Soluble Antigen Arrays Efficiently Deliver Peptides and Arrest Spontaneous Autoimmune Diabetes

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Antigen-specific immunotherapy (ASIT) offers a targeted treatment of autoimmune diseases that selectively inhibits autoreactive lymphocytes, but there remains an unmet need for approaches that address the limited clinical efficacy of ASIT. Soluble antigen arrays (SAgAs) deliver antigenic peptides or proteins in multivalent form, attached to a hyaluronic acid backbone using either hydrolysable linkers (hSAgAs) or stable click chemistry linkers (cSAgAs). They were evaluated for the ability to block spontaneous development of disease in a nonobese diabetic mouse model of type 1 diabetes (T1D). Two peptides, a hybrid insulin peptide and a mimotope, efficiently prevented the onset of T1D when delivered in combination as SAgAs, but not individually. Relative to free peptides administered at equimolar dose, SAgAs (particularly cSAgAs) enabled a more effective engagement of antigen-specific T cells with greater persistence and induction of tolerance markers, such as CD73, interleukin-10, programmed death-1, and KLRG-1. Anaphylaxis caused by free peptides was attenuated using hSAgA and obviated using cSAgA platforms. Despite similarities, the two peptides elicited largely nonoverlapping and possibly complementary responses among endogenous T cells in treated mice. Thus, SAgAs offer a novel and promising ASIT platform superior to free peptides in inducing tolerance while mitigating risks of anaphylaxis for the treatment of T1D.

Autoimmune diseases, including type 1 diabetes (T1D), are manifestations of tissue damage caused by autoreactive lymphocytes that have eluded mechanisms of central and/or peripheral tolerance. Antigen-specific immunotherapy (ASIT) aims to expose immune cells to relevant antigens under noninflammatory conditions to tolerize/desensitize specific populations of autoreactive lymphocytes (1). Depending on conditions of exposure (e.g., route, dose, and antigen-presenting cells [APCs] involved), engagement of autoreactive lymphocytes after ASIT can achieve antigen-specific tolerance through deletion, induction of anergy, and/or conversion to regulatory/suppressive populations.

Peptide-based ASITs focus on relevant epitopes, allowing exclusion of unnecessary or unwanted epitopes and inclusion of epitopes from multiple antigens. However, peptides tend to disperse rapidly, requiring relatively high doses to achieve proper engagement of T cells in vivo. Another limiting factor is that several peptides have been shown to cause anaphylactic reactions after repeated administration (2–5), and NOD mice seem more susceptible than other common strains (4). Issues of dispersion, requirement for high doses, and risk of anaphylaxis could be mitigated by using proper delivery systems that facilitate drainage to lymphoid tissues, control release of peptides, and minimize their degradation (6,7). Soluble antigen arrays (SAgAs) constitute a new class of ASIT comprising multiple copies of peptides chemically conjugated onto small hyaluronic acid (HA) chains and providing soluble, multivalent, and linear presentation and delivery of these peptides. Increased molecular weight minimizes wide dispersion into systemic circulation, which can occur for peptides, and instead facilitates drainage to

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lymph nodes (LNs). Their solubility and structure also distinguish SAgAs from particulate forms of delivery vehicles that characterize conventional (immunogenic) vaccines. The multivalent display of antigens by SAgAs enables high-avidity interactions (e.g., proteins linked on SAgAs with B cells), resulting in more effective anergy induction (8). Two forms of SAgAs were evaluated in this study, one in which the linkers for grafted peptides are hydrolysable (hSAgAs) and one in which the peptides are more stably linked by click chemistry (cSAgAs) (9).

We previously showed that SAgAs can ameliorate disease in the experimental autoimmune encephalomyelitis model of multiple sclerosis (10) and that SAgAs can efficiently engage diabetogenic T cells (9). In both patients with T1D and NOD mice, the list of targeted epitopes has been greatly extended from native epitopes to neoepitopes, including posttranslationally modified versions of native epitopes and hybrid peptides (11,12). These recently identified neoepitopes likely play a critical role in the pathogenesis of this disease (13,14). Indeed, these neoepitopes may not be presented during thymic selection of autoreactive T cells, and reactive T cells may therefore circulate at higher frequencies. Moreover, many diabetogenic T cells have a low affinity for native epitopes and may be more strongly engaged by modified epitopes (15) or alternatively by mimotopes (16,17). The therapeutic utility of hybrid insulin peptides (HIPs) and mimotopes has been demonstrated (16–18). In this study, we provide evidence that two SAgAs displaying 2.5HIP (11) and the p79 mimotope (19) are able, in combination, to prevent the onset of disease in NOD mice and that SAgAs, particularly cSAgA, make the delivery of peptides more effective and potentially safer.

RESEARCH DESIGN AND METHODS

Materials

We purchased HA sodium salt (molecular weight 16 kDa) from Lifecore Biomedical; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and N-hydroxysuccinimide from TCI Chemicals; 2-(N-morpholino)ethane-sulfonic acid sodium salt, tris(3-hydroxypropyltriazolylmethyl)amine, and sodium ascorbate from Sigma-Aldrich; and 11-azido-3,6,9-trioxaundecan-1-amine, N-hydroxysuccinimide, and copper (II) sulfate pentahydrate from Acros Organics. Alkyne-functionalized peptides bearing an N-terminal 4-pentynoic acid (homopropargyl) modification, hpP79 and aoP79, were obtained from Biomatik. Additionally, alkyne-functionalized peptides bearing an N-terminal alkyne polyethylene glycol group modification, 2.5HIP and a control p79 with two permuted amino acids inactive on BDC2.5 T cells, were purchased from PolyPeptide. All reagents were used as received without further purification.

Synthesis and Analytic Characterization of hSAgAs and cSAgAs

SAgAs were synthesized by conjugating ~10 peptides to a 16-kDa HA backbone. SAgAs used either a hydrolysable (fSAgA, $hSAgA_{p79}$, and $hSAgA_{2.5HIP}$) or nonhydrolysable ($cSAgA_{p79}$ and $cSAgA_{2.5HIP}$) linker and were produced as previously described (10,20). For uptake studies, FITC was conjugated directly to HA using stable click linker chemistry (two FITC molecules on average per SAgA molecule for both $hSAgA_{p79}$ and $hSAgA_{2.5HIP}$). Azide-functionalized HA, hSAgAs, and cSAgAs were characterized by nuclear magnetic resonance, dynamic light scattering, and circular dichroism, as previously reported (9). Peptide conjugation was determined through gradient reversed-phase high-performance liquid chromatography. Initial studies were focused on cellular effects using the hSAgA format; comparative studies of hSAgAs and cSAgAs then revealed a surprising enhancement of potency with cSAgAs, with greater disease protection in later preclinical studies. This motivated our studies exploring mechanistic differences between peptides in free form, those releasable from HA via hydrolysis (hSAgAs) and those more durably linked to HA (cSAgAs).

Mice

NOD (#001976; Jax), BDC2.5 (#004460; Jax), NOD.CD45.2 (#014149; Jax), and NOD.Foxp3/GFP mice (#025097; Jax) were obtained from The Jackson Laboratory. NOD.Foxp3/GFP mice were bred with BDC2.5 mice and NOD.CD45.2 mice to produce BDC2.5.Foxp3/GFP mice with CD45.2 and/or CD45.1 congenic markers for in vivo tracking. All mice were bred and maintained in the specific pathogen-free animal barrier facility of the Columbia Center for Translational Immunology. Both male and female transgenic/congenic mice (6–16 weeks of age) were used as donors for adoptive transfer experiments. Male and female NOD mice (8–12 weeks of age) were used as recipient mice for adoptive transfer and in vivo uptake studies. Female NOD mice (8 weeks of age) were used in studies involving treatments (preclinical) and MHC tetramers (Tetrs). All procedures were approved by the Columbia University Institutional Animal Care and Use Committee and performed in adherence with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

SAgA Uptake In Vivo

Mice were treated with subcutaneous injection of FITC-conjugated $hSAgA_{p79}$ and $hSAgA_{2.5HIP}$ in combination or separately. Several LNs and spleen were analyzed 6 and 12 h later for FITC signal in various immune populations. For in vitro uptake studies, splenocytes were analyzed at indicated time points after culture with FITC-conjugated hSAgAs. Cell populations were analyzed on a BD Fortessa using a combination of markers for major APC populations (Supplementary Table 1).

SAgA Treatments, Adoptive Transfer, and MHC Tetr Analysis of T-Cell Responses

In all studies, $SAgA_{mix}$ indicates a mix of two different SAgAs, each with one type of peptide. In preclinical studies, mice were treated with saline, HA, free peptide with or without HA, hSAgA, or cSAgA by subcutaneous injection

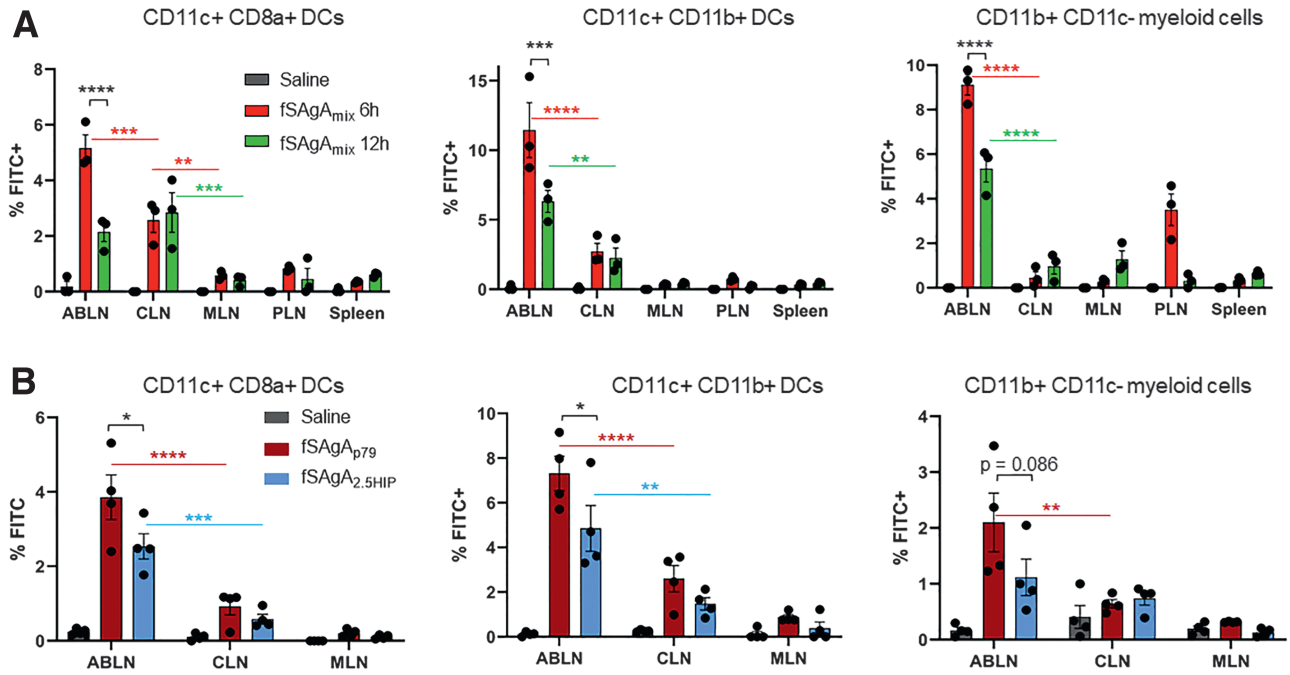

Figure 1— In vivo uptake of SAgAs by different APC populations in lymphoid tissues at varying distance from injection site. *A*: Percentage of cells that have taken up FITC-conjugated hSAgA (fSAgA) within CD11c$^+$ CD8a$^+$ resident dendritic cells (DCs; cDC1), CD11c$^+$ CD11b$^+$ migratory dendritic cells (cDC2), and other myeloid (CD11b$^+$ CD11c$^-$) cells 6 and 12 h after subcutaneous injection of 5 nmol fSAgA$_{mix}$ (equimolar mix of fSAgA with p79 or 2.5HIP) in the neck fold. *B*: Relative uptake of fSAgA$_{p79}$ versus fSAgA$_{2.5HIP}$ (injected at 5 nmol each) by cDC1, cDC2, and other myeloid cells 6 h after subcutaneous injection. Bars represent the mean ± SEM (n = 3–4 per group). Statistical analysis was performed using two-way ANOVA/Tukey for all panels. Other populations analyzed included CD3$^+$ T cells, B220$^+$ B cells and CD11c$^+$ B220$^+$ cells (including plasmacytoid DCs), which showed no or limited uptake of SAgAs in vivo. ABLN, pooled axillary and brachial LNs; CLN, cervical LNs; MLN, mesenteric LNs; PLN, pancreatic LNs.

(behind the neck) weekly starting at 8 weeks of age, and blood glucose levels were monitored weekly. Mice were considered diabetic when their glycemia exceeded 250 mg/dL in two consecutive readings 2 days apart. In adoptive transfer studies, mice were treated subcutaneously with a single dose of saline, free peptide(s), hSAgA, or cSAgA. Free peptides and SAgAs were always injected based on the same equimolar amount of peptides (1 nmol SAgA ~10 nmol peptides). Donor CD4$^+$ CD25$^-$ T cells from pooled LNs and spleen of BDC2.5 or BDC2.5.Foxp3/GFP mice (with CD45.2 congenic marker) were purified using the MojoSort Mouse CD4 T Cell Isolation Kit supplemented with biotinylated anti-CD25 (BioLegend) and then labeled with Violet Cell Proliferation Dye (eBioscience) as previously described (9). These antigen-specific T cells (0.5×10^6 to 1×10^6 cells) were injected intravenously on the same day as the treatment in all adoptive transfer studies, except in studies assessing persistence of antigen presentation, in which T cells were injected 1, 2, or 3 days after treatment with peptide or SAgA. Three days after T-cell transfer, spleen and various LNs were collected for analysis of T-cell responses by flow cytometry to assess surface markers and intracellular cytokines (Supplementary Table 2), along with GFP (Foxp3) and proliferation. Intracellular staining was performed after a 4-h incubation with PMA (0.1 μg/mL), ionomycin (40 μg/mL), brefeldin A (1.5 μg/mL), and monensin (1 μmol/L). To assess endogenous antigen-specific T-cell responses, NOD mice were treated with SAgAs or saline, and responding T cells were identified with I-A^{g7}/p79-Tetrs and/or I-A^{g7}/2.5HIP-Tetrs (Supplementary Table 3) after staining for 1 h at 37°C.

Statistical and Data Analyses

Statistical analysis was performed using GraphPad Prism's two-way ANOVA with Tukey post hoc tests or unpaired t tests, as indicated in each figure legend. Log-rank tests were used in studies of diabetes and anaphylaxis incidence. The threshold for statistical significance was $P < 0.05$ ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). Flow cytometric data were analyzed with FCS Express 7.

Data and Resource Availability

The data sets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request. Research resource identifiers for antibodies are provided in Supplementary Tables 1 and 2.

RESULTS

SAgAs Are Rapidly Taken Up by Professional APCs In Vivo Following a Diffusion Gradient

To assess uptake of SAgAs in vivo, we injected a mix of FITC-conjugated hSAgA$_{p79}$ and hSAgA$_{2.5HIP}$ and assessed

relative uptake by different immune cells in draining and distal LNs and spleen. SAgAs were clearly detected in subsets of migratory and resident $CD11c^+$ dendritic cells ($CD11b^+$ and $CD8a^+$) as well as in other myeloid populations ($CD11b^+$ $CD11c^-$) within 6 h (Fig. 1*A* and Supplementary Fig. 1*A* and *B*), but to a limited extent in other populations (data not shown). The sequence of the two peptides predicted lower solubility of $SAgA_{2.5HIP}$. To determine how this may influence uptake and in vivo diffusion, we compared the relative uptake of the two different SAgAs in vitro and in vivo. Uptake of $SAgA_{p79}$ was consistently greater than that of $SAgA_{2.5HIP}$ at two time points and in the above three APC populations in vitro (Supplementary Fig. 1*C* and *D*). The same consistent difference was observed in draining LNs in vivo (Fig. 1*B*). In all in vivo studies, uptake was highest in draining axillary and brachial LNs followed by cervical LNs (draining more remotely) and lowest in mesenteric and pancreatic LNs (distal) and spleen, indicating a gradient of biodistribution to lymphoid tissues.

SAgAs Elicit More Efficient Stimulation of Antigen-Specific T Cells Than Free Peptides In Vivo

Several T1D-relevant antigenic peptides were considered for SAgA formulation based on known reactivity of diabetogenic T cells, but some of them (e.g., InsB:9–23) were not advanced in SAgA form because of insufficient solubility. p79 and 2.5HIP were among the top candidate peptides that passed our selection criteria and were validated (9) for further investigation. Two forms of SAgAs (hSAgAs and cSAgAs, with peptides grafted using hydrolysable and nonhydrolysable linkers, respectively) were compared against equimolar amounts of free peptides for T-cell responses elicited in the aforementioned lymphoid tissues. NOD mice received BDC2.5 $CD4^+$ T cells, which react to both p79 and 2.5HIP peptides, and were treated with these two peptides in free form or one of the SAgA forms. Both forms of SAgAs induced greater clonal expansion, which was highest in draining LNs followed by distal LNs and spleen (Fig. 2*A* and *B*; only cSAgAs at lower doses), and a higher percentage of proliferated cells (Fig. 2*C* and *D*) compared with free peptides. At a 0.1-nmol dose, cSAgAs induced more proliferation and CD44 upregulation than hSAgAs; this difference disappeared at a 0.5-nmol dose (Fig. 2*A*–*F*). Surprisingly, CD44 was either less induced or downregulated in response to a 0.5-nmol dose of SAgAs relative to peptides (Fig. 2*F*). CD25 induction was most prominent with SAgAs, but only in draining LNs and at a 0.5-nmol dose (Supplementary Fig. 2*A* and *B*).

SAgAs Are Stronger Inducers of Tolerance-Associated Markers Than Free Peptides In Vivo

Both SAgAs induced checkpoint molecules Lag-3 (Supplementary Fig. 2*C* and *D*) and programmed death-1 (PD-1) (Supplementary Fig. 2*E*), whereas peptides induced none or few of these molecules (Supplementary Fig. 2*F*–*H*). Moreover, SAgAs induced pronounced upregulation of CD73, both in terms of percentage and level (Fig. 2*G* and *H* and Supplementary Fig. 2*G*), whereas free peptides did not. CD73 induction was consistently higher with cSAgAs than with hSAgAs. The difference in percentage of $CD73^+$ $FR4^+$ cells (defined as anergic) (21,22) was even more dramatic between free peptides and SAgAs (Supplementary Fig. 2*I*). Treatments had no major effects on recipient T cells (e.g., as shown for CD73/FR4) (Supplementary Fig. 2*J*), although for some measurements, we did observe some marginal upregulations resulting from the presence of responding antigen-specific T cells among recipient cells. Thus, SAgAs induce greater expression of tolerance-related markers CD73/FR4, Lag-3, and PD-1 than free peptides in vivo, in a manner consistent with the gradient of antigen uptake.

SAgAs Promote Foxp3 and Interleukin-10–Expressing T Cells at Different Doses

We assessed the potential of SAgAs to induce regulatory T-cell (Treg) populations, which consist primarily of $Foxp3^+$ and $interleukin\text{-}10^+$ ($IL\text{-}10^+$) Tregs (also known as Tr1). The BDC2.5 $CD4^+$ T cells used in most adoptive transfer experiments carried the Foxp3/GFP reporter. Both free peptides and SAgAs induced Foxp3 expression in donor T cells in a dose-dependent manner (Fig. 3*A* and *B* and Supplementary Fig. 3*A*). Foxp3 was upregulated only at the lower dose (0.1 nmol), and this was most pronounced at distal sites (Fig. 3*A*), where the dose was even lower, according to the antigen gradient. Similar effects were seen with treatment using 2.5HIP peptide or $hSAgA_{2.5HIP}$ only (Supplementary Fig. 3*B* and *C*); proliferation was comparable in all tested tissues (Supplementary Fig. 3*D*). We also assessed interferon-γ (IFN-γ) and IL-10 expression by responding T cells, which was detected only at the higher dose (0.5 nmol). SAgAs increased expression of IL-10 but not of IFN-γ relative to free peptides in donor T cells (Fig. 3*C* and Supplementary Fig. 3*E*), as well as in recipient T cells marginally in the spleen (Fig. 3*D*). Most $IL\text{-}10^+$ T cells did not coexpress IFN-γ (Fig. 3*C*). As a result, the ratio of IL-10 to IFN-γ was significantly higher with SAgAs than with free peptides in antigen-specific $CD4^+$ T cells (Fig. 3*E*) but was not changed in recipient polyclonal $CD4^+$ T cells (Fig. 3*F*). In sum, SAgAs induced $Foxp3^+$ Tregs at a lower dose and in distant lymphoid tissues and $IL\text{-}10^+$ Tr1 cells at a higher dose, in which case SAgAs were significantly more efficient than the same amount of peptides in free form.

SAgAs Prolong Persistence of Peptide Presentation In Vivo

We wondered if the differences in T-cell responses between SAgAs and free peptides could be linked to more prolonged presentation of peptides, indirectly reflecting an extended half-life of the peptides in vivo. Given the superior therapeutic potential of cSAgA (presented next), we compared free peptide versus cSAgA administration. To

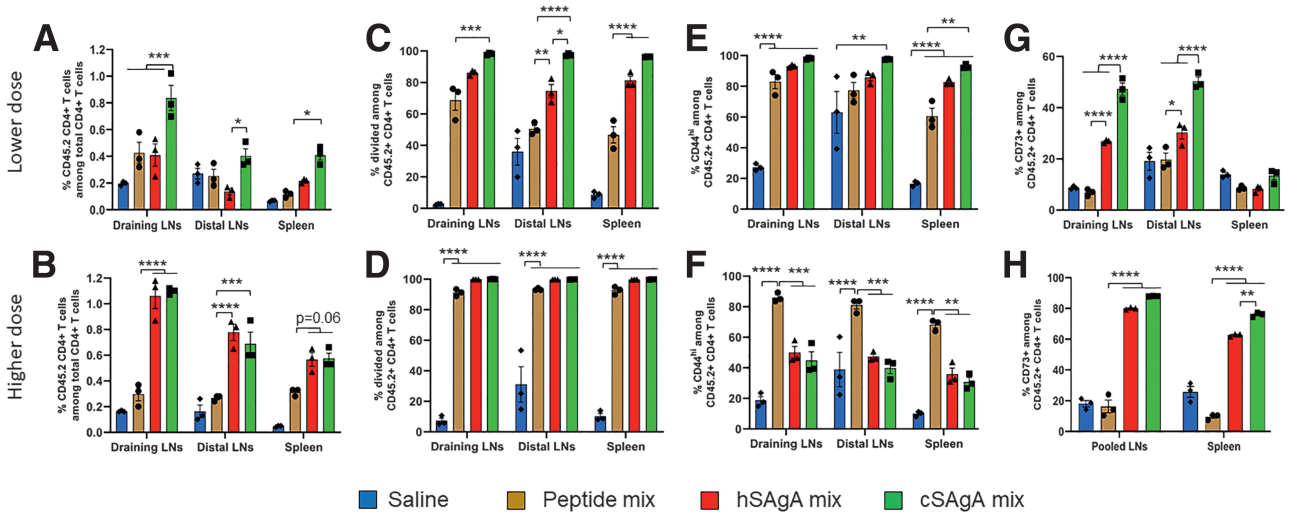

Figure 2— Response of adoptively transferred BDC2.5 CD4$^+$ T cells to SAgAs or free peptides in different lymphoid tissues. Responses were measured in LNs and spleen by flow cytometry 3 days after a single treatment at lower dose (1 nmol peptides and 0.1 nmol SAgAs; upper panels) or higher dose (5 nmol peptides and 0.5 nmol SAgAs; lower panels). Dose shown is for each peptide (p79 and 2.5HIP) in the mix. Analysis was done on CD45.2$^+$ CD4$^+$ T cells (donor BDC2.5 T cells), and data are presented as follows: percentages of donor CD45.2$^+$ cells among total CD4$^+$ T cells (*A* and *B*), divided cells (diluted proliferation dye) among donor T cells (*C* and *D*), CD44^{hi} among donor T cells (*E* and *F*), and CD73$^+$ among donor T cells (*G* and *H*). Draining LNs were pooled axillary, brachial, and cervical; distal LNs were pooled mesenteric and pancreatic (PLN); pooled LNs had all, including pancreatic. Bars represent the mean ± SEM from three mice per group. Statistical analysis was performed using two-way ANOVA/Tukey for all panels. Higher background response to saline in distal LNs is due to recognition of endogenous antigens by BDC2.5 T cells in PLNs. In recipient CD4$^+$ T cells, percentage of CD44^{hi} cells was 10–25%, and percentage of CD73$^+$ cells was <0.5%. There was no significant difference for both markers in recipient T cells between the four groups.

detect antigen presentation, antigen-specific donor T cells were transferred 1, 2, or 3 days after treatment with free peptide or SAgA at equivalent dose. Antigen presentation from free peptides decreased significantly each day based on proliferation and CD44 upregulation of donor T cells, whereas antigen presentation from SAgAs remained stable for at least 3 days in draining LNs (Fig. 3*G* and *H*). As a control, there was no difference and no change over time in pancreatic lymph nodes, where the presented antigen was predominantly from islets (Fig. 3*I*).

Two SAgAs in Combination Efficiently Prevent Diabetes With Fewer Adverse Effects Than Their Corresponding Free Peptides

Our preclinical evaluation of SAgAs to prevent disease in NOD mice was first done with hSAgAs. Treatment with a mixture of hSAgAs (hSAgA$_{mix}$; p79 and 2.5HIP at 2.5 nmol each) demonstrated complete protection until 18 weeks of age, whereas more than half of the control mice had diabetes by that time (Fig. 4*A*). However, some hSAgA$_{mix}$-treated mice started to develop anaphylaxis at 18 weeks (fatal for three mice), and the treatment was discontinued. After treatment discontinuation, protection was gradually lost, and mice developed diabetes, although with a significant delay relative to controls. Here, HA alone had a marginal protective effect, which was not seen in later experiments (e.g., Fig. 4*B*). In a follow-up study, we added a group with a lower dose (0.5 nmol of each hSAgA) and longer treatment period. Mice treated with 0.5 nmol hSAgA$_{mix}$ never developed anaphylaxis and had partial but not significant protection from disease, with half of the mice remaining diabetes free at 33 weeks (Fig. 4*B*). Incidence of disease remained low (70% mice were diabetes free by 36 weeks of age) in the 2.5-nmol group compared with saline and HA controls, suggesting that dose and prolonged dosing were important for long-lasting protection. However, anaphylaxis caused the loss of two mice at 16 weeks, and dosing was halved (Fig. 4*B*). At 22 weeks, anaphylaxis caused the loss of another three mice, at which point the treatment was ceased. In contrast, each single hSAgA tested separately (2.5 nmol each) had no significant effect on disease incidence on their own and did not induce anaphylaxis (Fig. 4*C*). Reminiscent of previous reports on other peptides, our peptide mix elicited an anaphylactic reaction, and we therefore ascertained whether the SAgA form of delivery was responsible. We treated NOD mice with a mix of free peptides, hSAgAs, or cSAgAs (all with p79 and 2.5HIP peptides), with dose tapering from 2.5 nmol of each SAgA to 1.5 nmol after five injections and to 0.5 nmol after four injections. Incidence of anaphylaxis was more severe with free peptides than with hSAgA$_{mix}$ (Fig. 4*D*), whereas cSAgA$_{mix}$ did not induce any lethal anaphylaxis (Fig. 4*D*). Mice treated with cSAgA$_{mix}$ did not develop diabetes during dosing at 2.5–1.5 nmol and had significantly reduced incidence of hyperglycemia during 0.5-nmol dosing, with 70% of mice diabetes free and healthy at 30 weeks of age (Fig. 4*E*). Thus, not only was SAgA not responsible for peptide-induced anaphylaxis, it minimized anaphylaxis. Overall, cSAgA treatment was most effective clinically while averting these adverse effects.

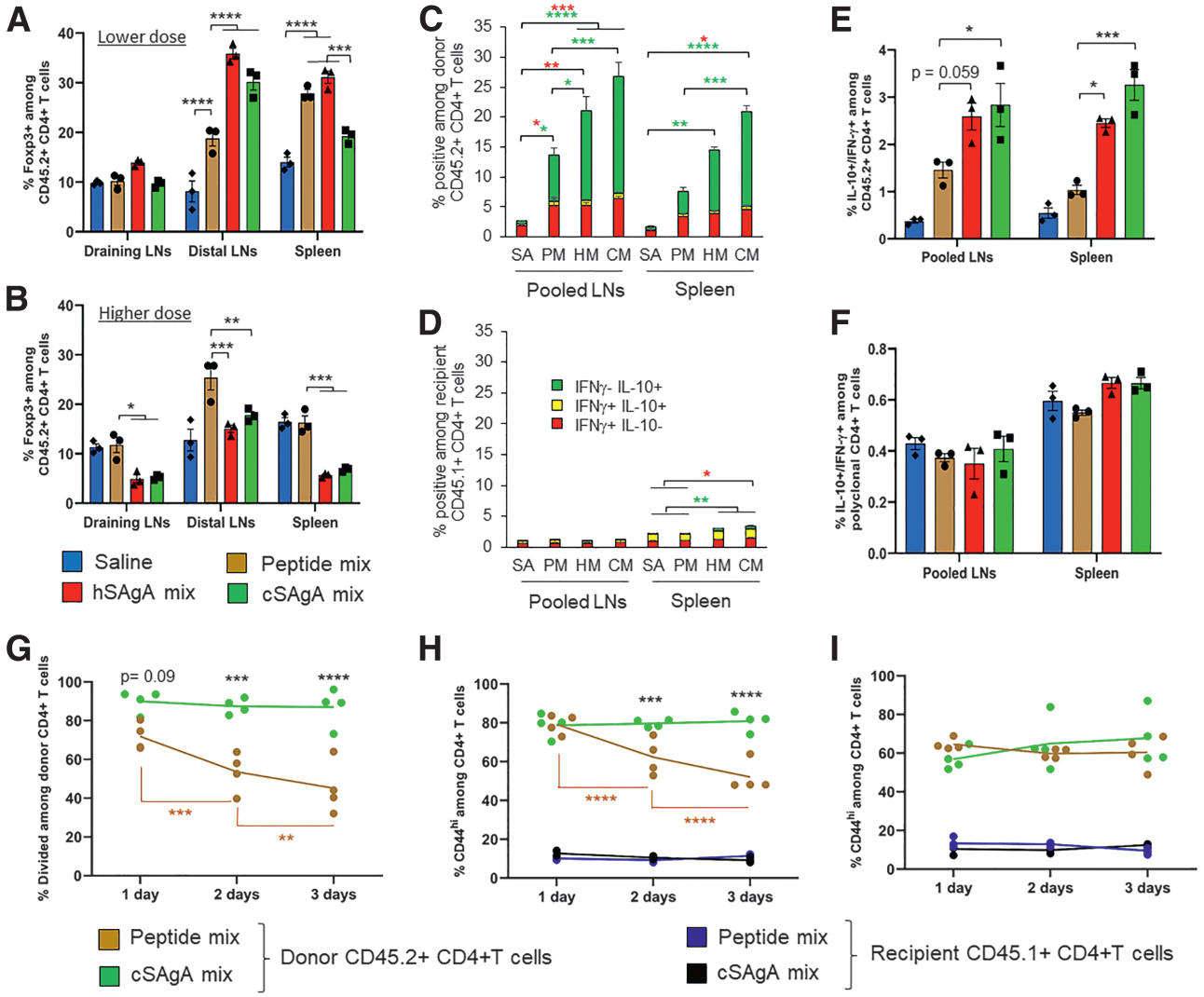

Figure 3— Response of adoptively transferred BDC2.5 CD4$^+$ T-cell to SAgAs or free peptides. Recipient mice received a single treatment at the lower dose (1 nmol peptides and 0.1 nmol SAgAs) (*A*) and higher dose (5 nmol peptides and 0.5 nmol SAgAs) (*B–I*). Dose given is for each peptide (p79 and 2.5HIP) in the mix. *A* and *B*: Percentage of Foxp3$^+$ (GFP$^+$) cells among CD45.2$^+$ CD4$^+$ donor T cells (expressing the Foxp3/GFP reporter) in draining LNs (axillary, brachial, and cervical), distal LNs (mesenteric and pancreatic [PLN]), and spleen by flow cytometry 3 days after treatment. Foxp3 induction significantly increased from draining to distal LNs (***peptides, ****hSAgAs and cSAgAs) and from draining LNs to spleen (****peptides and hSAgAs, ***cSAgAs) at the lower dose. *C–F*: Cytokine responses (to higher dose) were measured in pooled LNs and spleen by flow cytometry 3 days after treatment, after short PMA/ionomycin restimulation. Percentage of IFN-γ single positive (red), IFN-γ IL-10 double positive (yellow), and IL-10 single positive (green) is shown for donor CD45.2$^+$ CD4$^+$ T cells (*C*) and recipient CD45.1$^+$ CD4$^+$ T cells (*D*) in response to saline (SA), peptide mix (PM), hSAgA$_{mix}$ (HM), and cSAgA$_{mix}$ (CM). *E* and *F*: Ratio of percentages of IL-10$^+$ to IFN-γ$^+$ T cells among donor CD45.2$^+$ CD4$^+$ T cells (*E*) and recipient CD45.1$^+$ CD4$^+$ T cells (*F*). Bars represent the mean ± SEM from three mice per group. Pooled LNs comprised axillary, brachial, cervical, mesenteric, and PLNs. In recipient CD4$^+$ T cells, ratio of percentages of IL-10$^+$ to IFN-γ$^+$ T cells was 0.25–0.5 in LNs and 0.5–0.7 in spleen. No cytokine expression above background (based on recipient T cells) was detected with the lower dose. *G–I*: Persistence of antigen presentation in vivo at different time points after free peptide mix or cSAgA$_{mix}$ delivery at equivalent dose, measured based on proliferation (*G*) and CD44 upregulation (*H* and *I*) of donor (CD45.2$^+$) T cells adoptively transferred at these time points. Responses were measured 3 days later in draining (axillary, brachial, and cervical) LNs (*G* and *H*) and in PLNs (*I*). Statistical analysis was performed using two-way ANOVA/Tukey for all panels. *G* and *H*: Black stars show comparison between peptide mix and cSAgA$_{mix}$, whereas beige stars show comparison between time points.

SAgAs Upregulate Tolerogenic Markers and IL-10 in Endogenous Antigen-Specific T Cells

To address the immunologic effects of SAgAs on endogenous (polyclonal) antigen-specific T cells in NOD mice, we used MHC Tetrs to analyze p79-reactive CD4$^+$ T cells first. Their frequency was significantly increased after multiple doses, ranging from two to six doses (Supplementary Fig. 4*A* and *B* showing results after six doses of hSAgA$_{mix}$), and they persisted as late as 21 weeks after treatment was discontinued (Supplementary Fig. 4*C* and *D*). These antigen-specific T cells displayed marked upregulation of CD73 and FR4 in response to six weekly doses of hSAgA$_{mix}$, particularly with the 2.5-nmol dose relative to 0.5 nmol (Fig. 5*A* and *B*). Similarly, expression of PD-1 was also

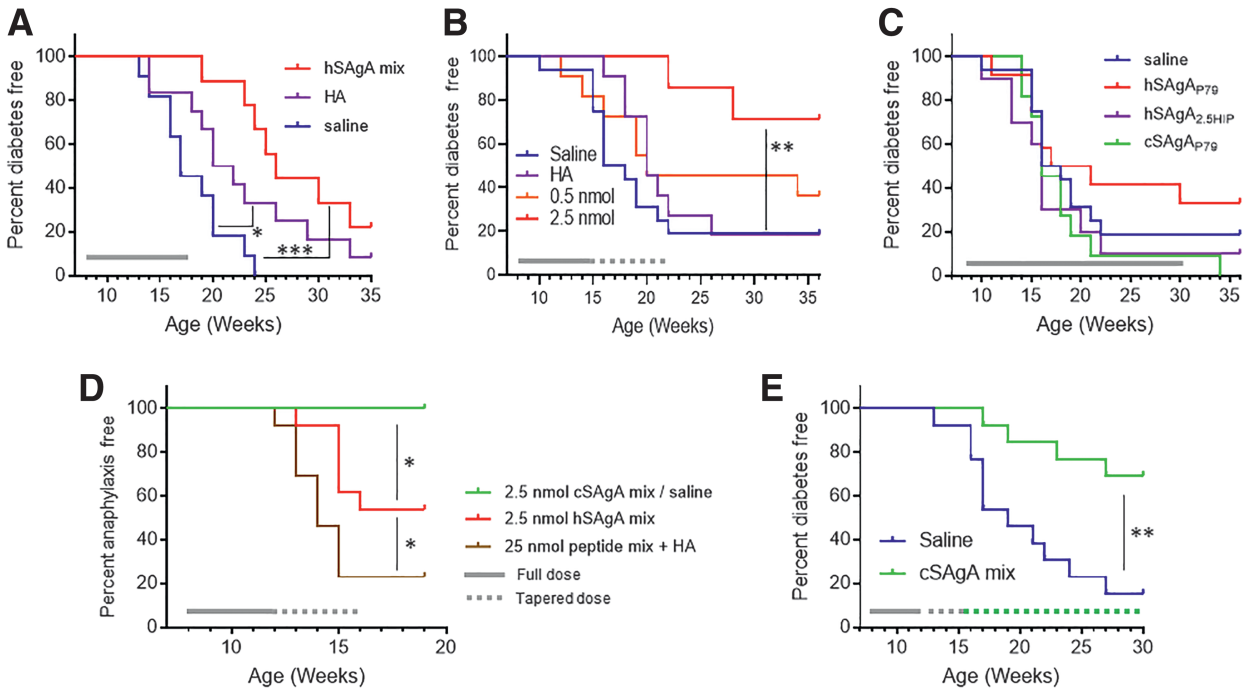

Figure 4—$SAgA_{p79}$ and $SAgA_{2.5HIP}$, when combined, protect mice from diabetes development and attenuate anaphylaxis caused by free peptides. *A*: Incidence of diabetes onset in NOD mice treated with saline ($n = 11$), 2.5 nmol HA ($n = 12$), or 2.5 nmol $hSAgA_{mix}$ each ($n = 9$ after three mice were lost as a result of anaphylaxis at 18 weeks). *B*: Incidence of diabetes onset in NOD mice treated with saline ($n = 16$), 5 nmol HA ($n = 12$), 0.5 nmol $hSAgA_{mix}$ each ($n = 12$) or 2.5 nmol $hSAgA_{mix}$ each ($n = 7$ after two mice lost as a result of anaphylaxis at 16 weeks and three mice at 22 weeks). Significance shown is between 2.5 nmol hSAgA and both saline and HA treatments. In this experiment, the difference between hSAgA and HA did not reach significance ($P = 0.078$). *C*: Incidence of diabetes onset in NOD mice treated with saline ($n = 16$), $hSAgA_{p79}$ ($n = 12$), $hSAgA_{2.5HIP}$ ($n = 12$), or $cSAgA_{p79}$ ($n = 12$) at 2.5 nmol each. Gray line indicates the period of weekly treatment at full dose; dashed line indicates a half-dose. Data shown in panels *B* and *C* are from the same experiment but shown separately to make the graphs less busy; only the control group is replicated between the two panels. *D* and *E*: Incidence of anaphylaxis (*D*) and incidence of diabetes (*E*) in NOD mice treated with saline ($n = 13$), peptide mix ($n = 13$), $hSAgA_{mix}$ ($n = 13$), or $cSAgA_{mix}$ ($n = 13$), with the mix of AVPRLWVRME and LQTLALWSRMD peptides at 2.5 nmol each (SAgAs) or 25 nmol each (free peptides). Dose tapering from 2.5 to 1.5 nmol for SAgAs (25–15 nmol for free peptides) was applied after five doses (dashed gray line) and to 0.5 nmol after four more doses (dashed green line). Statistical analysis was performed using log-rank test for all panels.

significantly increased (50% in treated mice as compared with 5% in control mice) (Fig. 5*C*). These effects were also evident after three weekly doses of $SAgA_{p79}$ (Fig. 5*D–F*); however, $cSAgA_{p79}$ was more potent than $hSAgA_{p79}$ at inducing CD73/FR4 (Fig. 5*D* and *E*) and PD-1 (Fig. 5*F*). CD73 upregulation persisted after 21 weeks of treatment interruption (Supplementary Fig. 4*E*). At the earliest time point tested (after two injections, 3 days apart), the effect of $hSAgA_{p79}$ on upregulation CD73/FR4 on p79-reactive T cells was limited, whereas that of $cSAgA_{p79}$ was already pronounced (Supplementary Fig. 4*F* and *G*). Although non-specific T cells preferentially express IFN-γ over IL-10, p79-reactive T cells comprised more IL-10^+ than IFN-γ^+ cells after six weekly treatments (Fig. 6*A–C*). Consistent with surface markers, frequency of cells expressing these cytokines increased with dose of hSAgAmix (Fig. 6*B*). After only three weekly treatments, the effect of $hSAgA_{p79}$ relative to control mice was not yet significant (Fig. 6*D–F*), whereas $cSAgA_{p79}$ had induced significantly more IL-10 single-positive cells among p79-reactive T cells than $hSAgA_{p79}$ in both LNs and spleen (Fig. 6*E*). The increase in IL-10 single-positive p79-Tetr$^+$ cells was most pronounced in LNs.

Antigen-Specific T-Cell Responses to p79 and 2.5HIP In Vivo Are Largely Nonoverlapping

The p79 and 2.5HIP peptides share similar COOH-terminal residues (LWVRME for p79 and LWSRMD for 2.5HIP), which are recognized by the BDC2.5 T-cell clone. Using an in vitro stimulation assay with NOD splenocytes spiked with BDC2.5 splenocytes (9), we found that $hSAgA_{2.5HIP}$ was ∼10-fold less active than $hSAgA_{p79}$ (Supplementary Fig. 5). We addressed whether endogenous p79- and 2.5HIP-reactive T cells were generally cross-reactive like the BDC2.5 clone. Using both p79-Tetr and 2.5HIP-Tetr, we tracked the response of both populations in response to $cSAgA_{p79}$, $cSAgA_{2.5HIP}$, and $cSAgA_{mix}$. First, p79-Tetr$^+$ and 2.5HIP-Tetr$^+$ T cells were largely nonoverlapping, and few T cells costained for both Tetrs, regardless of treatment (Fig. 7*A*). Second, clonal expansion was only significant for p79-Tetr$^+$ T cells in response to p79 alone or in the mix, but not to 2.5HIP alone (Fig. 7*B*), suggesting lack of cross-reactivity. Third, consistent with previous experiments,

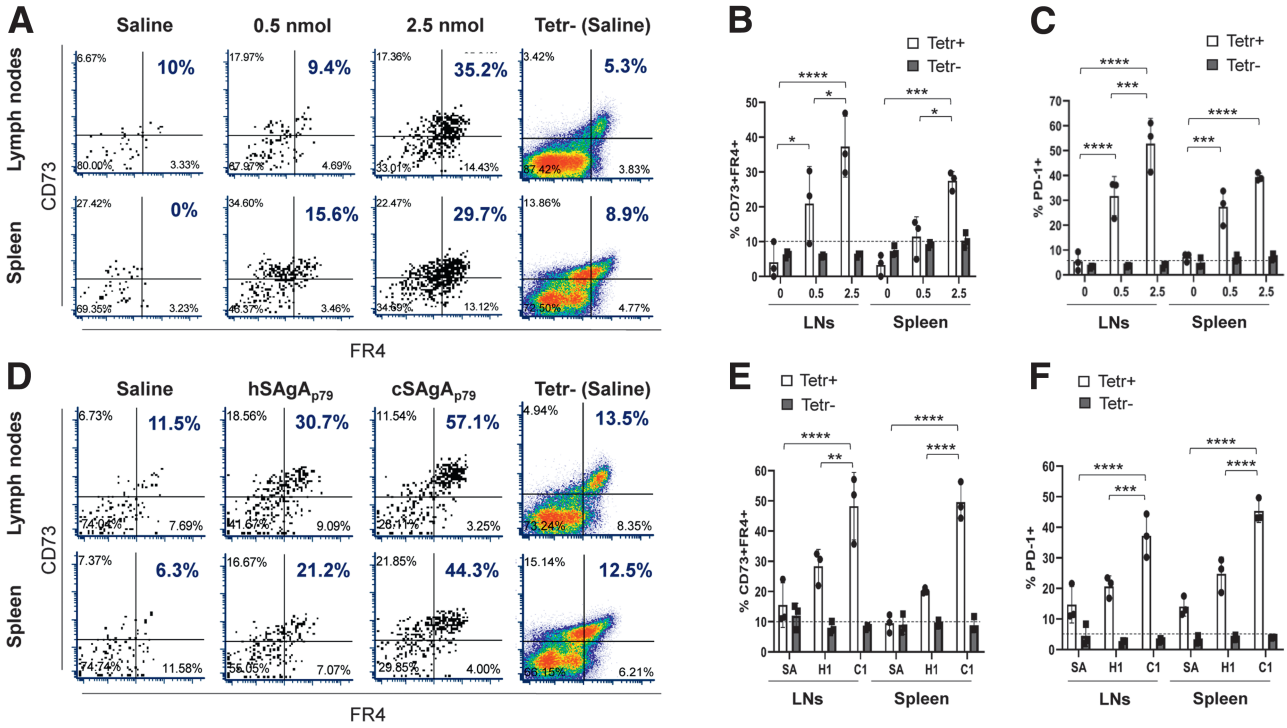

Figure 5—Tolerance-associated markers induced on antigen-specific T cells by SAgAs in vivo. *A*: Expression of CD73 and FR4 on p79-Tetr$^+$ and Tetr$^-$ CD4$^+$ T cells after 6 weekly doses of hSAgA$_{mix}$ (0.5 or 2.5 nmol each) analyzed 3 days after the last dose by flow cytometry. *B* and *C*: Percentage of CD73$^+$ FR4$^+$ cells (*B*) and PD-1$^+$ cells (*C*) among p79-Tetr$^+$ CD4$^+$ T cells and Tetr$^-$ CD4$^+$ T cells. 0, saline; 0.5, hSAgA$_{mix}$ (0.5 nmol each); 2.5, hSAgA$_{mix}$ (2.5 nmol each). *D*: Expression of CD73 and FR4 on p79-Tetr$^+$ and Tetr$^-$ CD4$^+$ T cells after 3 weekly doses of SAgAs (2.5 nmol) analyzed 3 days after the last dose by flow cytometry. *E* and *F*: Percentages of CD73$^+$ FR4$^+$ cells (*E*) and PD-1$^+$ cells (*F*) among p79-Tetr$^+$ CD4$^+$ T cells and Tetr$^-$ CD4$^+$ T cells. Data graphed in panels *B*, *C*, *E*, and *F* show the mean ± SEM of three mice per group. Statistical analysis was performed using two-way ANOVA/Tukey for all panels. SA, saline; H1, hSAgA$_{p79}$; C1, cSAgA$_{p79}$.

cSAgA$_{p79}$ strongly induced CD73$^+$ cells (Fig. 7*C* and *D*) and CD73$^+$ FR4$^+$ cells (Supplementary Fig. 6*A* and *B*). Upregulation of CD73/CD44 (Fig. 7*C* and *D*) and KLRG1 (Fig. 7*E* and *F*) was evident on both p79- and 2.5HIP-reactive T cells in response to their respective epitope, confirming that both populations were effectively and specifically stimulated by their respective antigens, although cSAgA$_{p79}$ was more potent. Upregulation of PD-1 was also evident in p79-Tetr$^+$ T cells, without reaching significance (Supplementary Fig. 6*C*). The response of p79-reactive T cells was dominated by IL-10$^+$ cells, whereas that of 2.5HIP-reactive T cells did not change and, in the spleen, was largely dominated by IFN-γ$^+$ cells (Fig. 8). In 2.5HIP-specific T cells, a majority of KLRG1$^+$ cells expressed IFN-γ, whereas in p79-reactive T cells, they did not (Supplementary Fig. 6*D*). No significant de novo induction of Foxp3$^+$ cells among p79- and 2.5HIP-reactive T cells was seen; however, stimulation of existing Foxp3$^+$ T cells (based on CD44 coexpression) was significant with cSAgA$_{mix}$, but not with the free peptide mix (Supplementary Fig. 7*A* and *B*). The free peptide mix had no significant effect on IL-10 induction or ratio of IL-10 to IFN-γ, unlike cSAgA$_{mix}$ (Supplementary Fig. 7*C* and *D*). Finally, an effect on the frequency and stimulation of CD4$^+$ T cells specific to other β-cell antigens was not seen (Supplementary Fig. 8).

DISCUSSION

SAgAs constitute a novel and effective antigen delivery platform that is soluble and avoids the pitfalls of particle-based approaches. The solubility and size of SAgAs promote drainage to lymphoid tissue, and their multivalency disarms autoimmune cells, making SAgAs uniquely suitable for tolerance induction (6,8,10,23,24). In fact, structural features of SAgAs harken back to seminal work pointing to soluble multimeric haptens <100 kDa as inhibitors of immunity and larger or insoluble multimers as instigators (25). Here, we show that SAgAs promote more effective and persistent engagement of autoreactive T cells than free peptides in vivo, particularly cSAgAs. This is in part attributed to the higher molecular weight of SAgAs, which enables more efficient drainage to lymphoid tissues. T-cell responses correlated with the extent of SAgA uptake, highest in draining LNs and lower in more distal lymphoid tissues. One exception was the induction of Foxp3, which was greatest with lower administered doses and at more distal sites. This observation is consistent with lower (subimmunogenic) doses of insulin mimotopes that preferentially induced Foxp3$^+$ Tregs (16,17). Over a certain threshold of stimulation, Foxp3 is no longer induced, and IL-10$^+$ Tr1 cells seem to take over. However, at a dose supporting IL-10 induction, cSAgAs (but not free peptides)

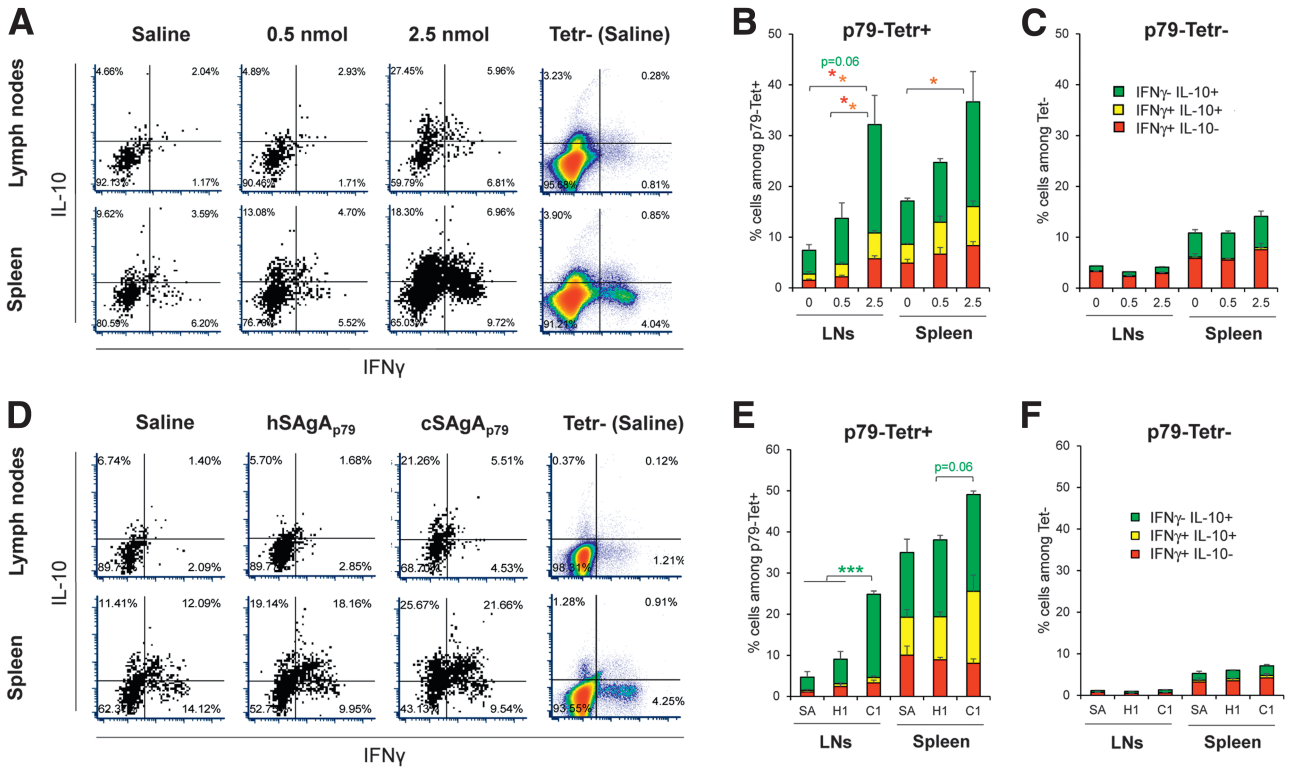

Figure 6—Cytokines induced on antigen-specific T cells by SAgAs in vivo. *A*: Expression of IFN-γ and IL-10 in p79-Tetr$^+$ and Tetr$^-$ CD4$^+$ T cells after 6 weekly doses of hSAgA$_{mix}$ (0.5 or 2.5 nmol each) analyzed 3 days after the last dose by flow cytometry. *B* and *C*: Percentages of IFN-γ and IL-10 single- and double-positive cells in p79-Tetr$^+$ CD4$^+$ T cells (*B*) and Tetr$^-$ CD4$^+$ T cells (*C*). 0, saline; 0.5, hSAgA$_{mix}$ (0.5 nmol each); 2.5, hSAgA$_{mix}$ (2.5 nmol each). *D*: Expression of IFN-γ and IL-10 on p79-Tetr$^+$ and Tetr$^-$ CD4$^+$ T cells after 3 weekly doses of SAgA (2.5 nmol) analyzed 3 days after the last dose by flow cytometry. *E* and *F*: Percentages of IFN-γ and IL-10 single- and double-positive cells in p79-Tetr$^+$ CD4$^+$ T cells (*E*) and Tetr$^-$ CD4$^+$ T cells (*F*). *B*, *C*, *E*, and *F*: Significant changes for IFN-γ$^-$ IL-10$^+$ populations are indicated with green stars, for IFN-γ$^+$ IL-10$^-$ populations with red stars, and for both IFN-γ$^+$ IL-10$^+$ populations with orange stars. Statistical analysis was performed using two-way ANOVA/Tukey for all panels. SA, saline; H1, hSAgA$_{p79}$; C1, cSAgA$_{p79}$.

can stimulate existing p79- and 2.5HIP-reactive Foxp3$^+$ CD4$^+$ T cells, which may contribute to overall protection.

A characteristic effect of SAgAs was the dramatic upregulation of CD73 on both adoptively transferred and endogenous antigen-specific T cells, particularly with cSAgAs, whereas this upregulation was not induced by free peptides. CD73 is an ectonucleotidase that, in concert with CD39, generates extracellular adenosine, an immunosuppressive metabolite (26). Upregulation of CD73 on T cells has been associated with disease protection in NOD mice (22), and T cells that coexpress CD73 and FR4 have previously been defined as anergic (21). Interestingly, it has been reported that such T cells may become regulatory if they also express Nrp-1 (21,27); therefore, Nrp-1 would be an important marker to follow in future SAgA studies. Furthermore, KLRG1 was found to be induced on both p79- and 2.5HIP-reactive CD4$^+$ T cells and PD-1 only on the p79-reactive ones (possibly requiring stronger stimulation). KLRG1 has recently emerged as a new checkpoint molecule for T cells and NK cells (28–30), and both KLRG-1 and PD-1 have been associated with anergic and exhausted-like T-cell phenotypes achieved during immunotherapy in patients with T1D (31–33). Our studies demonstrate the potential of SAgAs to improve delivery of self-peptides and the tolerogenic engagement of autoreactive T cells, potentially resulting in anergy and/or exhaustion of specific T cells. Because persistent antigen exposure may promote exhaustion, the prolonged antigen presentation observed after SAgA treatment may contribute to this result. If antigen presentation lasts close to a week, weekly SAgA treatment may indeed result in persistent antigen exposure.

Although both peptides stimulated BDC2.5 T cells, the responses of endogenous p79- and 2.5HIP-reactive T cells were largely nonoverlapping in staining and reactivity. Indeed, MHC-II Tetrs loaded with these two peptides identified discreet populations of CD4$^+$ T cells, and to our surprise, few cells were stained with both Tetrs, and cross-reactivity in T-cell responses was negligible. Moreover, SAgA$_{p79}$ or SAgA$_{2.5HIP}$ elicited different responses. The p79 mimotope is the most potent synthetic peptide, identified from a library of artificial peptides, that stimulates BDC2.5 T cells (22). In contrast, 2.5HIP is thought to be the most potent natural peptide for BDC2.5 T cells identified to date (14). Delivery of the same 2.5HIP peptide using nanoparticles was recently shown to efficiently

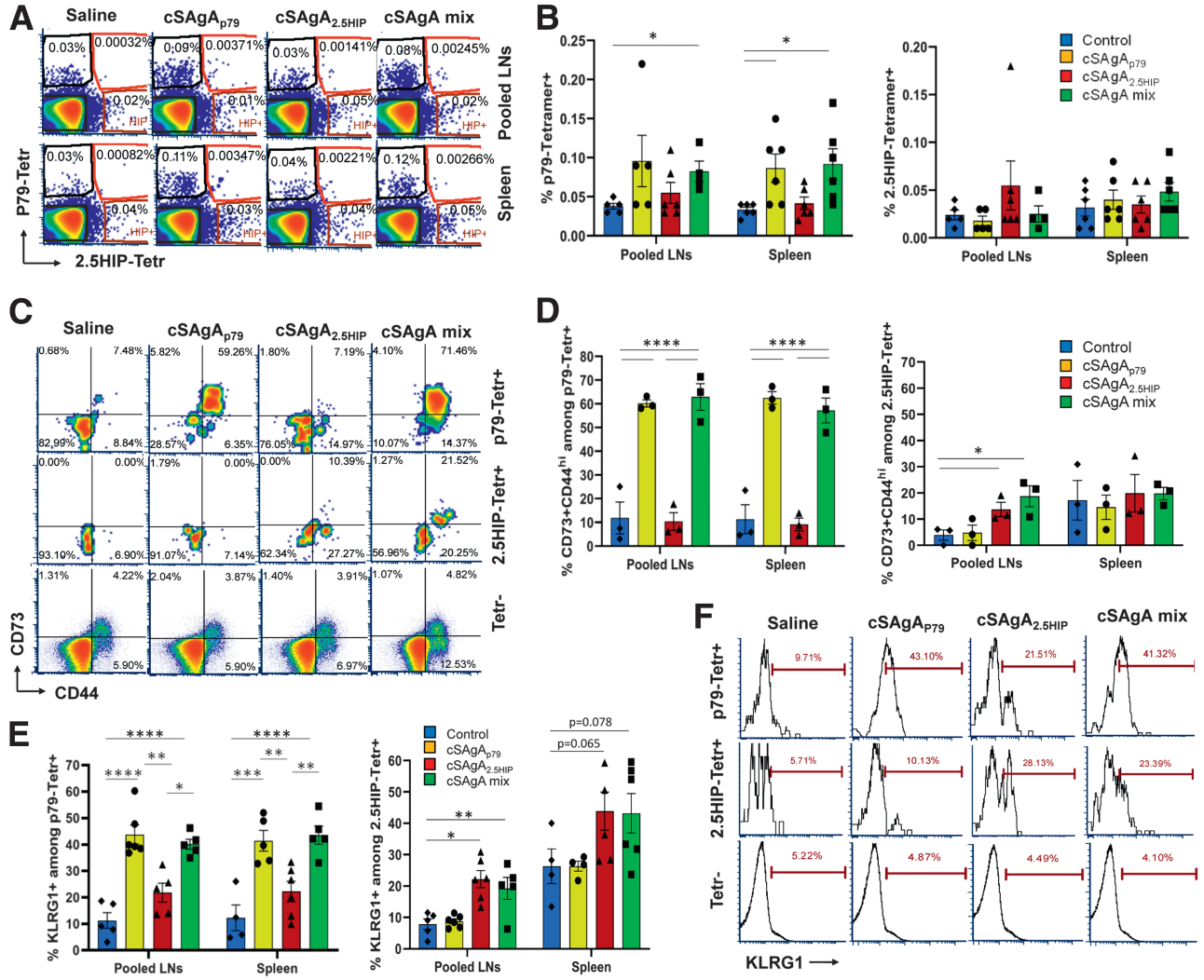

Figure 7—Differential response of p79- and 2.5HIP-reactive T cells to cSAgAs in vivo. *A* and *B*: Detection of p79-Tetr$^+$ and 2.5HIP-Tetr$^+$ T cells in NOD mice treated with saline, cSAgA$_{p79}$, cSAgA$_{2.5HIP}$, or cSAgA$_{mix}$ (2.5 nmol per antigen, 3 days after two doses), showing representative density plots (*A*) and percentage of Tetr$^+$ cells in bar graphs (*B*) from pooled LNs and spleen, all gated on CD4$^+$ T cells. Cells stained by both Tetrs represent only 3–5% of each single Tetr$^+$ population in all control and treatment groups. *C* and *D*: Expression of CD73 on p79-Tetr$^+$ and 2.5HIP-Tetr$^+$ CD4$^+$ T cells, showing representative density plots against CD44 from pooled LNs as example (*C*) and percentage of CD73$^+$ among Tetr$^+$ cells in bar graphs (*D*). *E* and *F*: Expression of KLRG1 on p79-Tetr$^+$ and 2.5HIP-Tetr$^+$ CD4$^+$ T cells, showing percentage of KLRG1$^+$ among Tetr$^+$ cells in bar graphs (*E*) and representative histograms from pooled LNs as example (*F*). All graphs (*B*, *D*, and *E*) show the mean ± SEM of four to six mice per group. Statistical analysis was performed using two-way ANOVA/Tukey (*D* and *E*, p79-Tetr) or unpaired *t* test (*B*, all; *D* and *E*, 2.5HIP-Tetr; ANOVA not significant).

prevent disease in NOD.SCID mice transferred with BDC2.5 T cells (monoclonal model of disease development) (21). Multiple administrations of SAgA$_{p79}$ led to substantial expansion of p79-reactive T cells, with a high proportion of CD73$^+$ FR4$^+$ cells and IL-10$^+$ Tr1 cells predominating over IFN-γ$^+$ Th1 cells. In contrast, the phenotype of 2.5HIP-reactive T cells remained largely unchanged after two doses, with a predominance of IFN-γ production over IL-10. The induced KLRG1$^+$ cells were primarily IFN-γ$^+$ in 2.5HIP-reactive T cells and IFN-γ$^-$ in p79-reactive T cells. There are multiple possible explanations for these disparate responses. First, when delivered as SAgAs, the p79 epitope is ∼10 times more active on BDC2.5 T cells than 2.5HIP in vitro. Second, the 2.5HIP peptide has lower water solubility than p79 (as determined by the PepCalc.com calculator), which can influence overall SAgA solubility and its biodistribution in vivo. Indeed, our data showing reduced uptake of FITC-SAgA$_{2.5HIP}$ may reflect more limited in vivo diffusion because of the difference in solubility. Third, 2.5HIP is a natural antigen in NOD mice; therefore, 2.5HIP-reactive T cells may have been more antigen experienced than p79-reactive T cells, which may explain the higher initial frequency of IFN-γ$^+$ cells among 2.5HIP-reactive T cells. Thus, the engagement of specific T cells to peptides from SAgA$_{p79}$ and SAgA$_{2.5HIP}$ may differ as a result of peptide

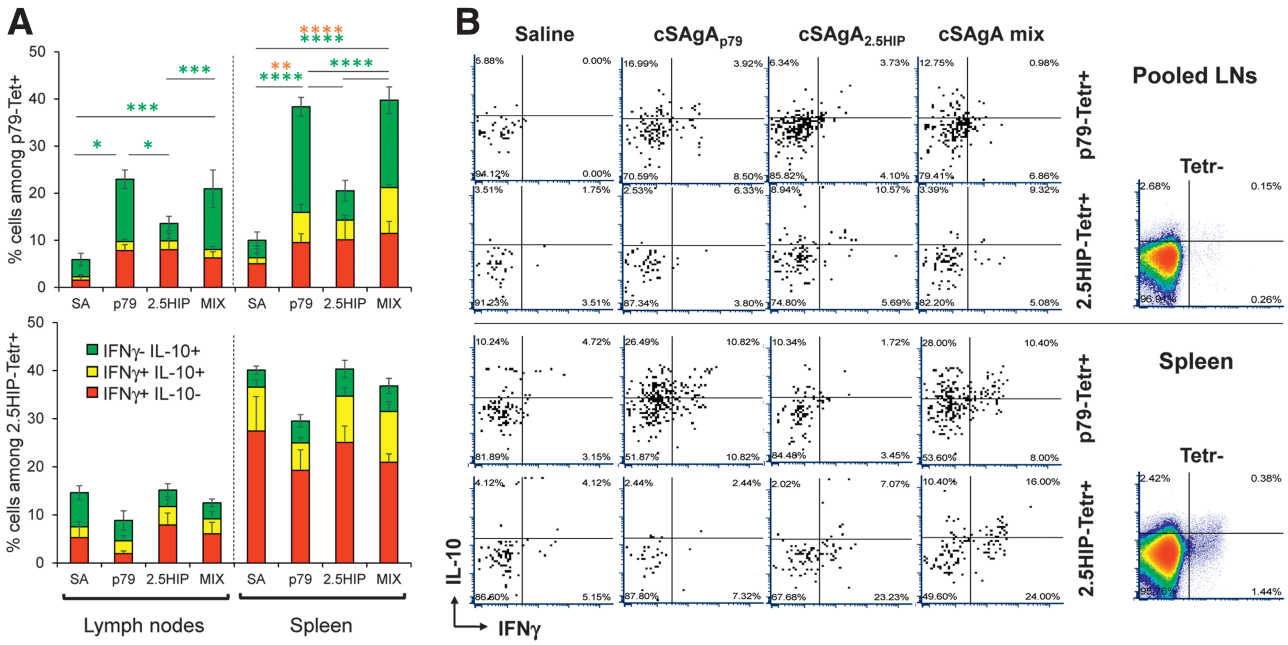

Figure 8—Differential cytokine response of p79- and 2.5HIP-reactive T cells to cSAgAs in vivo. Expression of IFN-γ and IL-10 on p79-Tetr$^+$ and 2.5HIP-Tetr$^+$ CD4$^+$ T cells from pooled LNs and spleen, showing percentages of IFN-γ single-positive, IL-10 single-positive, and IFN-γ IL-10 double-positive cells among Tetr$^+$ cells in stacked bar graph (*A*) and representative dot plots (*B*). Graphs (*A*) show the mean ± SEM of four to six mice per group. Statistical analysis was performed using two-way ANOVA/Tukey for all panels. Significant changes for IFN-γ$^-$ IL-10$^+$ populations are indicated with green stars, for IFN-γ$^+$ IL-10$^-$ populations with red stars, and for both IFN-γ$^+$ IL-10$^+$ populations with orange stars. Although staining of intracellular cytokines was compatible with Tetr$^+$ T cells, the harsher protocol for nuclear factor staining made it difficult to perform reliable detection of Foxp3 in Tetr$^+$ T cells.

properties (e.g., MHC binding affinity and T-cell receptor reactivity), solubility/dispersion properties affecting local antigen dose, and prior antigen exposure. This differential response to two peptides is interesting and highlights the importance of assessing the mechanisms of action over multiple antigens for the same delivery approach.

A differentiating feature of SAgAs is the apparent complementarity of SAgA$_{p79}$ and SAgA$_{2.5HIP}$ (at 2.5 nmol each) in arresting disease development in NOD mice. Treatment with SAgA$_{p79}$ or SAgA$_{2.5HIP}$ alone (2.5 nmol) had no significant effect on disease incidence. Even the mixture at 0.5 nmol each had an effect that was at least as good as individual hSAgAs at 2.5 nmol, suggesting that it is not a dose issue and that the two SAgAs may indeed synergize. The strong protection conferred by SAgA$_{mix}$ (with these two peptides) suggests that active suppression is likely involved through the complementary action of IL-10$^+$ Tr1 cells (induced by p79) and possibly Foxp3$^+$ Tregs (induced by the less stimulatory 2.5HIP) to regulate the other diabetogenic T-cell clones. Moreover, bystander suppression may also be mediated via CD73 by promoting a more immunosuppressive local environment with adenosine. Treatment with SAgAs did not lead to deletion of T cells reactive to both SAgA-linked peptides and other β-cell antigen-related epitopes. On the contrary, the repeated delivery of SAgAs substantially expanded antigen-specific T cells, and although their frequency dwindled over time, they remained detectable several months after cessation of treatment. This maintained population of antigen-specific T cells may be needed to regulate other T-cell populations. Clonal expansion and anergy are not incompatible and rather reflect two consecutive stages in the T-cell response over time (34,35). This study provides important insights into how different peptides with similar sequences, same delivery method, and same dose can have remarkably different effects on specific T cells in vivo.

Our studies demonstrate that 2.5HIP and p79, delivered as SAgAs, stimulate nonoverlapping endogenous T-cell populations and significantly block spontaneous disease development in NOD mice. The two epitopes may be synergistic in combination, and delivery of more epitopes may further enhance the efficacy of this ASIT approach. Anaphylactic reaction to administered peptides, including natural islet peptides, has been reported in NOD mice (2,3). Delivery as SAgAs could attenuate or negate such adverse effects that some peptides may elicit, and this novel property of SAgAs will be further investigated, given the important implications in peptide-based ASIT. Because the free peptides most strongly induced anaphylaxis, we posit that cSAgAs, with more stable linkers, are less likely to shed free peptides before uptake. When translating this approach to patients, epitopes may be customized based on their MHC haplotypes, autoantibodies, and other immune markers and delivered in combination to enhance the efficacy of this ASIT approach. Our studies support the

use of mimotopes and hybrid peptides to target disease-relevant T cells and possibly complement more conventional antigens. Thus, the SAgA platform is expected to enhance the delivery, efficacy, and possibly safety of peptide-based immunotherapy for the treatment of autoimmune diabetes.

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Author Contributions. R.F.-F. and R.J.C. designed experiments and wrote the first draft of the manuscript. R.F.-F. performed experiments and analyzed data. S.N.J., M.A.L., J.O.S., and C.B. produced and characterized SAgAs. M.K.-M. assisted with tissue isolation and analysis. R.L.B. validated and provided 2.5HIP-Tetr. R.J.C. directed the research. R.F.-F., S.N.J., R.L.B., J.O.S., C.B., and R.J.C. edited the manuscript. All authors approved the manuscript. R.F.-F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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