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Prevention of type 1 diabetes in mice by tolerogenic vaccination with a strong agonist insulin mimetope

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Type 1 diabetes (T1D) results from the destruction of insulin-secreting pancreatic β cells by autoreactive T cells. Insulin is an essential target of the autoimmune attack. Insulin epitopes recognized by diabetogenic T cell clones bind poorly to the class II I–Aα7 molecules of nonobese diabetic (NOD) mice, which results in weak agonistic activity of the peptide MHC complex. Here, we describe a strongly agonistic insulin mimetope that effectively converts naive T cells into Foxp3+ regulatory T cells in vivo, thereby completely preventing T1D in NOD mice. In contrast, natural insulin epitopes are ineffective. Subimmunogenic vaccination with strongly agonistic insulin mimetopes might represent a novel strategy to prevent T1D in humans at risk for the disease.
agonistic insulin mimetopes can convert naive T cells into protective Foxp3+ T reg cells and thereby prevent T1D.

Our results show that dominant tolerance to essential insulin epitopes indeed fails in T1D and that its establishment by Foxp3+ T reg cell conversion using a strong agonistic insulin mimetope is capable of completely preventing the onset of T1D in the nonobese diabetic (NOD) mouse.

RESULTS

Agonist and T reg cell conversion activity of insulin epitopes and mimetopes

T cells expressing a transgenic TCR specific for the insulin B chain epitope 9–23 presented in the context of I-Aβ MHC class II molecules (Jasinski et al., 2006) were used to determine the activities of several insulin epitopes and mimetopes (Fig. 1). These cells represent a subset of T cells that recognize the weakly agonistic natural epitope because high doses of the natural epitope are required to induce proliferation. The poor agonistic activity is likely to result from the weak binding of the natural insulin epitope to I-Aβ in the third MHC-binding register (Stadinski et al., 2010; Fig. 1 a, MHC-binding residues for HLA-A2).

(a) One-letter amino acid codes are shown for insulin B chain epitopes and mimetopes. MHC-binding residues are shown in subscript and in blue for binding register 3. Residues 9–11, which do not contribute to MHC binding, are shown in gray. (b) Analysis of proliferation of insulin B:9–23 TCR transgenic T cells stimulated with epitopes and mimetopes. MHC-binding residues are shown in subscript and are marked blue for the binding register 3) that is recognized by most T cell clones from diabetic mice, including the T cells used in this experiment (Stadinski et al., 2010). Accordingly, the arginine residue (R22) in the natural peptide that in this reading frame confronts another arginine in the positively charged p9 pocket of I-Aβ was replaced with glutamic acid (Fig. 1 a, R22E). Both the full-length and the core peptide with the mutated residue induced much stronger proliferation (~40-fold at 0.1 µg/ml), as well as conversion into Foxp3+ T reg cells (~3-fold) when used under appropriate subimmunogenic conditions, i.e., either applied by subcutaneous peptide infusion using osmotic minipumps (5 µg/d for 14 d) or by a subcutaneous single shot injection using DEC205 fusion antibodies (40 ng/mouse; Fig. 1). The mutated core peptide was labeled mimetope 3 and used in all further experiments.

T1D prevention and insulin autoantibodies

Insulin autoantibodies (IAAs) correlate with insulitis, the development of T1D, and T cell activation (Yu et al., 2000), the latter interfering with the effective conversion of T cells into Foxp3+ T reg cells (Fig. 2 a). We used the recently developed highly specific and sensitive method of a competitive IAA assay that has overcome previous problems in IAA detection (Babaya et al., 2009). In NOD mice, IAA can be detected as early as 15 d after birth (Reddy et al., 1988; Michel et al., 1989) with few mice exhibiting very high titers at the age of 4 wk, correlating with early onset of disease (Yu et al., 2000). To correlate IAA indices and possible effects of insulin epitopes and mimetopes on T1D prevention, IAA indices were determined in NOD mice at the age of 4 (before vaccination) and 6 wk (after vaccination). As shown in Fig. 2 there was indeed a correlation of IAA indices and development of diabetes, in that mice with higher indices developed diabetes earlier. Moreover, although the natural epitope afforded no protection, the mimetope 3 prevented the onset of T1D in the mice with moderate IAA (indices <1). Only in mice that already harbored high IAA with indices >1 at 4 wk of age was the mimetope 3 unable to completely prevent disease development. This is best explained by the assumption that mice with high indices of IAA already contain too many activated T cells that cannot be efficiently converted into Foxp3+ T reg cells (Fig. 2 a). Analysis of IAA before and after vaccination showed that subimmunogenic treatment with mimetope 3 substantially decreased IAA indices (Fig. 2 d), which is consistent with inducing immunosuppression. At 4 wk of age, ~95% of mice exhibited IAA with indices <1 (moderate levels of IAA), and therefore were included in further experiments.

The protection by mimetope 3 was studied for longer time periods (Fig. 3 a), and with varying doses, in the aforementioned mice. A dose of 5 µg/d of mimetope 3 protected mice for 40 wk (which is when animals were sacrificed for analyses), whereas the same dose of the natural epitope had very little beneficial effect in preventing diabetes development. NOD mice were protected from developing T1D for up to 70 wk when they were followed for longer periods.

Figure 1. Agonist activity of insulin B chain epitopes and mimetopes. (a) One-letter amino acid codes are shown for insulin B chain epitopes and mimetopes. MHC-binding residues are shown in subscript and in blue for binding register 3. Residues 9–11, which do not contribute to MHC binding, are shown in gray. (b) Analysis of proliferation of insulin B:9–23 transgenic T cells stimulated with epitopes and mimetopes. Results are the averages ± SEM of triplicate wells from four independent experiments. (c) Agonist and T reg cell conversion activity. In vivo T reg cell conversion using naive CD4+ insulin B:9–23 TCR transgenic T cells transferred into congenic NOD Thy1.1+ mice at 4 wk of age. Error bars show percentages of Foxp3+ T reg cells (% SEM from three independent experiments; n = 4 per group) at 2 wk after injection of cells and infusion of peptide. Peptide was delivered either by a 2-wk infusion period using osmotic minipumps or by a single i.p. injection of DEC205 fusion antibody. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus insulin B:9–23.
Figure 2. Efficacy of Foxp3+ T reg cell vaccination as a function of IAA levels at 4 wk. (a) Naive CD4+CD25−CD62LhiCD44loFoxp3GFP− cells or activated CD4+CD25−CD62LhiCD44hiFoxp3GFP− cells were subjected to in vitro conversion assays (α-CD3 + α-CD28) in the presence or absence (= control) of TGFβ. After 3 d, Foxp3 expression was analyzed by FACS. Figure shows a representative dot plot from four independent experiments performed in triplicates per treatment group. (b and c) NOD mice were infused with insulin B:9-23 peptide (b) or insulin mimetope 3 (c) at 4–6 wk of age. IAA indices were measured at 4 wk of age, before vaccination. Vertical lines indicate the lower detection limit for IAs. n = 12 for each group from three independent experiments. (d) IAA indices in NOD mice were measured at 4 wk of age (before vaccination) and at 6 wk of age (after vaccination). Nonvaccinated control mice were infused with PBS. Mice with IAA indices <1 were included. Bars show averages ± SEM of IAA indices. n = 8 from two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus insulin B:9-23.

Figure 3. Development of diabetes. (a) Cumulative diabetes incidence in NOD mice, nonvaccinated, vaccinated with insulin B:9-23 or insulin mimetope 3 at 4–6 wk of age. n = 12 from three independent experiments. (b) Cumulative diabetes incidence of NOD mice, nonvaccinated, vaccinated with insulin B:9-23 or insulin mimetope 3 at 12–14 wk of age. n = 6 from two independent experiments. **, P < 0.01; ***, P < 0.001 versus insulin B:9-23.

Figure 4. Dose response of insulin epitope and mimetope. (a) Cumulative diabetes incidence in NOD mice that were nonvaccinated, vaccinated with insulin B:9-23, or vaccinated by insulin mimetope 3 at 4–6 wk of age (doses 2.5–20 µg/d). n = 6 from two independent experiments. (b) In vivo T reg cell conversion using naive CD4+ insulin B:9-23 TCR transgenic T cells transferred into congenic NOD Thy1.1+ mice. Percentages of Foxp3+ T reg cells (± SEM, n = 6 from two independent experiments) at 2 wk after vaccination with indicated doses of peptide. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus insulin B:9-23.
When the natural epitope and the mimetope were titrated (Fig. 4), it became clear that daily doses of mimetope 3 (2.5 or 20 µg/d) applied at 4–6 wk of age were somewhat less effective in achieving complete protection from diabetes development than 5 and 10 µg/d doses, whereas none of the doses of the natural epitope afforded significant protection from disease (Fig. 4 a). This is consistent with results that were obtained by testing various doses on Foxp3+ T reg cell conversion; none of the doses of the natural epitope (including 40 µg/d) resulted in Foxp3+ T reg cell conversion exceeding 15% of naive T cells. Mimetope 3 at a dose of 5 and 10 µg/d yielded between 40 and 50% of converted Foxp3+ T reg cells, whereas a dose of 20 µg/d resulted in <30% conversion (Fig. 4 b).

We then tested whether the rapamycin analogue everolimus had an effect on the development of T1D in normal NOD mice and in mice that had received the natural epitope or mimetope 3. As shown in Fig. 5, everolimus indeed delayed the onset of T1D in otherwise untreated mice and mice receiving the natural epitope, whereas no T1D developed in mice that also received mimetope 3. These data suggest that the simultaneous application of everolimus and mimetope represents a powerful tool for the prevention of T1D (see insulitis scores in Fig. 6) because everolimus prevents the immediate activation of effector cells as long as naive T cells are not yet converted while enhancing the conversion of naive T cells into T reg cells in vivo.

It has been established that the extent of infiltration by mononuclear cells in pancreatic islets of NOD mice corresponds to the extent of glycemia. Mice that have been treated with the natural epitope exhibited mostly strong (malignant) infiltration, whereas mimetope 3–treated mice showed mainly perisulitis or partial insulitis with many morphologically intact β cells producing insulin, resulting in normoglycemia (Fig. 6 and data shown in Fig. 7). Quantitative assessment of a larger number of samples yielded insulitis scores as also shown in Fig. 6. Mimetope 3–vaccinated mice exhibited significantly lower insulitis scores in comparison to untreated mice or mice that had received the natural insulin epitope. The combined application of

Moreover, in mice with moderate levels of IAA, vaccination with mimetope 3 but not the natural insulin epitope was still effective in preventing the development of T1D, even when the peptide infusion was started in 12-wk-old mice (Fig. 3 b).
mimetope 3 and everolimus resulted in a further reduction of insulitis scores, underlining a superior effect in the prevention of T1D when used together (Fig. 6 and Fig. 7).

Dominant tolerance in normoglycemic mice
To test whether the observed protection was indeed caused by dominant tolerance executed by converted Foxp3\(^+\) T reg cells the number of Foxp3\(^+\) T reg cell was determined in several experimental mice. To this end, NOD or NOD Foxp3 GFP reporter mice that were untreated or injected with natural epitope or mimetope 3 were analyzed. As shown in Fig. 7 and Fig. 8 mice vaccinated with mimetope 3 always exhibited the highest number of Foxp3\(^+\) cells and insulin-

producing \(\beta\) cells, which was further enhanced when everolimus was co-injected. The data of percentages of Foxp3\(^+\) T reg cells in pancreas-infiltrating CD4\(^+\) T cells confirm the superior effect of the mimetope in inducing Foxp3\(^+\) T reg cells as seen from absolute numbers of T reg cells (Fig. 8). It is clear that injection of the natural epitope also yielded a modest increase in Foxp3\(^+\) cells that was obviously insufficient to protect from T1D (Fig. 3 and Fig. 4). In addition, in NOD Foxp3 GFP reporter mice vaccination with mimetope 3 resulted in the highest percentages and most GFP\(^+\) cells in pancreatic lymph nodes (Fig. 8 b).

Because activated Foxp3\(^+\) T reg cells do not home properly to pancreatic lymph nodes (Tarbell et al., 2004, 2007), we did not attempt to transfer T reg cells from vaccinated mice to assess dominant tolerance. Instead, we used a previously described protocol (Sundstedt et al., 2003; Tarbell et al., 2004, 2007) of transferring CFSE-labeled, insulin-specific T cells into differently vaccinated mice and monitoring their proliferation. If dominant tolerance was established, proliferation should be suppressed and recessive forms of tolerance like deletion or anergy should not result in suppression of proliferation because recessive tolerance induced by strong agonist ligands is preceded by proliferation (Lanoue et al., 1997). Fig. 9 shows that mimetope 3–vaccinated mice displayed suppression of proliferation of insulin-specific T cells when the proportion of cells with diluted CFSE label was determined (Fig. 9a-c). Suppression of insulin-specific effector cells in mimetope 3–treated mice could be detected in 8- and 30-wk-old mice (Fig. 9, a–c). In additional experiments, we transferred CFSE-labeled, polyclonal-activated CD4 effector T cells purified from pancreatic lymph nodes of newly diabetic mice into mimetope-vaccinated NOD mice. It became clear that, in contrast to the natural epitope, vaccination with the mimetope resulted in suppression of polyclonal effector cells (Fig. S1).

Finally, we determined the proliferative response of ex vivo cells from pancreatic lymph nodes from untreated, natural epitope–, or mimetope 3–vaccinated

![Figure 7. Analysis of Foxp3 expression in 30-wk-old NOD mice.](image)

**Figure 7.** Analysis of Foxp3 expression in 30-wk-old NOD mice. Foxp3 immunohistochemistry in pancreatic cryosections. (a) Co-stainings of CD4 (green) and Foxp3 (red) expression in the respective treatment groups (b–d). Representative co-stainings of insulin (green) and Foxp3 (red) expression in pancreatic sections of mice that were non-vaccinated (b), vaccinated with insulin B:9-23 (c), and vaccinated with insulin mimetope 3 (d). n = 8 per group from two independent experiments. Bars, 100 \(\mu\)m. (e) Quantification of Foxp3\(^+\) cells in pancreatic sections as described in b–d. Bars show Foxp3\(^+\) cells per high-power field (± SEM from two independent experiments; n = 8 per group). *, P < 0.05; **, P < 0.01 versus insulin B:9-23.
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NOD Foxp3 GFP reporter mice in the presence or absence of Foxp3+ T reg cells. As shown in Fig. 9, Foxp3+ T reg cells from mimetope 3–vaccinated mice completely suppressed proliferation of effector cells, reflecting an enrichment of insulin-specific cells within the Foxp3+ T reg cell population, whereas T reg cells from nonvaccinated animals or mice treated with the natural epitope were less suppressive. Importantly, the proliferation of effector cells in the absence of Foxp3+ T reg cells was similar in all mice, indicating the absence of recessive tolerance. When the cytokine response of effector cells was analyzed, it became clear that there were no differences in IL-10 production, whereas vaccination with mimetope 3 resulted in reduced secretion of IFN-γ and IL-17 (Fig. 10).

Collectively, these results indicate that in contrast to the natural insulin epitope, vaccination with mimetope 3 results in the highest number of stable Foxp3+ cells that suppress the proliferation of IFN-γ– and IL-17 cytokine-secreting, insulin-specific effector cells.

DISCUSSION

Vaccination with insulin or its mimetopes has been tested previously, to prevent or treat T1D using mucosal exposure or in the presence of adjuvant such as cholera toxin B subunit or IFA (Zhang et al., 1991; Daniel and Wegmann, 1996; Harrison et al., 1996; Bergerot et al., 1997; Chen et al., 2001;
Alleva et al., 2002; Aspord and Thivolet, 2002; Martinez et al., 2003). The goal of these attempts was not to induce Foxp3+ T reg cells, and they afforded only incomplete protection, perhaps because of the fact that such maneuvers shifted the Th1 to Th2 ratio (Alleva et al., 2002). This would explain why with our tolerogenic vaccination protocol we see a drop in IAA indices, whereas in previous vaccination protocols an increase was noted (Orban et al., 2010). However, none of the previously established protocols reached the level of protection noted in the approach presented here and ensuing clinical trials using natural insulin epitopes were largely unsuccessful.

The findings demonstrated here support the hypothesis that weakly agonistic self-ligands recognized by autoreactive T cells fail to efficiently induce dominant tolerance. We therefore propose a novel concept of extrathymic Foxp3+ T reg cell conversion in autoimmune disease settings by applying minute amounts of strongly agonistic mimotopes of critical autoantigens, such as insulin in T1D. In contrast, natural insulin epitopes with poor agonistic activity that are recognized by diabetogenic T cells, irrespective of the applied dose, are ineffective in inducing stable Foxp3+ T reg cells. The ability to convert naive T cells through appropriate antigenic stimulation under subimmunogenic conditions into Foxp3+ T reg cells offers a convenient strategy to induce antigen-specific dominant tolerance for several reasons. Foxp3+ T reg cell vaccination requires only a single epitope because the induced T reg cells suppress immune responses of T cells to antigen from the same source through bystander suppression (Verginis et al., 2008), whereby the vicinity to antigen presenting cells is relevant. An additional advantage is that this mode of tolerance can be easily induced in the mature immune system by sub-immunogenic delivery of antigen (Verginis et al., 2008; Daniel et al., 2010), i.e., in the absence of general immunosuppression that can have fatal consequences. The induced Foxp3+ T reg cells are stable (Polansky et al., 2008) and maintain their function even in an immunogenic context (Rubtsov et al., 2010) because reencounter of antigen causes neither loss of T reg cells nor loss of their activity (Klein et al., 2003).

Because the method is preventive, one should aim at a cohort of individuals with elevated risk of developing T1D. IAAs precede the development of T1D (Ziegler et al., 1989; Yu et al., 2000), i.e., most diabetes patients have IAA before T1D onset, therefore individuals with high-risk MHC alleles, such as HLA-DQ8 and DQ2, and moderate levels of IAA represent a well-defined population to vaccinate by this method.

The use of strongly agonistic ligands applied under subimmunogenic conditions results in complete protection from autoimmune disease development; this protection from autoimmune diabetes development has not previously been achieved with natural epitopes, irrespective of dose and mode of application, which is consistent with the finding that insulin-specific T cells in NOD mice exhibit specificity for poorly agonistic ligands (Stadinski et al., 2010). It is also consistent with the belief that high-affinity TCR ligands are the best inducers of T reg cells, although in these short-term experiments using models unrelated to autoimmunity only TCR.

Figure 10. Characterization of effector T cells isolated from pancreatic lymph nodes of NOD Foxp3-GFP reporter mice at 20 wk. The mice were nonvaccinated, vaccinated with insulin B:9-23, or insulin mimotope 3 at 4–6 wk. Cytokine expression in T effector cells after depletion of Foxp3+ T cells in response to the insulin mimotope 3 was analyzed by intracellular staining (a) or by ELISA (b). *, P < 0.05 versus control. Representative dot plots are shown. Data are representative of two independent experiments. *n = 4 per group. Expression of the lineage-specific transcription factors as assessed by intracellular staining is shown in c. Cells are gated on CD4+ T cells. Black solid line, nonvaccinated control; red solid line, insulin B:9-23 vaccinated; blue dotted line, insulin mimotope 3 vaccinated. Results are the means ± SEM from two independent experiments. n = 4 per group.
contact residues were modified (Gottschalk et al., 2010). The validity of the approach to create higher affinity TCR ligands appears to be limited to TCR transgenic systems (Gottschalk et al., 2010), as it is unlikely that a peptide that forms a higher affinity ligand for a given TCR will also form a higher affinity ligand for polyclonal T cell populations reacting with the same peptide.

Subimmunogenic doses of strongly agonistic mimetopes appear mandatory to achieve complete protection by immunosuppression. In contrast, high immunogenic doses of ligands lead to the activation of the PI3K–Akt–mTOR pathway (Merkenschlager and von Boehmer, 2010), which could interfere with extrathympic Foxp3 induction (Sauer et al., 2008). Recent studies show that Foxo proteins, which are inactivated by Akt, drive Foxp3 expression. These studies could explain the negative regulation of Foxp3 by the PI3K–Akt network (Merkenschlager and von Boehmer, 2010) after strong T cell activation. The best Foxp3+ T reg cell conversion is seen in T cells that undergo only limited proliferation, whereas higher doses of agonists result in more extensive proliferation and diminished Foxp3+ T reg cell conversion (Kretschmer et al., 2005). In support of these conclusions, robust T cell activation has been shown to negatively impact on the extrathympic T reg cell generation based on a cell-cycle dependent maintenance of a silenced state of the Foxp3 locus (Josefowicz et al., 2009). This mode of immunosuppression differs from methods that induced increased levels of IL-10 production by CD25+ cells (Sundstedt et al., 2003).

The NOD mouse appears to represent a faithful model of human disease because it is controlled by the same genetic factors, such as MHC class II genes (Suri et al., 2005, 2008) and levels of intrathympic insulin expression (Fugliese et al., 1997; Jaeckel et al., 2004). Epitopes recognized by human CD4 T cells include the insulin B:9-23 (Alleva et al., 2001) peptide, which is identical to that of mouse insulin. Such cells were reported in cohorts of people at risk of developing T1D and with recent onset of T1D, but not in control subjects (Alleva et al., 2001). Human class II HLA-DQ8 and DQ2 alleles bear striking resemblance to the mouse class II I-Aβ0 allele that confers susceptibility (Lee et al., 2001) with a positively charged p9 MHC-binding pocket that distinguishes the DQ8 and DQ2 alleles from other class II alleles that do not confer susceptibility. Thus, it seems reasonable to explore the possibility that the scenario as revealed in the NOD mouse also applies in human disease.

MATERIALS AND METHODS

Mice. Insulin (B:9-23) TCR transgenic RAG−/− NOD mice were previously described (Jasinski et al., 2006) and were bred in the facility of the Dana Farber Cancer Institute. 4-wk-old female NOD mice were purchased from Taconic. Thy1.1 congenic NOD mice and NOD Foxp3 GFP reporter mice were obtained from The Jackson Laboratory. Antigen-specific in vivo T reg cell conversion protocols, with or without everolimus treatment, were executed as previously described (Daniel et al., 2011). Before T reg cell conversion, mice were tested for IAA. Mice with high titers (indices ≥1) were excluded from analyses, as in these mice most insulin-specific T cells may already have been activated and hence resistant to T reg cell conversion. Diabetes development was monitored according to previously described protocols (Jaeckel et al., 2003, 2004). All animal care and procedures were executed according to National Institutes of Health guidelines and in accordance with the guidelines of the Animal Care and Use Committee of the Dana Farber Cancer Institute.

Histopathology of mouse NOD pancreata. Pancreata were fixed in Bouin’s solution and embedded in paraffin, and serial sections were stained with hematoxylin and eosin for general morphology. Histology and scoring of insulitis in pancreatic sections were executed as previously described (Jaeckel et al., 2004; Krishnamurthy et al., 2006). Pancreatic islets were assigned scores as follows: 0, intact islets/no lesions; 1, periislet infiltrates; 2, <25% islet destruction; 3, >25% islet destruction; 4, complete islet destruction.

Peptides. All peptides with amino acid sequences described in Fig. 1a were synthesized at New England Peptide.

IAA assay. Mouse high specificity/sensitivity competitive IAA assays in sera from NOD mice were performed in an ELISA format, as described previously (Babaya et al., 2009). In brief, high binding 96-well plates (Costar) were coated with human (recombinant) insulin (100 U/ml; Humulin; Lilly) overnight at 4°C. Unspecific-blocking was done using PBS containing 2% BSA for 2 h at room temperature. Preincubated serum (diluted 1:10) with or without insulin competition was added and incubated for 2 h at room temperature. After 4 wash steps, biotinylated anti-mouse IgG1 (Abcam), diluted 1:10,000 in PBS/BSA was added for 30 min at room temperature. After washing the plate, horseradish peroxidase–labeled streptavidin was added for 15 min. The plate was washed 5 times, and TMB substrate solution was added (OptEIA reagent set; BD). Each sample was run in duplicate with and without competition using human insulin. For each sample, an index was calculated based on the mean of the results.

Isolation of pancreatic islets and infiltrating T cells. Cells were purified from mouse pancreas after in situ pancreas perfusion with collagenase, pancreas digestion, and islet purification using previously described protocols (Trembleau et al., 1995; Szt et al., 2007; Li et al., 2009). Purified islets were washed and subsequently selected by hand or separated by Ficoll gradient.

In vitro T cell proliferation assay. Assays were performed in round-bottom 96-well tissue culture plates. CD11c+ DCs from NOD mice were used as antigen-presenting cells and were purified using anti-CD11c+ magnetic beads (Miltenyi Biotec; Daniel et al., 2011). Thymidine incorporation of stimulated CD4+ TCRβ−–enriched insulin B:9-23 TCR transgenic T cells or of T cells purified from pancreatic lymph nodes of nonvaccinated or vaccinated NOD Foxp3 GFP reporter mice in response to the insulin mimotope was analyzed by scintillation counting after the addition of [3H]thymidine for the last 12 h of a 72-h culture period.

Analyses of dominant tolerance. CD4+ insulin B:9-23 TCR transgenic T cells were labeled with CFSE at 2 µM and incubated for 4 min at 37°C in PBS plus 0.1% BSA. Labeled cells were adoptively transferred i.p. into T reg cell–vaccinated 8- or 30-wk-old NOD female mice, followed by i.p. co-injection with insulin B:9-23 peptide (15 µg/mouse). Analyses of CFSE dilution was done 4 d later in pancreatic lymph nodes using flow cytometry. Concentrations of IL-10, IFN-γ, and IL-17 were analyzed by mouse ELISA sets (OptEIA; BD or R&D Systems).

Immunohistochemistry. Immunohistochemistry was performed essentially as previously described (Weigmann et al., 2008), with the following changes: 10 µm cryosections from pancreatic specimens of NOD mice were stained for Foxp3 with rat antibody (FJK-16s; eBioscience), rabbit anti-insulin antibody (SantaCruz), and mouse anti-CD4 antibody (mAbs1312; Abcam). Tissues were fixed in ice-cold acetone, followed by methanol blocking, biotin/avidin blocking (Vector Laboratories), and protein-blocking reagent (Roche) for elimination of unspecific background staining. Sections were permeabilized with Perm-Medium B (ADG), followed by incubation with primary antibodies. Sections incubated with isotype-matched control antibodies served as negative controls.
control. Next, samples were incubated with biotinylated secondary IgG antibody (Dianova), incubated with streptavidin-HRP, and stained with tyramide-Cy3 or tyramide-FITC (Invitrogen) according to the manufacturer's instructions.

**Antibodies and flow cytometry.** mAbs specific for Thy1.1 (OX-7), Thy1.2 (53–21), CD4 (RM4-5), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD127 (ATR34), Foxp3 (FJK-16s), ROR-γ (AKFJKS-9), T-bet (eBio4B10), GATA3 (TWAJ), IL-17 (TC11-18H10) and IFN-γ (XMGl2.1) were purchased from BD, e Bioscience, or BioLegend and were used as biotin, FITC, PE, PE-Cy7, PerCP-Cy5.5, allophycocyanin, allophycocyanin-Cy7, Alexa Fluor 488 or Pacific blue conjugates. Enumeration of cells and acquisition are performed by using FACSARIA and FACS DIVA software (BD). Single-cell data analyses are done by the use of the FlowJo software (Tree Star, Inc.).

**Statistical analysis.** Data were analyzed by one-way ANOVA with Tukey's post hoc test or with the Holm-Sidak method for comparison of multiple columns. Wilcoxon signed rank test was used to analyze the impact of Treg cell vaccination on IAA levels before and after treatment. Cumulative incidence of diabetes was determined by Kaplan-Meier estimates, and statistical analysis of difference was determined by log-rank test. A value of P < 0.05 was considered significant. Analyses were done using SigmaStat 3.5 (Systat Software).

**Online supplemental material.** Fig. S1 shows the analysis of dominant tolerance to polyclonal effector T cells in vaccinated NOD mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110574/DC1.

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