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Peptide-MHC Class II Dimers as Therapeutics to Modulate Antigen-Specific T Cell Responses in Autoimmune Diabetes

Emma L. Masteller,* Matthew R. Warner,* Walter Ferlin,† Valeria Judkowski,‡ Darcy Wilson,‡ Nicolas Glaienhauser,† and Jeffrey A. Bluestone2*△

Type 1 diabetes is an autoimmune disorder caused by autoreactive T cells that mediate destruction of insulin-producing β cells of the pancreas. Studies have shown that T cell tolerance can be restored by inducing a partial or altered signal through the TCR. To investigate the potential of bivalent peptide-MHC class II/Ig fusion proteins as therapeutics to restore Ag-specific tolerance, we have developed soluble peptide I-Ag7 dimers for use in the nonobese diabetic mouse model of diabetes. I-Ag7 dimers with a linked peptide specific for islet-reactive BDC2.5 TCR transgenic CD4+ T cells were shown to specifically bind BDC2.5 T cells as well as a small population of Ag-specific T cells in nonobese diabetic mice. In vivo treatment with BDC2.5 peptide I-Ag7 dimers protected mice from diabetes mediated by the adoptive transfer of diabetogenic BDC2.5 CD4+ T cells. The dimer therapy resulted in the activation and increased cell death of transferred BDC2.5 CD4+ T cells. Surviving cells were hypoproliferative to challenge by Ag and produced increased levels of IL-10 and decreased levels of IFN-γ compared with cells from control I-Ag7 dimer-treated mice. Anti-IL-10R therapy reversed the tolerogenic effects of the dimer. Thus, peptide I-Ag7 dimers induce tolerance of BDC2.5 TCR T cells through a combination of the induction of clonal anergy and anti-inflammatory cytokines. The Journal of Immunology, 2003, 171: 5587–5595.

Type 1 diabetes is caused by autoreactive T cells that mediate the destruction of insulin-producing β cells of the pancreas. Therapeutic approaches designed to prevent or cure the disease through general immune suppression, such as treatments with cyclosporin A, anti-inflammatory cytokines, or Abs against proinflammatory cytokines or T cells, have been shown to be effective in animal models and, in some cases, human clinical trials, but also have a variety of undesirable side effects (1, 2). Thus, approaches that could selectively tolerize autoreactive T cells without disrupting the ability of the immune system to neutralize opportunistic pathogens would be highly advantageous.

T cell tolerance can be achieved by inducing a partial or altered signal through the TCR/CD3 complex using mAbs or altered peptide ligands (3, 4). Treatment of mice with anti-CD3 mAbs that have been altered to prevent binding to FcR prevents or reverses diabetes (5–7). Non-FcR-binding anti-CD3 mAbs were found to induce cell death and anergy in Th1 cells, which are associated with pathogenesis of diabetes while promoting survival/expansion of Th2 (8), and CD4+CD25+ regulatory T cells (9), which are associated with protection from disease. Based on the success in animal models, clinical trials were initiated using a humanized non-FcR-binding anti-CD3 mAb to treat patients with new-onset type 1 diabetes (10). A single course of anti-CD3 mAb treatment was found to arrest the deterioration of insulin production in the majority of treated patients. Anti-CD3 mAb treatment resulted in a transient reduction in the number of circulating lymphocytes and in detectable presence of IL-10 in the serum. Thus, the effect of anti-CD3 treatment may have been to shift the autoimmune response toward the production of protective or regulatory cytokines.

Recently, soluble ligands for TCRs have been developed to target Ag-specific T cells (11–13). These TCR ligands consist of engineered MHC molecules in multimeric form, some with covalently attached peptide Ags. Like mAbs directed against the TCR/CD3 complex, soluble bivalent peptide-MHC molecules are capable of delivering a signal through the TCR (14–17). These reagents may therefore provide for therapy to selectively induce tolerance in Ag-specific T cells involved in autoimmune responses. Recently, peptide-MHC multimers have been shown to be effective in delaying the onset and reducing the severity of collagen-induced arthritis in mice by induction of Ag-specific hyporesponsiveness (18). Furthermore, soluble peptide-MHC has been shown to be effective in treating diabetes in mice using a model double-transgenic system in which mice express the influenza virus hemagglutinin (HA)3 in pancreatic islets and a HA-specific TCR (19). However, these studies did not address the effect of peptide-MHC therapy in spontaneous autoimmune systems with naturally occurring Ags or in the presence of a diverse repertoire of autoreactive T cells.

To investigate the potential of peptide-MHC class II multimers in treating autoimmune diabetes, we have generated I-Ag7 dimers with covalently linked BDC2.5 mimotope peptides that are capable of identifying BDC2.5 TCR transgenic CD4+ T cells and BDC2.5 mimotope-peptide-reactive cells from nonobese diabetic (NOD) mice. The BDC2.5 CD4+ T cell clone originally isolated from a diabetic NOD mouse is highly diabetogenic when transferred into young NOD recipients (20). Transgenic NOD mice expressing the TCR α (Vα1)- and β (Vβ4)-chains of the BDC2.5 T cell clone

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Abbreviations used in this paper: HA, hemagglutinin; HEL, hen egg lysozyme; NOD, nonobese diabetic; Tg, transgene.

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develop insulin, and activated T cells from these mice transfer disease to NOD recipients (21). BDC2.5 T cells recognize an as yet unidentified islet Ag presented by I-A\(^{\beta}\) (22); however, by screening peptide libraries, several highly active decametides that stimulate BDC2.5 transgenic T cells were identified (23). In vivo administration of BDC2.5 mimotope I-A\(^{\beta}\) dimers prevented the onset of diabetes in mice receiving activated BDC2.5 TCR transgenic spleen cells. Treatment with BDC2.5 mimotope I-A\(^{\beta}\) dimers resulted in activation and increased cell death of the autoreactive T cells. More interestingly, the remaining autoreactive T cells became hypoproliferative to subsequent challenge to Ag and preferentially produced the anti-inflammatory cytokine, IL-10. Treatment of NOD mice with BDC2.5 mimotope I-A\(^{\beta}\) dimers resulted in the increased production of IL-10 upon rechallenge with Ag in vitro. More significantly, anti-IL-10R mAb reversed the tolerogenic effects of the dimer in vivo. In contrast, BDC2.5 mimotope peptide I-A\(^{\beta}\) dimers failed to prevent diabetes induced by the transfer of spleen cells from diabetic NOD mice or reverse diabetes in diabetic NOD mice. These results suggest that peptide-MHC class II multimers may serve as therapeutics to modulate Ag-specific T cell responses in autoimmune disease by inducing selective T cell anergy and deviating the residual autoimmune response toward production of anti-inflammatory cytokines, but that targeting a single specificity may be insufficient to treat diabetes in naturally occurring settings.

Materials and Methods

Mice

NOD mice were obtained from Taconic (Germantown, NY). NOD.BDC2.5 TCR transgenic mice (24) were obtained from D. Mathis (Harvard, Boston, MA). In our colony, <10% of the BDC2.5 TCR transgenic mice become diabetic unless bred into a T cell-deficient setting. NOD.TCR-\(\alpha\)-deficient mice were obtained from D. Serreze (The Jackson Laboratory, Bar Harbor, ME). All animals were bred and maintained under specific pathogen-free conditions at the University of California animal facility and treated in accordance with University of California animal care guidelines.

Peptides

Peptides were produced by the University of California Biomolecular Resource Center or the University of California HHMI Protein Facility. OVA peptide(223-239) consisted of amino acids IQSVYVAHAAEINAGR. Other peptides with acetylated N termini and amide C termini included: 1040–31, YVRLPWVME, specific for BDC2.5 T cells (23); and hen egg lysozyme(11–23), (HEL), AMKRKHGLNRYGGSY. In our experience, the N-acetylation of the peptides resulted in increased activity of the peptides, as measured in T cell proliferation assays.

Production of soluble bivalent peptide I-A\(^{\beta}\)/IgG2a fusion molecules and multimeric staining reagents

Constructs encoding the I-A\(^{\alpha}\) and I-A\(^{\beta}\) chains fused to acidic/basic leucine zipper sequences with the I-A\(^{\beta}\) acidic leucine zipper further fused to the Cc portion of mouse IgG2a were based on those previously published by Malherbe et al. (25). This template was used for introducing DNA sequences encoding the HEL peptide (aa 11–25) and the BDC2.5 mimotope peptide 1040-31 using overlapping PCR. For the empty I-A\(^{\beta}\) dimer, the cDNA encoding the signal peptide and extracellular domain of I-A\(^{\beta}\) were cloned by PCR from a plasmid construct provided by K. Wucherpfennig (Harvard) and inserted upstream of the basic leucine zipper. Constructs in the Drosophila vector pPHmHa-3 under the control of a metallothionein-inducible promoter were transfected into Drosophila S2 cells along with the selectable marker pS2neo and stable transfectants were selected, as described (25). Expression of I-A\(^{\beta}\) dimers was confirmed using ELISA with Ab pairs against the mouse IgG2a Fc portion (BD PharMingen, San Diego, CA) or the combination of the conformation-specific anti-I-A\(^{\beta}\) mAb, 10-2.16 (provided by A. Sant, University of Rochester, Rochester, NY), and a mAb against the IgG2a Fc portion. Peptide I-A\(^{\beta}\) dimers were purified over affinity columns using protein A (Life Technologies, Rockville, MD). Bound molecules were eluted using 0.1 M sodium citrate (pH 4.5). Fractions were neutralized with the addition of 2 M Tris (pH 9.0), dialyzed against PBS, and concentrated using Centricon concentrators (Millipore, Bedford, MA).

Protein concentration was determined by a bicinchoninic acid assay (Pierce, Rockford, IL). Complexing of peptide I-A\(^{\alpha}\)-dimer with Alexa 488-coupled protein A (Molecular Probes, Eugene, OR) for the production of multimeric staining reagents was performed, as originally described (25). For loading empty I-A\(^{\beta}\) dimers with peptides, 5 \(\mu\)g of dimer was incubated in 10 \(\mu\)l of PBS (pH 7.4) with peptide concentrations at 100–150 \(\mu\)M at 37°C for 17–22 h.

Production of HEL cell line

A 5-wk-old NOD mouse was immunized with 20 \(\mu\)g of HEL(11-25) peptide in CFA in a hind footpad and in the hind flanks. Seven days later, draining lymph nodes were harvested and processed into single cell suspensions. Cells were restimulated in vitro with 1 \(\mu\)M HEL peptide in the presence of irradiated NOD spleen cells in DMEM with standard supplements and 5% FCS. After the second round of in vitro stimulation, IL-2 was added to 20 U/ml. The specificity of the HEL line was demonstrated in T cell proliferation assays (data not shown).

T cell proliferation and responsiveness assays

For T cell proliferation assays using immobilized I-A\(^{\beta}\) dimers, flat-bottom 96-well plates were coated with rat anti-mouse IgG2a (BD PharMingen or Southern Biotechnology Associates, Birmingham, AL) in PBS by overnight incubation at 4°C and washed with PBS. Purified peptide I-A\(^{\beta}\) dimers at 2 \(\mu\)g/ml were added in 100 \(\mu\)l/well, incubated overnight at 4°C, and washed with PBS. BDC2.5 CD4\(^+\) T cells were enriched from spleen and lymph nodes to 95–98% purity by negative sorting with magnetic beads (Miltenyi Biotec, Auburn, CA). BDC2.5 T cells or the HEL T cell line were added at 5 \(\times\)10\(^4\) cells/well along with anti-CD28 mAb PV-1 at 1 \(\mu\)g/ml. [\(^{3}\)H]Thymidine at 1 \(\mu\)Ci/well was added during the last 8 h of a 48-h culture; cells were harvested onto glass fiber filters; and incorporated radioactivity was quantified in a beta scintillation counter (Topcount; PerkinElmer Life Sciences, Boston, MA).

T cells from dimer-treated mice, either total spleen or pancreatic lymph node cells plated at 2 \(\times\)10\(^6\) cells/well or, in some experiments, CD4\(^+\) T cells (enriched by negatively sorting with magnetic beads) plated at 5 \(\times\)10\(^4\) cells/well with 2 \(\times\)10\(^5\) irradiated NOD spleen cells were incubated with 1040-31 peptide at the indicated concentrations. For total spleen and lymph node preparations, BDC2.5 CD4\(^+\) T cells were determined to be at equivalent percentages between populations. [\(^{3}\)H]Thymidine addition and measurement of incorporation were performed, as indicated above.

Flow cytometry and fluorescence-activated cell sorting

For general detection of Ag-specific T cells, 1 \(\times\)10\(^6\) spleen, peripheral lymph node cells, or T cells from T cell lines were plated with 2.4G2 anti-Fc\(\gamma\) mAb supernatant, followed by incubation with peptide I-A\(^{\alpha}\)/AIDS ex88 protein A complexes for 1 h on ice in PBS supplemented with 2% BSA and 0.05% sodium azide. PerlCP or APC-conjugated anti-CD4 mAbs (BD PharMingen) were added during the last 30 min. Cells were then washed and analyzed on a FACSCalibur (BD Immunocytometry Systems, San Jose, CA). For cell-sorting experiments and decreased background staining in detection of rare cells in nontransgenic NODs, CD4\(^+\) T cells were first enriched using negative selection by magnetic bead separation (Miltenyi Biotec). Cells at 1 \(\times\)10\(^6\)/ml were stained with peptide I-A\(^{\alpha}\)-multimers at 100 \(\mu\)g/ml in PBS with 2% FCS for 1 h on ice. APC-conjugated anti-CD4 mAbs were added during the last 30 min. CyChrome-conjugated anti-CD8 and anti-B220 (BD PharMingen), Tri-color-conjugated anti-F4/80, biotin-conjugated anti-mouse NK cells (Catag, Burlington, CA), followed by CyChrome-conjugated streptavidin and Via-Probe (BD PharMingen) were also added during the last 30 min. Cells staining positive for these non-CD4\(^+\) T cell reagents were excluded from analysis.

For analysis of adaptively transferred BDC2.5 T cells, the additional mAbs were: FITC anti-V\(\beta\)4, PE anti-CD25, and PE CD62L (BD PharMingen). Annexin V-PE staining was performed following manufacturer’s recommendations (Southern Biotechnology Associates). Nonviable cells were excluded using Via-Probe (BD Pharmed). In vitro expansion of 1040-31-reactive cells from NOD mice

CD4\(^+\) T cells were enriched from spleen and lymph nodes by negative sorting using magnetic cell sorting. A total of 2 \(\times\)10\(^6\) CD4\(^+\) enriched cells was incubated with 5 \(\times\)10\(^5\) irradiated NOD spleen cells in 24-well plates in the presence of 10 \(\mu\)M 1040-31 peptide. After 6 days, cells were pooled, separated by Ficoll-Hypaque to remove dead cells, and stained for 1040-31- or OVA-reactive T cells.
Adoptive transfer experiments and treatment of mice with peptide I-A\(^{\beta}\)\(^{-}\) dimers

Whole spleen cells from 5- to 10-wk-old BDC2.5 TCR transgenic mice were reconstituted with 0.1 \(\mu\)M 1040-31 peptide for 5 days. On day 5, 1 \(\times\) 10\(^6\) cells were injected i.v. into cohorts of TCR-\(\alpha\)-deficient NOD mice 5–12 wk of age. Twenty-four hours following injection of activated BDC2.5 spleen cells, mice were injected retro-orbitally with PBS or 10 \(\mu\)g per injection of the indicated peptide I-A\(^{\beta}\)\(^{-}\) dimers. Mice received either a single series of three to four injections during the first 3–4 days following the initial transfer of BDC2.5 spleen cells or a single series of injections, followed by weekly injections of the indicated dimers at 10 \(\mu\)g per injection. Anti-IL-10R mAb 1B1.2 (provided by L. Chatenoud, Centre de l’Association Claude Bernard sur les Maladies Autoimmunes and Hopital Necker Enfants Malades Institut Fédératif de Recherche Necker Enfants-Malades, Paris, France) or control rat Ig (BW62) was given i.p. at a dose of 0.5 mg on days 1, 3, 5, and 7 after the transfer of BDC2.5 T cells. Glucose levels were determined from tail vein blood samples using Lifescan (One touch II; Lifescan, Milpitas, CA) glucose meters. Mice were considered diabetic after two consecutive measurements over 250 mg/dL.

Analysis of cytokine production by ELISA and intracellular cytokine staining

Total spleen or pancreatic lymph node cells from adoptively transferred recipient mice were stimulated in vitro with 0.1 \(\mu\)M 1040-31 peptide for 24 or 48 h, and cytokine levels were measured by ELISA using reagents purchased from Endogen (Cambridge, MA) and BD PharMingen. For intracellular cytokine staining, cells were treated with PMA (10 ng/ml), ionomycin (0.5 \(\mu\)M), and monensin (3 \(\mu\)M) for 3–4 h. Cells were harvested and stained with APC-conjugated anti-CD4 (BD PharMingen) and either p31 I-A\(^{\beta}\)/Alexa 488-protein A multimers or FITC-conjugated anti-V\(\beta\)4 (BD PharMingen). Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin, and stained with the indicated PE-conjugated mAbs (BD PharMingen).

Results

Production and characterization of peptide I-A\(^{\beta}\)\(^{-}\) dimers

To study islet-reactive T cells, we produced dimers of soluble peptide I-A\(^{\beta}\)\(^{-}\) in which the BDC2.5 mimotope peptide 1040-31 (23) was covalently linked to the N terminus of the I-A\(^{\beta}\)\(^{-}\)\(\beta\)-chain (hereafter referred to as p31 I-A\(^{\beta}\)\(^{-}\) dimers). A control dimer was also produced with HEL peptide (aa 11–25) covalently attached (HEL I-A\(^{\beta}\)). The transmembrane and cytoplasmic domains of the I-A\(^{\beta}\)\(\alpha\) and the I-A\(^{\beta}\)\(\beta\)-chains were replaced with leucine zipper dimerization domains to produce soluble molecules and to promote assembly of the \(\alpha\) and \(\beta\)-chains. The \(\alpha\)-chain was further modified by the addition of the murine IgG2a Fc domain allowing for the production of divalent molecules, as previously described (25). I-A\(^{\beta}\) dimers were produced in stably transfected Drosophila S2 cells and purified by affinity chromatography.

Peptide I-A\(^{\beta}\)\(^{-}\) multimers were examined for the ability to identify Ag-specific T cells in flow cytometry assays. Peptide I-A\(^{\beta}\)\(^{-}\) dimers were preincubated with Alexa 488-coupled protein A to enhance flow cytometric staining (25). p31 I-A\(^{\beta}\)\(^{-}\) multimers stained \(\sim\)95% of CD4\(^+\) T cells from BDC2.5 transgenic Tg\(^+\) (Tg\(^-\)) NOD mice (Fig. 1A), comparable to the number of BDC2.5 Tg\(^+\) T cells identified by an Ab to V\(\beta\)4 (data not shown). p31 I-A\(^{\beta}\)\(^{-}\) multimers stained relatively low numbers of CD4\(^+\) T cells from a HEL-specific NOD T cell line (1.7%). In contrast, HEL I-A\(^{\beta}\)\(^{-}\) dimers stained \(\sim\)18% of CD4\(^+\) T cells from the HEL line, but detected relatively low numbers of CD4\(^+\) cells from BDC2.5 Tg\(^-\) mice (0.7%). In addition to dimers with covalently linked peptides, empty I-A\(^{\beta}\)\(^{-}\) dimers capable of being loaded with various peptides in vitro were generated. Empty I-A\(^{\beta}\)\(^{-}\) reagents were demonstrated to be functional in flow cytometry experiments. Empty I-A\(^{\beta}\)\(^{-}\) multimers did not react with CD4\(^+\) T cells from either NOD or BDC2.5 TCR transgenic mice (0.8 and 1.5%, respectively); however, empty I-A\(^{\beta}\) multimers loaded with the 1040-31 peptide stained 94% of BDC2.5 CD4\(^+\) T cells. There was no readily apparent increase in staining of NOD CD4\(^+\) T cells (1.1%) as compared with background with this reagent (Fig. 1B).

Peptide I-A\(^{\beta}\)\(^{-}\) dimers were also tested for biological activity and specificity in T cell proliferation assays using CD4\(^+\) T cells isolated from BDC2.5 transgenic NOD mice and the HEL-specific T cell line. Peptide I-A\(^{\beta}\)\(^{-}\) dimers were immobilized in 96-well plates, and T cell proliferation was measured by \(^{3}H\)thymidine incorporation following 48 h of culture. p31 I-A\(^{\beta}\)\(^{-}\) dimers activated BDC2.5 Tg\(^+\) T cells, but failed to activate the HEL line above background levels (Fig. 1C). Similar activation of BDC2.5 T cells was observed with empty I-A\(^{\beta}\)\(^{-}\) dimers loaded with 1040-31, but not control peptides (data not shown). In contrast, HEL I-A\(^{\beta}\)\(^{-}\) dimers activated the HEL line, but failed to activate BDC2.5 Tg\(^+\) T cells. Empty unloaded I-A\(^{\beta}\)\(^{-}\) dimers failed to activate either population of T cells. The addition of Con A did not induce activation.
peptide on endogenous APCs (data not shown). Weekly treatment with p31 I-A<sup>87</sup> dimers was found to further enhance protection from diabetes. All mice receiving weekly treatment with p31 I-A<sup>87</sup> dimers were protected from the onset of diabetes throughout the course of treatment (Fig. 2B).

**In vivo treatment with p31 I-A<sup>87</sup> dimers induced T cell hyporesponsiveness**

Immune suppression/tolerance can be achieved by several mechanisms including prevention of TCR engagement by down-regulating or blocking the TCR, deletion of autoreactive T cells, or the induction of anergy. To determine how p31 I-A<sup>87</sup> dimers were preventing the onset of diabetes, recipient mice were analyzed 3 days following transfer. At this time point, mice had received two injections of peptide-MHC dimers or PBS, and all groups of mice were normoglycemic. Compared with control-treated mice, p31 I-A<sup>87</sup> dimer-treated animals showed slight increases in the numbers of BDC2.5 Tg<sup>T+</sup> T cells found in the spleens and pancreatic lymph nodes. BDC2.5 Tg<sup>T+</sup> CD4<sup>+</sup> cells in p31 I-A<sup>87</sup> dimer-treated mice ranged from 1 × 10<sup>6</sup> to 6 × 10<sup>5</sup>, averaging 3.4 × 10<sup>5</sup> in the spleen, and ranged from 0.3 × 10<sup>6</sup> to 2.6 × 10<sup>5</sup>, averaging 1.4 × 10<sup>5</sup> in the pancreatic lymph nodes. Total BDC2.5 Tg<sup>T+</sup> CD4<sup>+</sup> cells in control-treated mice ranged from 1 × 10<sup>5</sup> to 2.8 × 10<sup>5</sup>, averaging 1.5 × 10<sup>5</sup> in the spleens, and ranged from 0.26 × 10<sup>5</sup> to 1 × 10<sup>5</sup>, averaging 0.61 × 10<sup>5</sup> in the pancreatic lymph nodes.

There were no significant differences in cell numbers between control PBS-treated and control I-A<sup>87</sup> dimer-treated animals. In p31 I-A<sup>87</sup> dimer-treated mice, a minor percentage of BDC2.5 Tg<sup>T+</sup> T cells appeared to have down-regulated their TCR; however, the majority of cells retained expression of TCR, as determined by both p31 I-A<sup>87</sup> dimer and anti-V<beta>4 Ab staining (data not shown). At day 3 following transfer, BDC2.5 CD4<sup>+</sup> T cells in p31 I-A<sup>87</sup> dimer-treated mice were activated in both the spleen and pancreatic lymph nodes, as determined by increased CD25 and CD69 expression and decreased CD62L expression compared with BDC2.5 Tg<sup>T+</sup> CD4<sup>+</sup> cells isolated from PBS or control dimer-treated mice (Fig. 3A). BDC2.5 CD4<sup>+</sup> cells from p31 I-A<sup>87</sup> dimer-treated mice also showed an increase in cell size consistent with an activated phenotype (data not shown). At this time point, transferred BDC2.5 CD4<sup>+</sup> cells in PBS or control I-A<sup>87</sup> dimer-treated animals appeared to be resting in the spleen based on low levels of CD25 and CD69 expression. Transferred cells in the pancreatic lymph nodes of these animals expressed CD69 consistent with studies demonstrating that BDC2.5 Tg<sup>T+</sup> CD4<sup>+</sup> cells encounter cognate Ag in pancreatic lymph nodes (26), but in contrast to cells recovered from pancreatic lymph nodes of p31 I-A<sup>87</sup> dimer-treated mice, expressed lower levels of CD25 and had not fully down-regulated CD62L (Fig. 3A).

The increase in the activation phenotype of transferred cells in the p31 I-A<sup>87</sup> dimer-treated animals was accompanied by an increase in cell death. At day 3 following transfer, BDC2.5 CD4<sup>+</sup> cells from spleen and pancreatic lymph nodes of p31 I-A<sup>87</sup> dimer treated showed a greater percentage of cells staining positive for annexin V compared with cells from control-treated mice (Fig. 3B). However, the increased cell death did not result in total deletion of BDC2.5 CD4<sup>+</sup> cells. By days 8–10, the numbers of BDC2.5 CD4<sup>+</sup> cells had increased in both the spleen and pancreatic lymph nodes of p31 I-A<sup>87</sup> dimer-protected animals, averaging

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**FIGURE 2.** Treatment with p31 I-A<sup>87</sup> dimers prevents diabetes. A. Incidence of diabetes in groups of mice treated with a single series of treatment. Activated spleen cells from BDC2.5 TCR transgenic mice were transferred to TCR-α-deficient NOD mice. Recipient mice were treated with PBS, HEL I-A<sup>87</sup> dimers, or p31 I-A<sup>87</sup> dimers on days 1, 2, and 4 following transfer and followed for blood glucose levels. Data are from one of two experiments that gave similar results. Total mice in each group from both experiments are PBS, n = 6; HEL I-A<sup>87</sup> dimer, n = 3; p31 I-A<sup>87</sup> dimer, n = 6. B. Blood glucose levels of adoptively transferred recipient mice treated with a single series of injections, as described above, followed by weekly injections of PBS (n = 11), empty I-A<sup>87</sup> dimers (n = 4), HEL I-A<sup>87</sup> dimers (n = 8), or p31 I-A<sup>87</sup> dimers (n = 10). Treatment was stopped after mice gave two consecutive measurements above 250 mg/dl. None of the p31 I-A<sup>87</sup> dimer-treated mice developed diabetes during the course of treatment. Individual p31 I-A<sup>87</sup> dimer-protected mice were sacrificed at various time points throughout the course of the experiments for analysis.
Treatment with p31 I-A^d7 dimers activates autoreactive T cells and results in increased cell death. A, Pancreatic lymph node cells from mice treated with HEL or p31 I-A^d7 dimers were stained with anti-CD4, anti-Vβ4 (to identify BDC2.5 Tg^+ cells), and either anti-CD25 or anti-CD62L mAbs. Data shown are staining of CD4^+/Vβ4^+ gated cells. Similar results were found when using p31 I-A^d7 multimers in place of anti-Vβ4 mAbs to identify BDC2.5 transgenic T cells. B, Pancreatic lymph node cells from treated mice were stained with annexin V and anti-CD4 and anti-Vβ4 mAbs. Data shown are staining of CD4^+/Vβ4^-, 7-amino actinomycin D^- cells.

6.7 × 10^6 (±5.1 × 10^5) in the spleens and averaging 1.0 × 10^5 (±1.3 × 10^5) in the pancreatic lymph nodes. Unlike the early time points, the cells that were observed late did not appear to have an activated phenotype in that CD25 expression was negative (data not shown). At these later time points, the number of transferred cells in control-treated animals averaged 1.7 × 10^6 (±1.4 × 10^6) in the spleens and 2.2 × 10^5 (±1.7 × 10^5) in the pancreatic lymph nodes. To examine whether BDC2.5 CD4^+ cells in p31 I-A^d7 dimer-treated animals were responsive to Ag, the ex vivo response of transferred BDC2.5 cells to 1040-31 peptide was tested. Compared with equal numbers of BDC2.5 Tg^+ CD4^+ cells from control-treated mice, cells from the spleen and pancreatic lymph nodes from p31 I-A^d7 dimer-treated mice showed reduced proliferative responses to peptide (Fig. 4, A and B). Responsiveness could not be restored by the addition of IL-2.

Treatment with p31 I-A^d7 dimers generates IL-10-producing cells

Suppression/Tolerance of autoimmunity can also be achieved by deviating the immune response from a pathogenic Th1 response to a nonpathogenic or regulatory response. To investigate the effect of p31 I-A^d7 dimer treatment on cytokine production, splenic cells from treated animals were stimulated in vitro with 1040-31 peptide and the resulting supernatants were analyzed by ELISA. Cells from p31 I-A^d7 dimer-treated animals consistently produced increased levels of IL-10 and decreased levels of IFN-γ compared with PBS or HEL I-A^d7 dimer-treated animals (Fig. 5A). On average, compared with cells from control-treated animals, cells from p31 I-A^d7 dimer-treated animals produced increased levels of IL-4, but this result was more variable with cells from a minority of control-treated animals producing equivalent levels of IL-4.

There was little difference in IL-2 production between the different groups of mice. The increased production of IL-4 and IL-10 from p31 I-A^d7 dimer-treated mice was confirmed by intracellular cytokine staining with a greater percentage of these cells from both spleen and pancreatic lymph nodes staining for IL-4 and IL-10 compared with cells from PBS and control I-A^d7 dimer-treated mice (Fig. 5B and data not shown).

To determine whether IL-10 was involved in the suppression of diabetes mediated by p31 I-A^d7 dimer treatment in vivo, blocking anti-IL-10R mAb or control rat Ig mAb was administered to mice receiving activated BDC2.5 transgenic spleen cells and treatment with p31 I-A^d7 dimers. Anti-IL-10R mAb blocked the protective effect of p31 I-A^d7 dimer treatment, with mice becoming diabetic at the same rate as mice receiving control or no dimer treatment (Fig. 5C). Control Ig had no effect on p31 I-A^d7 dimer protection.

Detection of BDC2.5 mimotope-reactive cells in wild-type NOD mice

Having determined that peptide I-A^d7 dimer therapy was successful in preventing diabetes in a system in which the majority of T cells expressed a single TCR, we next wanted to test the effect of peptide-MHC class II multimer therapy in the more complex wild-type NOD background. Because the BDC2.5 mimotope 1040-31 was identified from a combinatorial peptide library and searches of databases revealed no precise matches (23), we first wanted to be sure that the p31 I-A^d7 dimers were able to target Ag-specific cells from a nontransgenic NOD background. NOD mice were immunized with 1040-31 or control OVA peptide (aa 323–339) emulsified in CFA, and CD4^+ cells from draining lymph nodes were analyzed for the presence of Ag-specific T cells. For the detection of rare cells, samples were also stained for B220, CD8, F4/80, and...
CD11c. Cells staining positive for these reagents were excluded from analysis, resulting in decreased background staining (compare background levels in Fig. 1A vs Fig. 6A). FACS analysis of draining lymph nodes detected an increase in the number of p31-reactive CD4⁺/H11001 T cells in p31-injected mice with numbers representing 0.34 ± 0.02% of CD4⁺/H11001 cells compared with levels of 0.04 ± 0.02% in nonimmunized mice and 0.11 ± 0.02% in mice immunized with OVA peptide (Fig. 6A). Conversely, OVA-loaded I-Ag7 dimers detected a population of cells representing 0.21 ± 0.01% of CD4⁺/H11001 T cells in OVA-immunized mice compared with 0.05 ± 0.01% in nonimmunized mice and 0.06 ± 0.01% in p31-immunized mice. Moreover, in vitro restimulation of cells from peptide-primed mice with immunizing peptide resulted in increased percentages of peptide-specific cells detected by peptide I-Ag7 dimers. After one round of in vitro stimulation, the percentage of CD4⁺/H11001 cells detected by p31 I-Ag7 dimers increased to 24% in 1040-31 peptide-stimulated cells (data not shown). The percentage of CD4⁺/H11001 cells detected by OVA-loaded I-Ag7 dimers increased to 12% in OVA peptide-stimulated cells.

To further substantiate that 1040-31-reactive CD4⁺/H11001 cells identified by p31 I-Ag7 dimers by flow cytometry were indeed specific for 1040-31 peptide, dimer-positive and dimer-negative cells from 1040-31/CFA-primed animals were sorted using FACS and then tested for reactivity to 1040-31 presented by APCs or to plate-bound p31 I-Ag7 dimers. p31 I-Ag7 dimer-positive, but not dimer-negative, CD4⁺/H11001 T cells were enriched from spleens and lymph nodes of NOD mice. Cells were stimulated in vitro with 1040-31 peptide (10⁻⁴ M) presented by irradiated NOD APCs. After 6 days, cells were pooled and live cells were collected on a Ficoll gradient and stained as indicated in Fig. 2A. Data shown are the percentage of either p31 I-Ag7 dimer-reactive cells or OVA-loaded I-Ag7 dimer-reactive cells detected compared with control.

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negative cells proliferated in response to 1040-31 peptide presented by NOD APCs (Fig. 6B). Furthermore, only p31 I-A\(^{e7}\) dimer-positive cells were reactive to plate-bound recombinant p31 I-A\(^{e7}\) (data not shown). These data demonstrated that cells capable of responding to 1040-31 exist in the NOD repertoire and that p31 I-A\(^{e7}\) dimers could detect most, if not all, 1040-31-reactive cells from immunized mice.

T cells from prediabetic and diabetic NODs respond to BDC2.5 mimotope peptides, 1040-63 and 1040-31, suggesting that BDC2.5 mimotope-reactive T cells have undergone a spontaneous expansion stimulated by self Ag in vivo (23) (data not shown). To determine whether p31 I-A\(^{e7}\) dimers could identify spontaneously arising 1040-31-reactive T cells, CD4\(^{+}\) T cells from nonimmunized NOD mice were stimulated in vitro with 1040-31 peptide and analyzed by flow cytometry. This approach has been shown to enhance detection of rare cells by peptide-MHC class II multimers by expanding the pool of preexisting Ag-specific cells (27–29).

Flow cytometry analysis of CD4\(^{hi}\) T cell populations from NOD mice stimulated with 1040-31 peptide showed an increase in the percentage of p31 I-A\(^{e7}\)-reactive cells, increasing to 0.22% from 0.04 \(\pm\) 0.02% detected in unprimed NOD T cells (Fig. 6, A and C). In contrast, CD4\(^{hi}\) cells reactive to OVA I-A\(^{e7}\) remained at approximately the same percentage in 1040-31-stimulated cells compared with freshly isolated T cells, 0.08% vs 0.05 \(\pm\) 0.01%, respectively (Fig. 6, A and C).

Effect of BDC2.5 mimotope I-A\(^{e7}\) dimers on endogenous BDC2.5 mimotope-reactive cells in NOD mice

To determine whether p31 I-A\(^{e7}\) dimer treatment had any effect on endogenous 1040-31 peptide-reactive cells, NOD mice were treated with p31 or HEL I-A\(^{e7}\) dimers. Spleen cells from treated mice were then challenged with 1040-31 or HEL peptide in vitro. IL-10 and IFN-\(\gamma\) were below detection in both groups upon challenge with HEL peptide (Fig. 7A). IL-10 was also undetectable in HEL I-A\(^{e7}\) dimer-treated cells in response to 1040-31 peptide. In contrast, IL-10 was detectable in p31 I-A\(^{e7}\) dimer-treated cells upon response to 1040-31 peptide. IFN-\(\gamma\) production in response to 1040-31 challenge was not decreased in cells from p31 I-A\(^{e7}\) dimer-treated animals compared with cells from control-treated mice (Fig. 7A). Treatment with p31 I-A\(^{e7}\) did not result in cells becoming anergic to Ag as spleen cells from p31 I-A\(^{e7}\) dimer-treated mice proliferated as well or better than cells from control-treated animals (Fig. 7B). Furthermore, treatment with p31 I-A\(^{e7}\) dimers failed to prevent diabetes induced by the transfer of spleen cells from diabetic mice and failed to reverse diabetes in newly diabetic NOD mice (data not shown).

Discussion

We have developed peptide I-A\(^{e7}\) dimers for use in the NOD model of autoimmune diabetes to explore the properties of peptide-MHC class II dimers as therapeutics to selectively inhibit the pathogenicity of diabetogenic T cells. We initially characterized the tolerogenic capabilities of peptide-MHC class II dimers on a previously primed autoimmune response using BDC2.5 TCR transgenic T cells. In vivo treatment with BDC2.5 mimotope-peptide-p31 I-A\(^{e7}\) dimers prevented diabetes caused by transferred BDC2.5 T cells. Treatment with p31 I-A\(^{e7}\) dimers resulted in the activation of transferred CD4\(^{+}\) T cells in both the pancreatic lymph nodes and the spleen, as determined by the increased expression of the activation markers CD25 and CD69 and the decrease in CD62L expression. Activation was accompanied by an increase in cell death, as determined by annexin V staining. An abortive form of activation that results in apoptosis has been described previously as a mechanism for inducing peripheral tolerance for both naïve and memory cells (30, 31). p31 I-A\(^{e7}\) dimer-induced activation and death of autoreactive T cells are also consistent with other studies of peptide-MHC class II multimers in which a soluble dimeric DR2-IgG fusion protein with a bound peptide from myelin basic protein was found to anergize a myelin basic protein-specific T cell clone through initial activation, followed by susceptibility to late stage apoptosis upon rechallenge with Ag (16). Clonal deletion alone cannot account for the protection from diabetes, in that increasing numbers of BDC2.5 transgenic CD4\(^{+}\) T cells were detected in the spleen, pancreases, and pancreatic lymph nodes in all protected animals examined as late as day 62 posttransfer. Upon rechallenge to Ag in vitro, cells from p31 I-A\(^{e7}\)-treated animals were hypoproliferative and produced increased levels of the anti-inflammatory cytokine IL-10 and decreased levels of inflammatory cytokine IFN-\(\gamma\) compared with cells from control-treated animal. Intracellular cytokine staining of cells ex vivo supported that splenic and pancreatic lymph node CD4\(^{+}\) T cells from p31 I-A\(^{e7}\) dimer-treated mice were producing increased IL-4 and IL-10 in vivo. Most importantly, treatment of mice with anti-IL-10R Ab reversed the tolerogenic effects of the dimer therapy. Thus, p31 I-A\(^{e7}\) dimer treatment promotes death of pathogenic cells while promoting the development of regulatory cells producing IL-10. This altered immune response results in down-modulation of the autoimmune response to the autoantigen. The regulatory cells are similar to type 1 regulatory T cells that have been shown to suppress the response of both naïve and memory T cells and Th1-mediated pathologies through bystander suppression (32). A previous study demonstrating the tolerogenic effect of HA peptide-MHC class II dimers in a system in which mice express a transgenic TCR specific for HA and express HA in pancreatic islets also attributed protection from diabetes to the induction of IL-10-producing regulatory T cells (19). Our studies extend these studies to demonstrate that the soluble bivalent
peptide-MHC can induce IL-10 production in disease-relevant spontaneously diabetogenic T cells and as importantly, previously activated T cell populations more like what would be encountered in the new onset autoimmune setting.

The development of peptide I-**A**^87^ dimers allowed studies to be performed in autoimmune prone NOD mice in a nontransgenic system. p31 I-**A**^87^ dimers were found to identify a small population of cells in wild-type NOD mice. Treatment of NOD mice with p31 I-**A**^87^ dimers resulted in the increased production of IL-10 from 1040-31-reactive cells, but did not result in decreased IFN-**y** production or in the induction of anergy. Furthermore, in preliminary studies, the p31 I-**A**^87^ dimer therapy was unable to prevent or delay diabetes caused by diabetogenic spleen cells from NOD mice or to reverse diabetes in newly diabetic NOD mice. There are several potential reasons why p31 I-**A**^87^ dimers were ineffective in these settings. One is that p31 I-**A**^87^ dimer-reactive cells may be present at too low of a frequency to overcome the diabetogenicity of other islet-reactive cells present. Multiple Ags are thought to be targeted in type 1 diabetes, and various autoreactive T cells are likely to be present. Peptide 1040-31-reactive cells were determined to be present in spleens of diabetic NOD mice by proliferation, but were below the level of detection by flow cytometry unless first expanded in vitro. Another recent study using BDC2.5 mimotope I-**A**^87^ tetramers also reported the BDC2.5 mimotope-reactive cells in NOD spleens at less than 0.3% of CD4^+^ cells (33). Treatment with p31 I-**A**^87^ dimers did alter the response of endogenous 1040-31-reactive cells, resulting in an increased production of IL-10 upon in vitro challenge. However, the level may have been too low to cause bystander suppression of other islet-reactive T cells. In the future, the effect of combinations of multiple peptide-MHC class II reagents can be tested. In NOD mice, ~50% of T cells infiltrating the pancreatic islets react with insulin, and 97% of the insulin-reactive T cells respond to the insulin peptide B(9–23) (34). Another possibility why p31 I-**A**^87^ dimers were ineffective in inducing tolerance in NOD mice is that cross-reacting cells may respond more anti-CD3 like while retaining the ability to target Ag-specific T cells. Further studies in spontaneous autoantigen-induced models of autoimmunity will be important to evaluate fully the therapeutical potential of peptide-MHC multimers.

References


