.45 T1D onset is highly correlated with a loss of B cell anergy, and broad B cell depletion using rituximab has proven clinically effective for preserving beta cell function in new onset patients while B cell suppression was maintained.47 Taken together, these observations highlight a fundamental role for B cells in both the development and treatment of this disease. Though the islet destruction hallmark of T1D is classically regarded as T cell-driven, B cells leverage upstream functions such as potent antigen-presentation and cytokine production to direct the T cell pathology.45,48,49 While past clinical breakthroughs with rituximab in T1D are compelling, practical application of such severe, chronic, and global immunosuppression is utterly inadequate for a disease that predominately affects children.45,50 The field has collectively been left with a need for B cell-targeted therapies that act in a specific manner.45

Here, we reported the generation and characterization of new 4-arm PEG-Ins molecules with potential to selectively target self-reactive B cells in T1D patients, as demonstrated using the VH125 mouse models.17−19,21,23−26 We synthesized a derivative of human insulin containing a reactive alkyne in a one-step process for precisely modifying the free B29 lysine ε-
amino group in the presence of the two free α-amino groups (amino termini on the A and B chains) (Figure 1). The specific nature of the reaction was based on the relative nucleophilicity of each of the deprotonated amino groups (pKₐ of the α-amino groups is approximately 8, whereas the pKₐ of the ε-amino group is 10). By performing the reaction at pH greater than 10.75 in a nonaqueous solvent, both the α- and ε-amino groups were deprotonated. Since the ε-amino group is intrinsically more nucleophilic, we drove the major product formation to a single modification at the ε-amino group. Importantly, the modified insulin retained its secondary structure compared to native insulin (Figure 3), and constructs synthesized from this modified autoantigen were still able to bind autoreactive B cells (Figure 4a).

We selected a 20 kDa 4-arm PEG polymer for our present application as a homogeneous starting material with a defined number of reactive handles. We fabricated PEG-Ins conjugates by grafting Ins-Alk onto 4-arm PEG backbones at discrete valencies. Due to the high reactivity of insulin–alkyne, only a slight excess (0.1–0.3 equiv.) of the stoichiometric amounts of insulin were needed to conjugate to the 4-arm PEG backbone. For constructs that were not fully occupied (<4 insulin molecules per 4-arm PEG), the products with an average of either 1, 2, or 3 insulin molecules per backbone did contain other valencies (Figure S2). In contrast, the fully substituted 4-arm PEG-Ins was more homogeneous since all free sites were occupied. PEG-Ins conjugates demonstrated a suppressive capacity against ex vivo VH125 splenocytes in several orthogonal assays and over multiple incubation time periods. We observed two consistent populations of CD19+ B cells (CD19hi+ and CD19lo+) throughout our ex vivo assays. CD19hi+ B cells showed a higher capacity to mobilize calcium (Figure 4b) and expressed higher levels of surface BCR (Figure S5). These changes, in addition to calcium inertia, are hallmarks of anergic B cells.56,57 Anergic B cells are described in other autoimmune settings, where CD19hi+ populations present a cell state primed for canonical B cell effector functions, including antibody production.31,52 CD19lo+ cells, conversely, are not able to propagate adaptive immunity.51 While CD19lo+ can indicate plasmablasts,53 these antibody-secreting cell types upregulate CD79b and CD86.43,55 The CD19lo+ population observed here exhibited decreased CD79b expression (Figure 6) and maintained CD86 expression (Figure S5). These changes, in addition to calcium inertia, are hallmarks of anergic B cells.36,56 Anergic B cells are susceptible to an apoptotic fate upon antigen stimulation;57 it is possible that pretreatment of these cells with PEG-Ins constructs could have propelled them toward apoptosis, as even ionomycin did not evoke a response in our studies (Figure 4).

The VH125 mouse models employed here have been heavily studied in the context of B cell anergy.31,38 Here, we observed that PEG-Ins conjugates (namely PEG-Ins(3) and PEG-Ins(4)) skewed VH125 splenic B cells from the CD19hi+ phenotype to the CD19lo+ state after 5 and 24 h of incubation (Figure 4, Figure S5), and those CD19 phenotype changes were proportional to the total prevalence of IBCs within the VH125 models.31,33 Importantly, these changes were not observed with the control 4-arm PEG molecules lacking conjugated insulin, suggesting that the effects were antigen specific. While PEG-Ins(3) and PEG-Ins(4) most potently altered the B cell phenotype after 5 and 24 h, a resazurin analysis after 72 h revealed an increasing cell metabolism with increasing PEG-Ins valency (Figure 6d). A cytokine analysis supplemented the resazurin findings by illustrating largely consistent secretion levels between treatment groups (Figure 6e). IL-2 and IL-17 levels did not increase, suggesting the absence of a pro-inflammatory response.59 However, IL-10 levels were not significantly increased by PEG-Ins conjugates, suggesting that potent tolerogenesis was not achieved but perhaps, rather, the disarmament of IBCs.15 The statistically significant increase of IL-6 levels induced by PEG-Ins(4) supports antigen-specific B cell-targeted activity.60

Our biological assays revealed apparent valency-dependent trends, where monovalent insulin and PEG-Ins(1) often exhibited similar stimulatory effects, while PEG-Ins(4) consistently exhibited hallmarks of IBC inactivation (Figure 4, Figure S5, and Figure 6). One aspect to consider for the interpretation of these data is that construct dosing was fixed at a 5 μg mass for all experiments as an “excess” quantity based on prior multivalent antigen array studies.38 While the overall concentration was fixed at 10 μg/mL, the concentration of insulin molecules varied between conjugate groups (PEG-Ins(1, 2, 3, or 4)), complicating the analysis of the valency-dependent behavior. Future studies will explore dose titrations and compare PEG-Ins multimers on a molar-insulin and molar-construct basis to reveal insulin valency-dependent features.26 Nonetheless, there was a clear benefit for insulin multimerization in this study (Figure 4, Figure 5, and Figure 6).

## CONCLUSION

We capitalized on pKₐ differences among lysine residues on human insulin to reliably and selectively install alkyne handles at the B29 residue using a controlled reaction pH. Modified Ins-Alk enabled the facile synthesis of PEG-Ins multivalent arrays with different insulin valencies that did not compromise biophysical properties or antigenicity. PEG-Ins conjugates showed suppressive immunomodulation ex vivo by skewing pathogenic VH125 B cells to an anergic phenotype. Future work will explore the potency and in vivo performance of PEG-Ins conjugates in the context of T1D and its mouse models.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.0c00782.

Raw mass spectral data for Ins-Alk and its fragments (Figure S1); RP-HPLC showing Ins-Alk and 4-arm PEG-Ins constructs (Figure S2); MALDI-TOF data for 4-arm PEG-N3, Ins-Alk, and 4-arm PEG-Ins constructs (Figure S3); circular dichroism signatures for insulin, Ins-Alk, and 4-arm PEG-Ins variants (Figure S4); CD86 MFI for CD19+ B cells treated with 4-arm PEG-Ins constructs (Figure S5); changes to CD19+CD11c+ B cells after incubation with 4-arm PEG-Ins constructs (Figure S6); CD79b, CD86, and CD19+ cellular responses following treatment with variants (Figure S7); CD19+CD11c+ cellular changes following incubation with 4-arm PEG-Ins constructs (Figure S8); and cytokine assessment resulting from various treatments (Figure S9) (PDF)
ACKNOWLEDGMENTS

The authors declare no competing financial interest.

REFERENCES


(18) Hartwell, B. L.; Pickens, C. J.; Leon, M.; Berkland, C. Multivalent Soluble Antigen Arrays Exhibit High avidity Binding and
Modulation of B Cell Receptor-Mediated Signaling to Drive Efficacy against Experimental Autoimmune Encephalomyelitis. Biomacromolecules 2017, 18 (6), 1893−1907.


(23) Sestak, J. O.; Fakhari, A.; Badawi, A. H.; Siahaan, T. J.; Berkland, C. Structure, size, and solubility of antigen arrays determines efficacy in experimental autoimmune encephalomyelitis. AAPS J. 2014, 16 (6), 1185−1193.


