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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 characterized by T cell-driven pathology. Administration of autoantigenic peptides provides a strategy to selectively target the pathogenic T cell response. Indeed, treatment with β cell peptides effectively prevents T1D in NOD mice. However, the efficacy of peptide immunotherapy generally wanes as β cell autoimmunity progresses and islet inflammation increases. With the goal of enhancing the efficacy of peptide immunotherapy, soluble (s)IAβ7-Ig dimers covalently linked to β cell autoantigen-derived peptides were tested for the capacity to suppress late preclinical T1D. NOD female mice with established β cell autoimmunity were vaccinated i.v. with a short course of sIAβ7-Ig dimers tethered to peptides derived from glutamic acid decarboxylase (GAD)65 (sIAβ7-pGAD65). Treatment with sIAβ7-pGAD65 dimers and the equivalent of only ~7 μg of native peptide effectively blocked the progression of insulitis and the development of diabetes. Furthermore, suppression of T1D was dependent on β cell-specific IL-10-secreting CD4+ T cells, although the frequency of GAD65-specific FoxP3-expressing CD4+ T cells was also increased in sIAβ7-pGAD65 dimer vaccinated NOD mice. These results demonstrate that MHC class II-Ig dimer vaccination is a robust approach to suppress ongoing T cell-mediated autoimmunity, and may provide a superior strategy of adjuvant-free peptide-based immunotherapy to induce immunoregulatory T cells. The Journal of Immunology, 2009, 183: 4809–4816.

Various 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 successful 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).

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-sMHCHI-Ig) fusion proteins (19–23). These recombinants consist of the extracellular domains of the MHCI α- and β-chains supported by an Ig scaffold. A peptide is tethered to the sMHCHI 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-sMHCHI-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-sMHCHI-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 sIAβ7-Ig dimers covalently linked to β cell-derived peptides could suppress a late preclinical stage of T1D in NOD mice.
Materials and Methods

Mice

NOD/LtJ, NOD.IL-10<sup>−/−</sup>, and NOD.CB17.Prdck<sup>sIA</sup>-Ig (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.

sIA<sup>7</sup>-Ig dimer expression, purification, and vaccination

sIA<sup>7</sup>-Ig dimers were engineered as previously described (25, 26). Briefly, I<sup>A</sup>α<sup>7</sup> α- and β-chain extracellular domains were attached to fous and jun leucine zippers, respectively. The I<sup>A</sup>α-chain was further modified with a murine IgG2a Fc domain to establish a divalent scaffold. Leucine residues at positions 234 and 235 in the IgG2a hinge region were substituted with alanines to prevent binding to Fc<sub>RI</sub> and Fc<sub>RII</sub> and activation of APC (27, 28). Peptide epitopes were covalently linked to the N terminus of the I<sup>A</sup>β<sup>7</sup>-β-chain by a flexible thrombin-GGGS linker. cDNAs encoding the sIA<sup>7</sup>-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 pHygro, and transfectants selected in hygromycin-containing Schneider’s medium. sIA<sup>7</sup>-Ig dimer protein expression was induced by 500 μM CuSO<sub>4</sub> 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 sIA<sup>7</sup>-Ig dimers prepared in 200 μl of PBS on three consecutive days. Three weeks later, a second course of three injections of sIA<sup>7</sup>-Ig dimer was administered. I<sup>A</sup>α<sup>7</sup>-Ig dimers were also administered intraperitoneally, i.e., 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

sIA<sup>7</sup>-Ig dimers were multimierized using Alexa Fluor 647-coupled protein A (Molecular Probes and Invitrogen) for FACS. Cells were incubated with sIA<sup>7</sup>-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–TFG-β (PE) or anti–IL-10 (FITC) Abs (eBioscience).

In some experiments single cell suspensions were cultured with plate-bound anti-CD3 (2 μg/ml) and soluble anti-CD28 (2 μg/ml) Abs in the presence of recombinant murine IL-2 (10 ng/ml) for 2 days, and then stimulated with PMA (5 ng/ml) and ionomycin (500 ng/ml) in the presence of recombinant α <sup>7</sup> (10 μg/ml) for 5 h. Cells were stained with sIA<sup>7</sup>-Ig multimers at room temperature for 1 h, followed by anti-CD3 (PeCy7), anti-CD4 (PacBlue), and anti–TFG-β (PerCP) Abs on ice for 30 min, and then fixed, permeabilized, and intracellularly stained with anti–IL-10 (FITC) and anti-FoxP3 (PE) Abs (eBioscience). Data were acquired on a Cyan flow cytometer (DakoCytomation) and analyzed using Summit software (DakoCytomation).

Islet isolation

Pancreas samples were perfused with 0.2 mg/ml Liberase (Roche) and digested for 30 min at 37°C. Islets were purified via ficoll gradient, hand-picked and counted. For FACS analyses of islet infiltrating T cells, isolated islets were dissociated into a single cell suspension using enzyme-free cell dissociation solution (Sigma-Aldrich). Lymphocytes infiltrating the islets were collected and cellular debris removed by 70-μm nylon filters.

ELISPOT and ELISA

ELISPOT was conducted as described (29). Briefly, splenocytes (5 × 10<sup>6</sup>/well) or cells from the pancreatic lymph nodes (PLN; 2 × 10<sup>7</sup>/well) were resuspended in 1 ml of Iscove’s Modified (BioWhittaker) and cultured on ELISPOT plates (Millipore) coated with anti-IFN-γ, anti–IL-4, or anti–IL-10 Abs (BD Pharmingen) for 48 h at 37°C. Cells were added at a final concentration of 20 μg/ml. Plates were washed, incubated with the appropriate biotinylated anti-mouse cytokine Abs and streptavidin-HRP (BD Pharmingen), and spot forming units developed with 100 μM sodium acetate buffer containing 0.3 mg/ml 3-amino-9-ethylcarbazole (Sigma-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 × 10<sup>6</sup>) were injected i.p. into 5- to 8-wk-old NOD.scid mice either: 1) alone, 2) with splenocytes (5 × 10<sup>6</sup>), or 3) CD4<sup>+</sup> T cells (5 × 10<sup>6</sup>) purified by negative selection from sIA<sup>7</sup>-Ig dimer treated NOD female mice. In some experiments NOD.scid recipients were injected twice weekly with 300 μg of anti–TFG-β Ab (ID11.16.8) over a period of 4 wk. In our hands this protocol effectively neutralizes TGF-β in vivo. Splenocytes (5 × 10<sup>6</sup>) from diabetes-free sIA<sup>7</sup>-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, χ-square test, and Student’s t test were used. Findings were considered significant with values for ρ < 0.05.

Results

sIA<sup>7</sup>-GADp217 or sIA<sup>7</sup>-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-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 sIA<sup>7</sup>-Ig dimer vaccination is a more efficient strategy of peptide-based immunotherapy.

sIA<sup>7</sup>-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 sIA<sup>7</sup>-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 sIA<sup>7</sup>-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 sIA<sup>7</sup>-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 sIA<sup>7</sup>-HEL (Fig. 2A). In contrast, the majority of NOD mice treated with sIA<sup>7</sup>-HEL, GADp217 (8/10; p = 0.0017 vs untreated mice ch-square) or sIA<sup>7</sup>-GADp290 (9/10; p = 0.0003 vs untreated mice χ-square) dimers remained diabetes-free (Fig. 2A). These results demonstrate that administration of sIA<sup>7</sup>-GADp217 or sIA<sup>7</sup>-GADp290, but not sIA<sup>7</sup>-HEL, efficiently prevents diabetes at a late preclinical stage of T1D.

Nondiabetic 35-wk-old NOD female mice treated with sIA<sup>7</sup>-GADp217 or sIA<sup>7</sup>-GADp290 were examined for islet infiltration. Histological analysis of pancreas samples showed a significantly reduced frequency and severity of insulitis in nondiabetic 35-wk-old NOD female mice treated with sIA<sup>7</sup>-GADp217 or sIA<sup>7</sup>-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 sIA<sup>7</sup>-GAD65-treated NOD mice was analogous to that of untreated 12-wk-old NOD female mice.
Therefore sIA<sup>87</sup>-Ig dimer treatment prevents diabetes by suppressing the progression of islet infiltration in a β cell peptide-specific manner.

**sIA<sup>87</sup>-GADp217 and sIA<sup>87</sup>-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<sup>87</sup>-Ig dimer treatment, adoptive transfer experiments were conducted. Twelve-week-old NOD female mice were treated with sIA<sup>87</sup>-GADp217 as described, and splenocytes harvested 3 wk after the last injection. Splenocytes from sIA<sup>87</sup>-GADp217- or sIA<sup>87</sup>-HEL treated groups were then mixed with splenocytes prepared from diabetic NOD donors and i.p. injected into NOD.scid recipients. Mice were monitored for diabetes. As expected, transfer of diabetogenic splenocytes alone or mixed with splenocytes from sIA<sup>87</sup>-HEL treated NOD donors induced diabetes in all of the recipients (Fig. 3). In contrast, the onset of diabetes was significantly delayed in NOD.scid mice receiving an equal mixture of splenocytes from sIA<sup>87</sup>-GADp217 treated and diabetic animals (p = 0.002) (Fig. 3). Notably, transfer of splenocytes prepared from sIA<sup>87</sup>-GADp217 treated mice alone failed to induce diabetes in NOD.scid recipients (0/6), indicating a lack of pathogenic T effectors (Fig. 3). The lack of diabetogenic activity and

**FIGURE 1.** Diabetes is prevented in NOD mice at a late preclinical stage of T1D with multiple and high doses of “native” GAD65 peptides in adjuvant. A, Groups of n = 8 NOD female mice 12 wk of age received a total of three i.p. injections of 200 μg of peptide in IFA over a 20-day period. Diabetes was monitored. *, p < 10<sup>-3</sup> by Kaplan-Meier log-rank test for NOD mice coinjected with GADp217 and GADp290 vs NOD mice receiving only GADp217 or GADp290 or left untreated (UnTx). B, PLN were harvested from individual NOD female mice (n = 4) 3 wk after the last injection of peptide in IFA as in A, and the frequency of IFN-γ-, IL-4-, and IL-10-secreting T cells in response to 20 μg/ml peptide was measured by ELISPOT. Error bar represents mean ± SEM. **, p < 10<sup>-3</sup> by Student’s t test, for NOD mice coinjected with GADp217 and GADp290 vs NOD mice treated with GADp217 or GADp290 only.

**FIGURE 2.** sIA<sup>87</sup>-GADp217 and sIA<sup>87</sup>-GADp290 treatment prevents diabetes and blocks the progression of insulitis at a late preclinical stage of T1D. A, Twelve-wk-old NOD female mice were treated with sIA<sup>87</sup>-GADp217 (n = 10), sIA<sup>87</sup>-GADp290 (n = 10), sIA<sup>87</sup>-HEL (n = 8), or left untreated (UnTx, n = 10), and diabetes was monitored. *, p ≤ 0.0015 by Kaplan-Meier log-rank test, for sIA<sup>87</sup>-GADp217 vs sIA<sup>87</sup>-HEL treated or untreated mice and †, p = 0.0003, for sIA<sup>87</sup>-GADp290 vs sIA<sup>87</sup>-HEL treated or untreated mice. B, Insulitis in the pancreases of nondiabetic NOD female mice treated with sIA<sup>87</sup>-GADp217 (n = 5) and sIA<sup>87</sup>-GADp290 (n = 6) was evaluated at 35 wk by histological analysis. In addition, insulitis in the pancreases of untreated (UnTx) nondiabetic NOD female mice 12 wk (n = 4) and 35 wk (n = 5) of age were compared; and diabetic NOD female mice treated with sIA<sup>87</sup>-HEL (n = 4). A minimum of 30 islets was examined for each mouse, p < 10<sup>-3</sup> by one-way ANOVA test for frequency and severity of insulitis in sIA<sup>87</sup>-GADp217 or sIA<sup>87</sup>-GADp290 treated NOD mice vs 35-wk-old untreated NOD female mice.
the suppressive effect of splenocytes suggested that the protection induced by sIA\textsuperscript{\alpha\gamma}-GADp217 treatment was mediated by active immunoregulation.

Next, the nature of the T cell response induced by the sIA\textsuperscript{\alpha\gamma}-Ig dimers 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 in response to a panel of \beta cell peptides was measured via ELISPOT in the PLN and spleen. Cultures established from the sIA\textsuperscript{\alpha\gamma}-GADp217 and sIA\textsuperscript{\alpha\gamma}-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\textsuperscript{\alpha\gamma}-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\textsuperscript{\alpha\gamma}-GADp217 and sIA\textsuperscript{\alpha\gamma}-GADp290 treated groups vs sIA\textsuperscript{\alpha\gamma}-HEL treated mice (Fig. 4A), indicating epitope spread among IL-10-secreting aTreg cells. TGF-\beta1 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\textsuperscript{+} T cells that bind sIA\textsuperscript{\alpha\gamma}-GADp217 multimer and expressed intracellular IL-10 was detected in the spleen, PLN, and islets of sIA\textsuperscript{\alpha\gamma}-GADp217 treated but not sIA\textsuperscript{\alpha\gamma}-HEL treated mice (Fig. 4, B and C). These results demonstrate that protection mediated by sIA\textsuperscript{\alpha\gamma}-GADp217 and sIA\textsuperscript{\alpha\gamma}-GADp290 treatment corresponds with the induction of IL-10- but not IL-4- or TGF-\beta1-secreting aTreg cells found in the spleen, PLN, and islets.

sIA\textsuperscript{\alpha\gamma}-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\textsuperscript{\alpha\gamma}-Ig dimer treatment increased FoxP3-expressing Treg cells was investigated. The frequency of "bulk" FoxP3-expressing CD4\textsuperscript{+}CD25\textsuperscript{+} 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\textsuperscript{\alpha\gamma}-GADp217 and sIA\textsuperscript{\alpha\gamma}-HEL treated NOD mice (see supplemental Fig. 1).\textsuperscript{4} No sIA\textsuperscript{\alpha\gamma}-GADp217 multimer binding CD4\textsuperscript{+} T cells that expressed FoxP3 were detected in the spleen, PLN, MLN, and islets of sIA\textsuperscript{\alpha\gamma}-HEL treated NOD mice. Conversely, an increased frequency of sIA\textsuperscript{\alpha\gamma}-GADp217 multimer-binding FoxP3-expressing CD4\textsuperscript{+} 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\textsuperscript{\alpha\gamma}-GADp217 (Fig. 5). Furthermore, the majority (~90%) of these FoxP3-expressing CD4\textsuperscript{+} T cells expressed surface TGF-\beta1 (Fig. 5A), whereas none expressed intracellular IL-10 (data not shown). These results demonstrate that sIA\textsuperscript{\alpha\gamma}-Ig dimer vaccination induces or expands FoxP3-expressing Treg cells in a peptide-specific manner.

IL-10 is necessary for protection induced by sIA\textsuperscript{\alpha\gamma}-Ig dimer vaccination

Because sIA\textsuperscript{\alpha\gamma}-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\textsuperscript{null}) or wild-type NOD mice were vaccinated with sIA\textsuperscript{\alpha\gamma}-GADp217 as described. Three weeks after the last injection CD4\textsuperscript{+} T cells were purified via negative selection. CD4\textsuperscript{+} 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 spleen cells only developed diabetes (Fig. 6A). In marked contrast, CD4\textsuperscript{+} T cells from NOD mice treated with sIA\textsuperscript{\alpha\gamma}-GADp217 effectively blocked the transfer of diabetes; 5 of 6 NOD.scid recipients remained diabetes-free (Fig. 6A). In contrast, the mixture containing CD4\textsuperscript{+} T cells from sIA\textsuperscript{\alpha\gamma}-GADp217 treated NOD.IL-10\textsuperscript{null} mice failed to prevent the transfer of diabetes (Fig. 6A). These results demonstrate that IL-10-secreting aTreg cells induced by sIA\textsuperscript{\alpha\gamma}-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\textsuperscript{\alpha\gamma}-Ig dimer vaccination was determined. Wild-type and NOD.IL-10\textsuperscript{null} female mice received sIA\textsuperscript{\alpha\gamma}-GADp217 injections as described, and 3 wk after the final injection, tissues were harvested and T cells stained with sIA\textsuperscript{\alpha\gamma}. GADp217 multimer ex vivo. As demonstrated in Fig. 6B, no significant difference in the frequency of sIA\textsuperscript{\alpha\gamma}-GADp217 multimer binding CD4\textsuperscript{+} T cells expressing FoxP3 was detected in the spleen, PLN, MLN, and islets of NOD and NOD.IL-10\textsuperscript{null} female mice vaccinated with sIA\textsuperscript{\alpha\gamma}-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\textsuperscript{\alpha\gamma}-GADp217-induced protection. To confirm the latter, a coadptive transfer experiment was conducted. CD4\textsuperscript{+} T cells were isolated from the PLN of NOD female mice treated at 12 wk of age with sIA\textsuperscript{\alpha\gamma}. GADp217 or sIA\textsuperscript{\alpha\gamma}-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\textsuperscript{+} T cells from sIA\textsuperscript{\alpha\gamma}-GADp217 but not sIA\textsuperscript{\alpha\gamma}-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\textsuperscript{\alpha\gamma}-GADp217 dimer vaccination (Fig. 6C). Altogether these results demonstrate that protection induced by sMHCII-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.

\textsuperscript{4} The online version of this article contains supplemental material.
Discussion

Administration of peptide-sMHCII recombinants has been shown to tolerate 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 cell autoimmunity 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, coinnoculation 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. 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 dimers treated mice. 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+ T cells (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 tolerogenic effect. For instance, significant expansion of GADp217-specific CD4+ 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+ T cells (9), which in turn was reflected by the inability of the native GAD65 peptides to prevent diabetes in NOD.IL10null mice (9). In contrast, protection induced by sIAg7-GADp217 or sIAg7-GADp290 vaccination was dependent on IL-10-secreting CD4+ T cells. These results are consistent with findings made by Casares et al. (24) demonstrating that sIE2-HA dimer vaccination elicits IL-10-secreting Treg 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 Treg cells, but also Treg-specific for other β cell peptides (Fig. 4A). The relative potency of IL-10-secreting Treg 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 Treg 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. sMHCI-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 sMHCI-IIg 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 aTreg 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 aTreg cells is also achieved by administration of soluble peptide-linked single chain recombinants consisting of the α1 and β1 domains of MHCI-I (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 proteoliop protein reversed in mice experimental autoimmune encephalomyelitis induced by a whole spinal cord homogenate via induction of proteoliop protein-specific IL-10- (and IL-13-) secreting aTreg cells (44). This finding further argues that soluble peptide-MHCIII 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-sMHCI-IIg dimer treatment is a robust approach to suppress late preclinical β cell autoimmunity via induction of immunoregulatory T effector cells. This work also provides rationale for using sMHCI-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 sMHCI-Ig dimers to induce IL-10-secreting aTreg cells is particularly relevant in view of work by Peakman 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


