Suppression of Ongoing T Cell-Mediated Autoimmunity by Peptide-MHC Class II Dimer Vaccination

Li Li, Zuoan Yi, Bo Wang and Roland Tisch

*J Immunol* 2009; 183:4809-4816; Prepublished online 14 September 2009;
doi: 10.4049/jimmunol.0901616
http://www.jimmunol.org/content/183/7/4809

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2009/09/14/jimmunol.0901616.DC1

**References**
This article cites 49 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/183/7/4809.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Suppression of Ongoing T Cell-Mediated Autoimmunity by Peptide-MHC Class II Dimer Vaccination

Li Li, Zuoan Yi, Bo Wang, and Roland Tisch

Tissue-specific autoimmune diseases such as type 1 diabetes (T1D) are mediated by pathogenic T cells (1–3). Considerable effort has been devoted to developing therapeutic approaches to target autoreactive T cells, and prevent or suppress tissue-specific autoimmunity. Strategies based on administration of immunosuppressant drugs, and Abs specific for T cells have been successfully used in experimental models, and in some instances the clinic (4–7). However, these approaches fail to discriminate between T cells specific for self- and foreign Ags, and compromise the normal function of the immune system to varying degrees. Peptide-based immunotherapies offer an approach to selectively target autoreactive T cells, leaving the remainder of the immune system intact (8). Approaches of peptide immunotherapy that induce IL-4- or IL-10-secreting adaptive immunoregulatory CD4+ T (aTreg) cells have proven to be effective for autoimmune diseases in which multiple autoantigens are targeted by T cells (9–11). Once established aTreg cells traffic to the relevant tissues and suppress, via cytokine secretion, the differentiation or function of pathogenic T effector cells in an Ag-independent manner (12).

Various tissue-specific autoimmune diseases such as type 1 diabetes (T1D) are mediated by pathogenic T cells. Peptide-MHC class II (pMHCII)-Ig fusion proteins (19–23) have been successfully used in experimental models, and in some instances the clinic (4–7). These recombinants consist of the extra-cellular domains of the MHCII α- and β-chains supported by an Ig scaffold. A peptide is tethered to the MHCII β-chain ensuring that each bivalent fusion molecule presents a peptide, which binds T cells directly independent of APC. Studies by Casares et al. (24) using a monoclonal TCR transgenic model targeting the neo-β cell autoantigen hemagglutinin (HA), provided initial evidence that peptide-MHCII-Ig vaccination can be effective in treating autoimmunity. Administration of sIE4-Ig dimers linked to a HA peptide was found to delete HA-specific T effectors and reverse diabetes in treated mice expressing HA in β cells. sIE4-Ig dimer vaccination also induced HA-specific aTreg cells (24). Nevertheless, whether peptide-MHCII-Ig vaccination can block autoimmunity mediated by pathogenic effector T cells with multiple specificities has yet to be established. Accordingly, we tested whether administration of sIA67-Ig dimers covalently linked to β cell-derived peptides could suppress a late preclinical stage of T1D in NOD mice.

Studies in the NOD mouse, a spontaneous model of T1D, demonstrate that administration of β cell peptides induces aTreg cells, and suppresses differentiation of type 1 T effector cells that mediate destruction of the insulin-producing β cells (9, 10, 13). Peptide immunotherapy is effective at early stages of disease progression but efficacy is generally limited at late preclinical stages of T1D when the frequency of pathogenic type 1 T effectors is high, and the proinflammatory milieu is well established in the β cell containing islets (14, 15). In addition to the stage of disease progression at which treatment is initiated, other factors influence the efficacy of peptide immunotherapy including dose and route of administration, the use of adjuvant, the binding affinity of peptides to MHC molecules, and in vivo peptide stability. For instance, peptides are rapidly cleared from the circulation and inefficiently presented by APC in vivo, which limits therapeutic efficacy (16–18).

One approach to overcome these limitations has been the engineering of peptide-soluble MHC class II-Ig (peptide-sMHCII-Ig) fusion proteins (19–23). These recombinants consist of the extra-cellular domains of the MHCII α- and β-chains supported by an Ig scaffold. A peptide is tethered to the MHCII β-chain ensuring that each bivalent fusion molecule presents a peptide, which binds T cells directly independent of APC. Studies by Casares et al. (24) using a monoclonal TCR transgenic model targeting the neo-β cell autoantigen hemagglutinin (HA), provided initial evidence that peptide-MHCII-Ig vaccination can be effective in treating autoimmunity. Administration of sIE4-Ig dimers linked to a HA peptide was found to delete HA-specific T effectors and reverse diabetes in treated mice expressing HA in β cells. sIE4-Ig dimer vaccination also induced HA-specific aTreg cells (24). Nevertheless, whether peptide-MHCII-Ig vaccination can block autoimmunity mediated by pathogenic effector T cells with multiple specificities has yet to be established. Accordingly, we tested whether administration of sIA67-Ig dimers covalently linked to β cell-derived peptides could suppress a late preclinical stage of TID in NOD mice.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901616
Materials and Methods

Mice

NOD/LtJ, NOD.IL-10−/−, and NOD.CB17.Prdcdscid1 (NOD.scid) mice were maintained and bred under specific-pathogen free conditions. Mice were diagnosed as diabetic with blood glucose measurements ≥250 mg/dl on three successive days as determined by an Autokit Glucose assay (WAKO). In our colony NOD female mice 12 wk of age typically exhibit elevated blood glucose levels (e.g., ~180–200 mg/dl). All procedures were reviewed and approved by the University of North Carolina Institutional Animal Care and Use Committee.

sIAγ7-Ig dimer expression, purification, and vaccination

sIAγ7-Ig dimers were engineered as previously described (25, 26). Briefly, IAγ chain α- and β-chain extracellular domains were attached to fas and jun leucine zippers, respectively. The IAβ chain was further modified with a murine IgG2a Fc domain to establish a divalent structure. Leucine residues at positions 234 and 235 in the IgG2a hinge region were substituted with alanine to prevent binding to FcγRI and FcγRII and activation of APC (27, 28). Peptide epitopes were covalently linked to the N terminus of the IAβ-β chain by a flexible thrombin-GGGGS linker. cDNAs encoding the sIAγ7-Ig chains were subcloned into the pmT-Bip vector (Invitrogen) and transgene expression driven by a metallothionein-inducible promoter. Expression vectors were cotransfected via calcium phosphate into Drosophila S2 cells with pEG202 and, and transfectants selected in hygromycin-containing Schneider’s medium. sIAγ7-Ig dimer protein expression was induced by 500 μM CuSO4 for 7–10 days and purified by affinity chromatography on a protein A column (GE Bioscience).

Twelve-wk-old NOD female mice were i.v. immunized with 50 μg of sIAγ7-Ig dimers prepared in 200 μl of PBS on three consecutive days. Three weeks later, a second course of three injections of sIAγ7-Ig dimer was administered. In some experiments, 12-wk-old NOD female mice received three i.p. injections of 200 μg of peptide emulsified in 0.1 ml of IFA over a 3-wk period. Mixtures of peptides were also prepared in 0.1 ml of IFA.

FACS analysis

sIAγ7-Ig dimers were multimerized using Alexa Fluor 647-coupled protein A (Molecular Probes and Invitrogen) for FACS. Cells were incubated with sIAγ7-Ig multimers at room temperature for 1 h, followed by anti-CD3 (FITC), and CD4 (PacBlue) Abs (eBioscience) staining on ice for 30 min. Cells were then fixed, permeabilized, and intracellularly stained with anti-CD4 (PacBlue), and anti-TGF-β1 (AbD Serotec) for FACS. Cells were incubated with 10 μg/ml of PBS on three consecutive days. An ImmunoSpot plate reader (Aldrich) and 0.015% hydrogen peroxide. An ImmunoSpot plate reader (Cellular Technology) was used to count spot forming unit per well. For ELISA, cells were cultured in 96-well microtiter plates, stimulated with peptide as described, and culture supernatant was harvested after 48 h. TGF-β was measured via ELISA per the manufacturer’s instructions (R&D Systems).

T cell adoptive transfers and histopathology

Splenocytes prepared from diabetic NOD donors (5 × 10⁶) were injected i.p. into 5- to 8-wk-old NOD.scid mice either: 1) alone, 2) with splenocytes (5 × 10⁶), or 3) CD4⁺ T cells (5 × 10⁵) purified by negative selection from sIAγ7-Ig dimer treated NOD female mice. In some experiments NOD.scid recipients were injected twice weekly with 300 μg of anti-TGF-β-Ab (ID11.16.8) over a period of 4 wk. In our hands this protocol effectively neutralizes TGF-β in vivo. Splenocytes (5 × 10⁴) from diabetes-free sIAγ7-Ig dimer treated NOD female mice alone were also i.p. injected into NOD.scid mice. Pancreases were harvested, and fixed with 10% formalin. Serial cross-sections separated by 150 μm were cut and stained with H&E.

Statistical analyses

Statistical analyses were performed using GraphPad Prism software. Incidence of diabetes was compared by Kaplan-Meier log-rank test. One-way ANOVA test, χ²-square test, and Student’s t test were used. Findings were considered significant with values for p ≤ 0.05.

Results

sIAγ7-GADp217 or sIAγ7-GADp290 dimers suppress ongoing β cell autoimmunity and prevent diabetes in NOD female mice

Previous work by our group (9) and a more recent analysis (Fig. 1A) demonstrated that coadministration of glutamic acid decarboxylase (GAD)65-specific peptides spanning amino acid residues 217 to 236 (GADp217) and residues 290 to 309 (GADp290) prepared in IFA suppressed β cell autoimmunity in NOD female mice at a late preclinical stage of T1D and prevented diabetes. Lack of diabetes in vaccinated NOD mice correlated with an increased frequency of GADp217- and GADp290-specific T cells secreting IL-4 but not IL-10 (Fig. 1B). Nevertheless, protection was induced only after multiple injections with high doses (e.g., 200 μg each injection) of the respective peptides in IFA. Furthermore, administration of either GADp217 or GADp290 alone failed to prevent diabetes (Fig. 1B) (9). With this in mind, we investigated whether sIAγ7-Ig dimer vaccination is a more efficient strategy of peptide-based immunotherapy.

sIAγ7-Ig dimers were tested which contained covalently linked GADp217 and GADp290, and the non-self hen egg lysozyme (HEL) epitope 12–26. T cell binding and stimulation by the respective sIAγ7-Ig dimers were verified (data not shown). Twelve-wk-old NOD female mice, representing a late preclinical stage of T1D, received three i.v. injections of 50 μg of sIAγ7-Ig in PBS over 3 days, followed by another three injections 3 wk later, and diabetes monitored up to 35 wk of age. Each 50-μg injection of sIAγ7-Ig was equivalent to 1.1 μg of native peptide. No significant difference in the time of onset or frequency of diabetes was detected in NOD mice left untreated or receiving sIAγ7-Ig HEL (Fig. 2A). In contrast, the majority of NOD mice treated with sIAγ7-Ig, GADp217 (8/10; p = 0.0017 vs untreated mice χ-square) or sIAγ7-Ig, GADp290 (9/10; p = 0.0003 vs untreated mice χ-square) dimers remained diabetes-free (Fig. 2A). These results demonstrate that administration of sIAγ7-GADp217 or sIAγ7-GADp290, but not sIAγ7-HEL, efficiently prevents diabetes at a late preclinical stage of T1D.

Nondiabetic 35-wk-old NOD female mice treated with sIAγ7-Ig, GADp217 or sIAγ7-GADp290 were examined for islet infiltration. Histological analysis of pancreas sections showed a significantly reduced frequency and severity of insulitis in nondiabetic 35-wk-old NOD female mice treated with sIAγ7-GADp217 or sIAγ7-Ig GADp290 compared with a group of untreated, 35-wk-old NOD female mice (Fig. 2B). Interestingly, the frequency and severity of insulitis in the 35-wk-old sIAγ7-GAD65-treated NOD mice was analogous to that of untreated 12-wk-old NOD female mice
Therefore sIA\textsuperscript{aG7}-Ig dimer treatment prevents diabetes by suppressing the progression of islet infiltration in a β cell peptide-specific manner.

\textit{sIA}\textsuperscript{aG7}-GADp217 and sIA\textsuperscript{aG7}-GADp290 vaccination blocks T1D progression by αTreg cell induction}

Treatment with self-peptide can mediate T cell tolerance by clonal anergy or deletion. Alternatively, self-peptide-specific αTreg cells can be induced that traffic to the site of inflammation, and suppress the differentiation or activity of pathogenic T effectors. To determine whether active immunoregulation was established by sIA\textsuperscript{aG7}-Ig dimer treatment, adoptive transfer experiments were conducted. Twelve-week-old NOD female mice were treated with sIA\textsuperscript{aG7}-GADp217 as described, and splenocytes harvested 3 wk after the last injection. Splenocytes from sIA\textsuperscript{aG7}-GADp217- or sIA\textsuperscript{aG7}-HEL treated groups were then mixed with splenocytes prepared from diabetic NOD donors and i.p. injected into NOD.\textsuperscript{scid} recipients. Mice were monitored for diabetes. As expected, transfer of diabetic splenocytes alone or mixed with splenocytes from sIA\textsuperscript{aG7}-HEL treated NOD donors induced diabetes in all of the recipients (Fig. 3). In contrast, the onset of diabetes was significantly delayed in NOD.\textsuperscript{scid} mice receiving an equal mixture of splenocytes from sIA\textsuperscript{aG7}-GADp217 treated and diabetic animals (p ≤ 0.002) (Fig. 3). Notably, transfer of splenocytes prepared from sIA\textsuperscript{aG7}-GADp217 treated mice alone failed to induce diabetes in NOD.\textsuperscript{scid} recipients (0/6), indicating a lack of pathogenic T effectors (Fig. 3). The lack of diabeticogenic activity and
the suppressive effect of splenocytes suggested that the protection induced by sIA\(^\varepsilon\)-GADp217 treatment was mediated by active immunoregulation.

Next, the nature of the T cell response induced by the sIA\(^\varepsilon\)-GAD was studied. NOD female mice 12 wk of age were treated as above, and 3 wk after the last injection the frequency of IL-4-, IL-10-, and IFN-\(\gamma\)-secreting T cells was measured via ELISPOT in the PLN and spleen. Cultures established from the sIA\(^\varepsilon\)-GADp217 and sIA\(^\varepsilon\)-GADp290 treated mice exhibited a significant increase in the frequency of p217- and p290-specific IL-10- but not IL-4-secreting T cells, respectively, compared with sIA\(^\varepsilon\)-HEL-injected animals (Fig. 4A). In addition, IL-10-secreting T cells specific for proinsulin (B24-C36) and insulin B chain (p9-23) were increased in the PLN of sIA\(^\varepsilon\)-GADp217 and sIA\(^\varepsilon\)-GADp290 treated groups vs sIA\(^\varepsilon\)-HEL-treated mice (Fig. 4A), indicating epitope spread among IL-10-secreting aTreg cells. TGF-\(\beta\)1 was not detected in supernatants of peptide-pulsed cultures established from the spleen and PLN of any of the treatment and control groups as measured by ELISA (data not shown). In addition, a significant increase in the frequency of CD4\(^+\) T cells that bind sIA\(^\varepsilon\)-GADp217 multimer and expressed intracellular IL-10 was detected in the spleen, PLN, and islets of sIA\(^\varepsilon\)-GADp217 treated but not sIA\(^\varepsilon\)-HEL treated mice (Fig. 4, B and C). These results demonstrate that protection mediated by sIA\(^\varepsilon\)-GADp217 and sIA\(^\varepsilon\)-GADp290 treatment corresponds with the induction of IL-10- but not IL-4- or TGF-\(\beta\)-1-secreting aTreg cells found in the spleen, PLN, and islets.

sIA\(^\varepsilon\)-GADp217 vaccination increases the frequency of GADp217-specific FoxP3-expressing Treg cells

Because FoxP3-expressing Treg cells play a key role in regulating self-tolerance (30–32), whether sIA\(^\varepsilon\)-GAD treatment increased FoxP3-expressing Treg cells was investigated. The frequency of “bulk” FoxP3-expressing CD4\(^+\)CD25\(^+\) T cells in the spleen, PLN, mesenteric lymph nodes (MLN) and islets and in vitro suppressor function of these Treg cells were similar in sIA\(^\varepsilon\)-GADp217 and sIA\(^\varepsilon\)-HEL treated NOD mice (see supplemental Fig. 1).\(^4\) No sIA\(^\varepsilon\)-GADp217 multimer binding CD4\(^+\) T cells that expressed FoxP3 were detected in the spleen, PLN, MLN, and islets of sIA\(^\varepsilon\)-HEL treated NOD mice (Fig. 5). Conversely, an increased frequency of sIA\(^\varepsilon\)-GADp217 multimer-binding FoxP3-expressing CD4\(^+\) T cells was detected in the islets, and to a lesser extent the PLN and spleen but not the MLN of NOD mice treated with sIA\(^\varepsilon\)-GADp217 (Fig. 5). Furthermore, the majority (~90%) of these FoxP3-expressing CD4\(^+\) T cells expressed surface TGF-\(\beta\)1 (Fig. 5A), whereas none expressed intracellular IL-10 (data not shown). These results demonstrate that sIA\(^\varepsilon\)-Ig dimer vaccination induces or expands FoxP3-expressing Treg cells in a peptide-specific manner.

IL-10 is necessary for protection induced by sIA\(^\varepsilon\)-Ig dimer vaccination

Because sIA\(^\varepsilon\)-GADp217 treatment induced IL-10-expressing T cells, the relative contribution of these aTreg cells in mediating protection was examined. Twelve-week-old female NOD mice lacking IL-10 expression (NOD.IL-10\(^{-/-}\)) or wild-type NOD mice were vaccinated with sIA\(^\varepsilon\)-GADp217 as described. Three weeks after the last injection CD4\(^+\) T cells were purified via negative selection. CD4\(^+\) T cells were then mixed with splenocytes prepared from diabetic NOD donors, transferred into groups of NOD. scid recipients, and the onset of diabetes monitored. All of the NOD.scid mice receiving the diabetogenic splenocytes only developed diabetes (Fig. 6A). In marked contrast, CD4\(^+\) T cells from NOD mice treated with sIA\(^\varepsilon\)-GADp217 effectively blocked the transfer of diabetes; 5 of 6 NOD.scid recipients remained diabetes-free (Fig. 6A). In contrast, the mixture containing CD4\(^+\) T cells from sIA\(^\varepsilon\)-GADp217 treated NOD.IL-10\(^{-/-}\) mice failed to prevent the transfer of diabetes (Fig. 6A). These results demonstrate that IL-10-secreting aTreg cells induced by sIA\(^\varepsilon\)-Ig dimer vaccination play a key role in suppressing the function of established pathogenic T effectors.

Next, whether IL-10 was required for the induction or expansion of FoxP3-expressing Treg cells by sIA\(^\varepsilon\)-Ig dimer vaccination was determined. Wild-type and NOD.IL-10\(^{-/-}\) female mice received sIA\(^\varepsilon\)-GADp217 injections as described, and 3 wk after the final injection, tissues were harvested and T cells stained with sIA\(^\varepsilon\). GADp217 multimer ex vivo. As demonstrated in Fig. 6B, no significant difference in the frequency of sIA\(^\varepsilon\)-GADp217 multimer binding CD4\(^+\) T cells expressing FoxP3 was detected in the spleen, PLN, MLN, and islets of NOD and NOD.IL-10\(^{-/-}\) female mice vaccinated with sIA\(^\varepsilon\)-GADp217. These results indicated that induction/expansion of peptide-specific FoxP3-expressing Treg cells was independent of endogenous IL-10, and that these immunoregulatory effectors play only a limited role in sIA\(^\varepsilon\)-GADp217-induced protection. To confirm the latter, a coadaptor transfer experiment was conducted. CD4\(^+\) T cells were isolated from the PLN of NOD female mice treated at 12 wk of age with sIA\(^\varepsilon\). GADp217 or sIA\(^\varepsilon\)-HEL dimer, and then injected with diabetogenic splenocytes into NOD.scid recipients. One group of recipients was treated with a neutralizing anti-TGF-\(\beta\) Ab. As expected, CD4\(^+\) T cells from sIA\(^\varepsilon\)-GADp217 but not sIA\(^\varepsilon\)-HEL dimer treated NOD mice blocked the transfer of diabetes (Fig. 6C). Notably, administration of anti-TGF-\(\beta\) Ab had no effect on the protection mediated by Treg cells induced by sIA\(^\varepsilon\)-GADp217 dimer vaccination (Fig. 6C). Altogether these results demonstrate that protection induced by sMHCI-Ig dimer treatment is primarily mediated by IL-10-secreting aTreg cells, and that Treg cells expressing TGF-\(\beta\) and FoxP3 have only a limited role.

\(^4\) The online version of this article contains supplemental material.
Discussion

Administration of peptide-sMHCII recombinants has been shown to tolerize pathogenic T cells in mono-specific models of autoimmunity (24), including collagen induced arthritis (22), experimental autoimmune uveitis (33, 34), and experimental autoimmune encephalomyelitis (35). In these studies, prevention or suppression of induced autoimmunity has been associated with establishing hyporeactivity in the pathogenic peptide-specific T effector cells, or eliciting peptide-specific Treg cell reactivity. The current work provides evidence that sMHCII-Ig dimer vaccination effectively suppresses a diverse repertoire of established autoreactive T effector cells via induction of IL-10-secreting aTreg cells. The former is demonstrated by sIAg7-Ig dimer vaccination blocking T1D at a late preclinical stage of T1D in NOD mice (Figs. 1 and 2). Based on dose and relative efficacy of a given epitope to mediate protection, sIAg7-Ig dimer treatment was found to be more potent than administering the corresponding native GAD65 peptides (Fig. 1A) (9). Coinjection of a total of 600 µg of each native GADp217 and GADp290 prepared in IFA prevented diabetes in 12-wk-old NOD female mice, and when injected individually neither peptide was protective (Fig. 1A) (9). Furthermore, coinjection of up to 600 µg of each soluble GADp217 and GADp290 (e.g., in the absence of adjuvant) also had no protective effect in 12-wk-old NOD female mice (R. Tisch, unpublished results). In contrast, the equivalent of only 7 µg total of native GAD65 peptide was sufficient to block diabetes using the sIAg7-Ig dimers, and either sIAg7-GADp217 or sIAg7-GADp290 alone did so equally well (Fig. 2). A single round of three injections of sIAg7-GADp217 or sIAg7-GADp290 had only a limited effect on the development of diabetes, indicating that the second set of sMHCII-Ig dimer injections is required to “boost” the immunoregulatory response (L. Li and R. Tisch, unpublished results).

The robust nature of sMHCII-Ig dimer vaccination is likely due to a variety of parameters. In vivo induction of aTreg cells is expected to be more efficient following treatment with sMHCII-Ig dimers relative to native peptide. For instance, a number of factors influence the efficacy of injected native peptide to stimulate aTreg cell induction/expansion including the binding affinity of the peptide for the MHCII molecule, the stability of the peptide-MHCII complex, and the type and number of APC presenting the peptide.

![Figure 4](https://www.jimmunol.org/)

**Figure 4.** sIAg7-GADp217 and sIAg7-GADp290 vaccination induces IL-10-secreting aTreg cells. Groups of *n* = 5 12-wk-old NOD female mice were treated with sIAg7-Ig and 3 wk after the second course of injections T cell reactivity assessed. A, Spleen and PLN suspensions were examined via ELISPOT, and the frequency of T cells secreting IFN-γ, IL-4, and IL-10 determined in response to 20 µg/ml peptide. For a given peptide used for in vitro stimulation, comparisons were made with cultures established from sIAg7-HEL dimer treated mice. *, *p* = 0.002; †, *p* = 0.005; ‡, *p* = 0.01; **, *p* = 0.0003; ††, *p* = 0.002; ‡‡, *p* = 0.005; and §, *p* = 0.005 by Student’s *t* test. Error bar represents mean ± SEM. B, Representative FACS data of the frequency of sIAg7-GADp217 multimer-staining CD4+ T cells expressing intracellular IL-10 cultured from the islets following anti-CD3 and anti-CD28 Ab stimulation of sIAg7-GADp217 and sIAg7-HEL dimer treated (Tx) mice. C, Frequency ± SD of sIAg7-GADp217 multimer-staining CD4+ T cells expressing intracellular IL-10 cultured from the spleen, PLN, MLN, and islets following anti-CD3 and anti-CD28 Ab stimulation of *n* = 5 individual NOD mice treated with sIAg7-GADp217 or sIAg7-HEL dimers. For a given tissue, comparisons were made between sIAg7-GADp217 vs sIAg7-HEL dimer treated mice. *p* = 0.048, spleen; †, *p* = 0.003, PLN; ‡, *p* = 0.01, MLN; and §, *p* = 0.005 by Student’s *t* Test. *p* = 0.02 by one-way ANOVA test, for frequency of sIAg7-GADp217 multimer-staining IL-10-expressing CD4+ T cells in sIAg7-GADp217 vs sIAg7-HEL dimer treated NOD mice.
However, these factors are negated by the use of sMHCII-Ig dimers because the peptide is covalently linked and the dimer complex directly binds T cells. Direct binding of sMHCII-Ig complexes would also be expected to enhance clonal anergy/deletion of CD4⁺/H₁₁₀₀₁ T cells at a sufficient dose (20, 22). Under the conditions used in this study, however, sIAg7-Ig-induced clonal anergy/deletion appears to have a minimal (if any) role in the tolergenic effect. For instance, significant expansion of GADp217-specific CD4⁺/H₁₁₀₀₁ T cells (5-fold) was detected in the islets of sIAg7-GADp217 vs sIAg7-HEL treated NOD mice (Fig. 4B).

Another likely parameter contributing to the potency of sMHCII-Ig dimer vaccination is the nature of Treg cells that are induced or expanded. For instance, protection induced in NOD mice by coinjection of native GADp217 and GADp290 in IFA correlated with an increased frequency of IL-4- but not IL-10-secreting CD4⁺/H₁₁₀₀₁ T cells (Fig. 1B) (9), which in turn was reflected by the inability of the native GAD65 peptides to prevent diabetes in NOD.IL-10null mice (9). In contrast, protection induced by sIAg7-GADp217 or sIAg7-HEL vaccination was dependent on IL-10-secreting CD4⁺/H₁₁₀₀₁ T cells. These results are consistent with findings made by Casares et al. (24) demonstrating that sIEHA dimer vaccination elicits IL-10-secreting aTreg cells in vivo. IL-10-secreting effector cells are a particularly potent subset of aTreg cells by regulating the responses of naive and memory T cells, and suppressing Th1 cell-mediated pathologies through bystander suppression mediated by local release of IL-10 (36–38). IL-10 also inhibits the activation and function of APC such as dendritic cells (39–41). IL-10-treated dendritic cells gain a “tolerogenic” phenotype and preferentially promote the development of aTreg cells (40–42). These direct
and indirect effects of IL-10 would be expected to amplify the immunoregulatory response induced by sIAg7-Ig dimer vaccination and may explain the abrupt block in the progression of insulitis in protected NOD mice (Fig. 2B). Indeed, sIAg7-p217 and sIAg7-GADp290 treatment induced not only GAD65-specific IL-10-secreting aTreg cells, but also aTreg-specific for other β cell peptides (Fig. 4A). The relative potency of IL-10-secreting aTreg cells may also explain why sIAg7-p217 or sIAg7-GADp290 alone suppressed ongoing β cell autoimmunity (Fig. 2A), whereas coinjection of native GADp217 and GADp290 in IFA was needed to similarly prevent diabetes onset (Fig. 1A). Injection of both native GAD65-derived peptides in IFA likely is required to induce a sufficient frequency of IL-4-secreting CD4+ T cells, which is limiting with either peptide alone (Fig. 1B). Interestingly, an increase in GADp217-specific FoxP3- and TGF-β1-expressing Treg cells was also detected in the PLN and islets of sIAg7-GADp217 vaccinated NOD mice (Fig. 5). Although protection mediated by sIAg7-Ig dimer was dependent on induction of IL-10-secreting aTreg cells, β cell-specific FoxP3-expressing Treg cells would also be expected to contribute to immunoregulation within the PLN and islets. However, the increase in GADp217-specific FoxP3-expressing Treg cells (Fig. 6B) was insufficient to suppress diabetogenic T effectors in the absence of IL-10-secreting aTreg cells (Fig. 6A), and neutralizing TGF-β had no effect on the regulatory function of CD4+ T cells from sIAg7-p217 vaccinated NOD mice (Fig. 6C).

Based on our findings and the findings of other studies (23, 24), properties intrinsic to sMHCII-Ig dimers favor differentiation of naïve T precursors toward IL-10-secreting aTreg cells. sMHCII-Ig dimers may also preferentially expand established IL-10-expressing aTreg cells. For instance Liu and colleagues (43) have detected GAD65-specific IL-10-expressing CD4+ T cells in the spleens of unmanipulated 8-wk-old NOD mice. However, the identity of the peptide bound by sMHCII-Ig dimer also appears to be a key factor determining the efficacy of a given recombinant. For instance, the Bluestone group (23) reported that vaccinating young NOD mice with a sIAg7-Ig dimer complexed with a mimetic peptide recognized by TCR transgenic BDC2.5 CD4+ T cells (sIAg7-p31) failed to induce αTreg cells that prevent diabetes in an adoptive transfer model. Similarly, we found that 12-wk-old NOD female mice injected with sIAg7-Ig dimer containing a different BDC2.5 mimetic peptide continued to develop diabetes (L. Li and R. Tisch, unpublished results). Nevertheless, preliminary findings demonstrate that vaccination with sIAg7-Ig dimers complexed with other β cell-derived autoantigens such as proinsulin, induce IL-10-secreting aTreg cells and block β cell autoimmunity in 12-wk-old NOD female mice (R. Tisch, unpublished results). Investigation of the biochemical and transcriptional signaling events transduced in CD4+ T cells will provide important insight into the events that regulate aTreg differentiation by sIAg7-Ig dimers. It is noteworthy that induction of αTreg cells is also achieved by administration of soluble peptide-linked single chain recombinants consisting of the α1 and β1 domains of MHCII (34, 35, 44). These recombinant TCR ligands directly bind to TCR independent of CD4, and have been found to also induce T cell hyperreactivity. Similar to our findings, a recent report showed that vaccination with recombinant TCR ligands linked to peptides derived from proteolipid protein reversed in mice experimental autoimmune encephalomyelitis induced by a whole spinal cord homogenate via induction of proteolipid protein-specific IL-10- (and IL-13-) secreting aTreg cells (44). This finding further argues that soluble peptide-MHCII vaccination may in general prove to be a highly effective approach of peptide immunotherapy to preferentially promote aTreg cell reactivity and suppress ongoing autoimmunity.

In summary, our findings indicate that peptide-sMHCII-Ig dimer treatment is a robust approach to suppress late preclinical β cell autoimmunity via induction of immunoregulatory T cell effectors. This work also provides rationale for using sMHCII-Ig dimer vaccination as a mode of adjuvant-free peptide immunotherapy in the clinic. For instance, HLA-DR4 or HLA-DQ8 recombinants covalently linked to known β cell-derived peptides restricted to the corresponding HLA molecules can be used to induce IL-10-secreting aTreg cells. The apparent intrinsic property of sMHCII-Ig dimers to induce IL-10-secreting aTreg cells is particularly relevant in view of work by Peukman and colleagues (45). This group demonstrated an increased frequency of HLA-DR4-restricted β cell peptide-specific CD4+ T cells secreting IL-10 in HLA-matched healthy control vs diabetic individuals. A role for IL-10-secreting Treg cells has also been implicated in other tissue specific autoimmune diseases such as multiple sclerosis (46). The relatively low dose of total native peptide required to induce protection also has important safety implications in view of murine and clinical studies reporting anaphylaxis following administration of high doses of soluble peptide (47–49). Currently, efforts are ongoing in the laboratory to assess the properties of such HLA sMHCII-Ig dimers.

Disclosures
The authors have no financial conflict of interest.

References
32. Haxhinasto, S., C. Benoist, and D. Mathis. 2007. Regulatory T-cell differentia-
30. Muller, S., L. Adorini, A. Juretic, and Z. A. Nagy. 1990. Selective in vivo inhi-
29. Wong, C. P., L. Li, J. A. Frelinger, and R. Tisch. 2006. Early autoimmune de-
28. Arnold, P. Y., K. M. Vignali, T. B. Miller, N. L. La Gruta, L. S. Cauley,
22. Luo, L., C. M. Cullen, M. L. Delay, S. Thornton, L. K. Myers, E. F. Rosloniec, 
10. Wong, C. P., L. Li, J. A. Frelinger, and R. Tisch. 2006. Early autoimmunity de-

4816 PEPTIDE-BASED IMMUNOTHERAPY OF AUTOIMMUNE DIABETES
SUPPLEMENTARY FIGURE 1. Analysis of the frequency and suppressive capacity of Treg cells from sIA^87^-GADp217 versus sIA^87^-pHEL dimer treated (Tx) NOD female mice. (A) The frequency of CD4^+ T cells expressing FoxP3^+ ex vivo in the spleen, PLN, MLN and islets. (B) The suppressive capacity of purified CD4^+CD25^+ T cells from the spleen of sIA^87^-GADp217 or sIA^87^-pHEL dimer treated NOD female mice was tested. CD4^+CD25^- responder T cells (T-eff; 5x10^4) were cultured with titrated numbers of CD4^+CD25^+ Treg in the presence of irradiated T cell-depleted spleen cells (5x10^4) and 5 μg/ml soluble anti-CD3 Ab. Mean [^3]H]thymidine incorporation indicated as CPM in triplicate wells. The ratios indicated represent the ratios of Teff:Treg.