Combination Therapy Using IL-2/IL-2 Monoclonal Antibody Complexes, Rapamycin, and Islet Autoantigen Peptides Increases Regulatory T Cell Frequency and Protects against Spontaneous and Induced Type 1 Diabetes in Nonobese Diabetic Mice

Jean N. Manirarora and Cheng-Hong Wei

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Combination Therapy Using IL-2/IL-2 Monoclonal Antibody Complexes, Rapamycin, and Islet Autoantigen Peptides Increases Regulatory T Cell Frequency and Protects against Spontaneous and Induced Type 1 Diabetes in Nonobese Diabetic Mice

Jean N. Manirarora and Cheng-Hong Wei

Regulatory T cells (Treg) play a crucial role in the maintenance of self-tolerance. In this study, we sought to expand Ag-specific Tregs in vivo and investigate whether the expanded Tregs can prevent or delay the development of type 1 diabetes (T1D) in the NOD mouse model. NOD mice were treated with a combination of IL-2/anti–IL-2 Ab complex, islet Ag peptide, and rapamycin. After the combined treatment, CD4+CD25+Foxp3+ Tregs were significantly expanded in vivo, they expressed classical Treg markers, exerted enhanced suppressive functions in vitro, and protected against spontaneous development of T1D in NOD mice. Moreover, treated mice were almost completely protected from the adoptively transferred, aggressive form of T1D caused by in vitro–activated cytotoxic islet Ag-specific CD8 T cells. Protection from T1D was transferrable by Tregs and could be attributed to reduced islet infiltration of immune cells as well as the skewing of the immune response toward a Th2 cytokine profile. This new method of peripheral immune regulation could potentially contribute to development of novel immunotherapeutic strategies to prevent the development of T1D or to promote tolerance to islet transplants without using immunosuppressive drugs for long terms. The Journal of Immunology, 2015, 195: 5203–5214.

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The online version of this article contains supplemental material.

Abbreviations used in this article: GAD65, 65-kDa isoform of glutamic acid decarboxylase (GAD65), and islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP) play pivotal pathogenic roles during the development of T1D in NOD mice (2, 3) and in humans (4–8).

There is currently no cure for T1D, and the disease is treated with life-long exogenous insulin replacement. Allogeneic islet transplantation has been performed experimentally in animal models and humans with promising results, but this investigational measure is not yet practical in regular clinical practice. Moreover, allogeneic islet transplants can provoke immune response, and long-term immunosuppressive drugs are needed to protect the transplanted islets (9, 10). Even when the transplanted islets are from monozygotic twins, they are still susceptible to the recurrent autoimmunity-mediated destruction (11). β cell regeneration therapies via genetic reprogramming have also been intensively pursued as a therapy; however, a recent study shows that the genetically reprogrammed, liver-derived insulin-producing cells are also susceptible to autoimmune destruction in settings of murine models of T1D (12). Therefore, it is critical to understand the recurrent autoimmune mechanisms leading to β cell destruction and to find immunotherapeutic strategies that specifically target autoimmune anti-islet responses to achieve tolerance to islet Ags without long-term use of immunosuppressive drugs.

One effective mechanism of peripheral tolerance involves the control of autoreactive T cells by naturally occurring CD4+ CD25+Foxp3+ regulatory T cells (nTregs). Tregs can potentely suppress the activation and effector function of other immune cells, including CD4 and CD8 T cells, B cells, NK cells, macrophages, and dendritic cells (13). Previous studies in mice have shown that nTregs play a major role in the control of a variety of autoimmune diseases (14, 15). Recently, Tregs have also been investigated in clinical trials to prevent/treat T1D, graft-versus-host diseases, and organ transplant rejection (16). Notably, it is well established in several models that Ag-specific Tregs are more effective than polyclonal Tregs in immunosuppression in vitro and control of autoimmunity in vivo (e.g., T1D, arthritis, multiple sclerosis, arthritis, colitis) (17–24); particularly, injection of small numbers of Ag-specific Tregs selectively expanded in vitro can reverse diabetes even after disease onset (17, 18). However, whether Ag-specific Tregs can be expanded in vivo, whether these cells retain their immunosuppressive function following expansion, and the stability of their Foxp3 expression have not yet been elucidated.

IL-2 is an important factor for the survival, expansion, and function of Tregs in vivo (25–28). The administration of IL-2/anti–IL-2 mAb (clone JES6-1) complexes in vivo has been shown to
increase polyclonal Treg numbers in lymphoid organs (29). It has also been reported that the malignant target of rapamycin inhibitor rapamycin can selectively inhibit proliferation of CD4⁺ effector T cells, enhance the expansion of Tregs (30, 31), and maintain the stability of expanded Tregs (32, 33). Therefore, we hypothesized that a combined treatment comprising an islet Ag (BDC2.5 cognate minemotope), IL-2/IL-2 mAb complexes, and rapamycin could act in synergy to expand Ag-specific Tregs in lymphoid organs.

In this study, we explored this new method to expand Ag-specific Tregs in NOD mice. The phenotype and function of expanded Tregs, as well as their capacity to protect against spontaneous and induced diabetes development in NOD mice, were investigated.

Materials and Methods

Mice

Female NOD/ShiLtJ, NOD SCID, CD8 TCR transgenic NOD 8.3, CD4 TCR transgenic NOD B2C2.5, and NOD.Thy1.1⁺/+ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The B2C2.5 TCR transgenic CD4⁺ T cells specifically recognize the I-Aβ⁻ restricted epitope derived from an islet Ag (34), whereas the 8.3 TCR transgenic CD8⁺ T cells specifically recognize the K⁻restricted IGRP206⁻214 epitope derived from the islet Ag IGRP (35). NOD Thy1.1⁺/+ BDC2.5 and NOD Thy1.1⁺/+ 8.3 mice were bred in a specific pathogen-free facility at the Food and Drug Administration Center for Biologics Evaluation and Research (Bethesda, MD). All of the mouse colonies were maintained according to Institutional Animal Care and Use Committee guidelines, and all animal protocols and procedures were approved by the Institutional Animal Care and Use Committee.

Expansion of CD4⁺CD25⁺ regulatory cells in vivo

IL-2/anti-IL-2 mAb (JES5-1) complexes were administered as previously reported (36). Briefly, 100 μg anti-IL-2 mAb (eBioscience, San Diego, CA; clone JES6-1, catalog no. 16-7022-85) was mixed in vitro with recombinant murine IL-2 (20 μg) (PeproTech, Rocky Hill, NJ) in 200 μl HBSS (Mediatech, Herndon, VA) supplemented with 1% heat-inactivated FBS (HyClone, Logan, UT), and incubated at 4°C for 30 min. After incubation, the volume of the mixture was brought to 2 ml with HBSS/1% FBS. Six-week-old female NOD mice were treated with one i.v. injection of 100 μg BDC2.5 TCR mimotope peptide (37) (amino acid sequence RVRLPLWVRME) for T cell expansion treatment. For simplicity, this treatment with BDC2.5 mimotope peptide, IL-2/anti-IL-2 mAb complexes, and rapamycin is referred to as standard combined treatment procedure. Each injection was a 100-μl volume. Mice were euthanized 3 d after the last injections (day 5) to assess the phenotype and functions of the expanded regulatory T cells. In some experiments, other islet autoantigen peptides were used, such as I-Aβ⁻ GAD65 5204–5345 (amino acid sequence SRLSKVAVKRMEMYGTT) (38) and I-Aβ⁻ insulin B chain 9–23 (amino acid sequence SLYEALTYLCGERC) (39). Additionally, a control non-islet autoantigen peptide I-Aβ⁻ myelin basic protein 74–85 (also called I-B3 cognate peptide) (amino acid sequence SRLPGCHMYKDS) (40) was also used.

Isolation of CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ responder T cells

Lymphocytes were obtained from lymph nodes and spleens and processed in HBSS supplemented with 2% FBS. CD4⁺ T cells were first negatively selected and purified using the mouse CD8⁺ T cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. CD4⁺CD25⁻ regulatory cell purity was consistently >90% as assessed by staining with anti-CD4, anti-CD25 Abs followed by flow cytometry analysis.

Isolation of CD8⁺ 8.3 T cells

Lymphocytes were obtained from lymph nodes and spleens of NOD 8.3 mice, or NOD.Thy1.1⁺/+ 8.3 mice, and processed in HBSS supplemented with 2% FBS, CD8⁺ T cells were negatively selected and purified using the mouse CD8⁺ T cell isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. The purity of CD8⁺ 8.3 T cells was usually >90%, as determined by flow cytometry.

T cell activation and CFSE assay

For T cell proliferation assay, CD8 T cells were cultured and labeled with CFSE (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. The CFSE-labeled Thy1.1⁺CD8⁺ 8.3 T cells (1 x 10⁶/well in 96-well round-bottom plates) were cultured for 3 d at 37°C in 5% CO₂ in the presence of anti-CD3/CD28 Dynabeads (Life Technologies, Grand Island, NY) (bead/T cell ratio 2:1) and murine IL-2 (PeproTech) at 2 x 10⁵ U/ml with or without treated CD4⁺CD25⁺ Tregs or untreated CD4⁺CD25⁻ Tregs at a series of titrated ratios (from 1:1 to 1:3 of Treg/8.3 T cell ratio). After 3 d, cells were harvested and labeled with fluorochrome-conjugated anti-CD4, anti-CD8, anti-CD90.1 (Thy1.1), and anti-CD90.2 (Thy1.2) Abs (BD Biosciences, San Diego, CA) for 20 min at 4°C and then washed twice. Cells were analyzed by flow cytometry using a FACSVerse I (Becton Dickinson, San Jose, CA).

For T cell activation before adoptive transfer, CD8⁺ 8.3 T cells (2 x 10⁶/well in 24-well plates) were cultured for 3 d at 37°C in 5% CO₂ in the presence of anti-CD3/CD28 Dynabeads (Life Technologies) at a 1:2 cell/bead ratio and murine IL-2 (PeproTech) at 2 x 10⁵ U/ml. After 3 d, activated T cells were harvested, separated from the beads with paramagnetic Dynabeads (i.v.) into 6-wk-old recipient NOD mice at 2 x 10⁶ T cells per mouse.

Flow cytometry analysis

For T cell subset analysis, cells were labeled with fluorochrome-conjugated anti-CD4, anti-CD8, anti-CD69, anti-CD62L, anti-CD44, and anti-glucocorticoid-induced TNFR (GITR) Abs (BD Biosciences) for 20 min at 4°C and then washed twice.

For non-T cell subset analysis, the cells were labeled with fluorochrome-conjugated anti-TCRαβ, anti-CD3, anti-CD11b, anti-CD11c, and anti-CD19 Abs (BD Biosciences) for 20 min at 4°C and then washed twice. For intracellular Foxp3 and CTLA-4 labeling, cells were labeled with fluorochrome-conjugated anti-CD4, anti-CD8, and anti-CD25 Abs (BD Biosciences), fixed and permeabilized, and then labeled with either fluorochrome-conjugated anti-Foxp3 (eBioscience) or fluorochrome-conjugated anti-CTLA-4 (eBioscience) Abs, according to the manufacturers’ instructions.

For intracellular cytokine staining, splenocytes were stimulated for 6 h with a ready-to-use polyclonal cell activation mixture containing the PMA, a calcium ionophore (ionomycin), and a protein transport inhibitor (brefeldin A) (BD Pharmingen, San Diego, CA). Cells were labeled with fluorochrome-conjugated anti-CD4, anti-CD8, and anti-CD44 Abs, fixed and permeabilized, and then labeled with fluorochrome-conjugated anti-IFN-γ (eBioscience) Abs according to the manufacturers’ instructions. Cells were analyzed using a FACSVerse II (Becton Dickinson) flow cytometer.

BDC/I-Aβ⁻ tetramer and tetramer staining

The PE-labeled BDCI- Aβ⁻ tetramer (I-Aβ⁻/RTPLWVRME) was synthesized by the National Institutes of Health Tetramer Core Facility at Emory University (Atlanta, GA). Cells from the spleens or pancreatic lymph nodes were stained with the BDC tetramer and then with surface markers (CD4, CD25), followed by intracellular staining for Foxp3.

Assessment of diabetes incidence

The NOD mice (females, 4–6 wk old) were randomly assigned into different groups using GraphPad software (http://www.graphpad.com/quickcalcs/randomize1.cfm). All mice were tested weekly for urine glucose starting at 10 wk of age using Keta-Diastix reagent strips for urinalysis (Bayer, Elkhart, IN). When the urine glucose measurement reached 300 mg/dl the mice were tested for blood glucose until 32 wk of age using Keta-Diastix reagent strips for urinalysis (Bayer, Elkhart, IN). When the urine glucose measurement reached 300 mg/dl the mice were tested for blood glucose until 32 wk of age using Keta-Diastix reagent strips for urinalysis (Bayer, Elkhart, IN).

Prevention of spontaneous diabetes

Six-week-old female NOD mice were left untreated or treated with standard combined treatment. Urine/blood glucose was assessed weekly starting at 10 wk.

Alternatively, 6-wk-old female NOD mice (or NOD B2C2.5 mice where indicated) were left untreated or treated with standard combined treatment. Three days after the last injections, the mice were sacrificed and CD4⁺CD25⁻ Tregs or CD4⁺CD25⁺ effector cells were purified from lymph nodes and spleens. Then, 1 x 10⁷ Tregs or effectr T cells were adoptively transferred into the recipient NOD mice.
transferred (i.v.) into 6-wk-old female NOD mice. Urine/blood glucose was assessed weekly starting at 10 wk. Mice with two consecutive blood glucose readings of >300 mg/dl were defined as diabetic.

Prevention of diabetes induced by adoptive transfer of activated CD8+ 8.3 T cells

Six-week-old NOD mice were left untreated or treated with standard combined treatment. At day 5, the mice were injected i.v. through the tail vein with $2 \times 10^6$ CD8+ 8.3 T cells activated for 3 d in vitro at 37°C in 5% CO2 in the presence of anti-CD3/CD28 Dynabeads (Life Technologies) (bead/T cell ratio 2:1) and murine IL-2 (PeproTech) at $2 \times 10^3$ U/ml. Urine/blood glucose was assessed daily.

H&E staining and histological assessment

Harvested pancreatic tissues were fixed in 10% formalin/PBS and embedded in paraffin, and 5-μm sections were prepared. Sections were stained with H&E and examined by microscopy.

Statistical analysis

Data are presented as means ± SD and were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Statistical analysis was performed using a Student t test for comparison between two groups and ANOVA for more than two groups. A p value <0.05 was considered significant. T1D disease incidence data were plotted using.
FIGURE 2. Transferred effector cells do not covert to Tregs, and the treatment enriches Ag-specific Tregs. (A) Thy1.1⁺CD4⁺CD25⁻ T cells (Tconvs) and Thy1.1⁺CD4⁺CD25⁺ T cells (Tregs) were purified from Thy1.1⁺ BDC2.5 NOD mice, CFSE labeled, and transferred to recipient NOD mice (n = 3/group); the recipient mice were treated with IL-2 complexes, rapamycin, and BDC2.5 cognate peptide for 3 d as described in Materials and Methods. On day 5, mice were sacrificed and the cells were isolated from spleen, stained with Abs against Thy1.1, CD4, and Foxp3, and analyzed by FACS. Cells are gated on CD4 and Thy1.1 double-positive cells to view only the transferred T cells. Shown are representative data of two independent experiments with similar results. (B) Female NOD mice were either left untreated or treated with standard combined treatment (n = 6/group). On day 5, mice were sacrificed and the cells were isolated from spleens, stained with Abs against CD4, CD25, Foxp3, and BDC-I-A<sup>g7</sup> tetramer, and analyzed by FACS. Shown are data compiled from two independent experiments. (C) Female NOD mice were either left untreated or treated with the indicated treatment (n = 3/group). On day 5, mice were sacrificed and the cells were isolated from pancreatic lymph nodes, stained with Abs against CD4, CD25, Foxp3, and BDC-I-A<sup>g7</sup> tetramer, and analyzed by FACS. **p < 0.01, ****p < 0.0001.
the Kaplan–Meier plot, and the statistical analysis was performed using the log rank analysis.

**Results**

*Tregs are expanded in recipients after the combined treatment*

To test the hypothesis that a combination of islet Ag peptide, IL-2/anti–IL-2 mAb complexes, and rapamycin can selectively expand Ag-specific Tregs in vivo, we first evaluated the effect of different treatments on Treg expansion in various lymphoid organs. With one i.v. injection of BDC2.5 peptide at day 0 and three daily i.p. injections (days 0, 1, and 2) of IL-2/anti–IL-2 mAb complexes and rapamycin we observed substantial expansion of CD4+Foxp3+ T cells on day 5 after start of treatment. Both endogenous CD4+Foxp3+ Tregs and transferred Thy1.1+ BDC2.5 TCR transgenic CD4+Foxp3+ T cells expanded equally in the spleen (Fig. 1A, 1C, 1E), pancreatic lymph nodes (Fig. 1B, 1D, 1F), and mesenteric lymph nodes (data not shown). The expansion of CD4+Foxp3+ T cells was less marked in the spleen (Fig. 1A, 1C, 1E) compared with the pancreatic lymph node (Fig. 1B, 1D, 1F). Additionally, only the combination of BDC2.5 peptide, IL-2/IL-2 mAb complexes, and rapamycin yielded a significant difference in Treg expansion in the pancreatic lymph node compared with untreated control (Fig 1B, 1D, 1F). In the pancreas, the combined treatment also significantly expanded Tregs compared with untreated control (Supplemental Fig. 1). Taken together, these data indicate the possibility of Ag-specific stimulation and expansion of Tregs by the combined treatment. We therefore decided to use this combination of IL-2/IL-2 mAb complexes, rapamycin, and islet peptide Ag as standard combined treatment in subsequent experiments.

To understand whether the observed increase of Tregs is due to effector T cell conversion into Tregs or simply due to the expansion of nTregs, or both, CFSE-labeled Thy1.1+CD4+CD25+ T cells (Tregs) and CFSE-labeled Thy1.1+CD4+CD25- T cells (effector T cells) were adoptively transferred into two separate groups of

**FIGURE 3.** Phenotype of the expanded Tregs in treated mice. Spleen cells were isolated from NOD mice untreated or treated with BDC2.5 peptide, IL-2/IL-2 mAb, and rapamycin for 3 d (n = 2/group). Cells were labeled with anti-CD4, anti-CD25, anti-GITR, and anti-CD62L Abs then with anti-Foxp3 or anti–CTLA-4 Abs and analyzed by FACS (A-I). Results shown are representative of one of three independent experiments with similar results. *p < 0.05, **p < 0.01, ***p < 0.001.
NOD mice, followed by the standard combined treatment. As shown in Fig. 1G and 1H, the Tregs expanded more robustly in the Treg transfer group than the group that received T effector cells, suggesting that the combined treatment preferentially leads to substantial expansion of Tregs rather than the conversion of CD4+CD25− conventional T cells into Tregs.

Furthermore, as shown in Fig. 2A, when CFSE-labeled Thy1.1+ CD4+CD25+ BDC2.5 T cells (Tregs) were adoptively transferred into NOD mice and treated with the combined treatment, all of the transferred CD4+CD25+ Tregs divided and remained Foxp3+. In contrast, when CFSE-labeled Thy1.1+CD4+CD25− BDC2.5 T cells (conventional T cells [Tconvs]) were adoptively transferred into NOD mice and treated with the combined treatment, very few (~5%) of the transferred CD4+CD25− T cells became Foxp3+, strongly suggesting that effector T cells do not convert into Foxp3+ cells under the condition of combined treatment in this model system. Therefore, we think that the increase of Tregs led to an increased percentage of CD44+CD62Lhi central memory cells in the expanded Tregs compared to the nTreg group. Treg transfer group than the group that received T effector cells, suggesting that the combined treatment preferentially leads to substantial expansion of Tregs rather than the conversion of CD4+CD25− conventional T cells into Tregs.

To assess whether the combined treatment indeed enhances Ag-specific Tregs or not, after treatment, Tregs were stained with H-2k7 DX5+ and analyzed by FACS. As shown in Fig. 3, the expansion of Tregs was not Ag specific. Furthermore, as shown in Fig. 2A, when CFSE-labeled Thy1.1+ CD4+CD25+ BDC2.5 T cells (Tregs) were adoptively transferred into NOD mice and treated with the combined treatment, all of the transferred CD4+CD25+ Tregs divided and remained Foxp3+. In contrast, when CFSE-labeled Thy1.1+CD4+CD25− BDC2.5 T cells (conventional T cells [Tconvs]) were adoptively transferred into NOD mice and treated with the combined treatment, very few (~5%) of the transferred CD4+CD25− T cells became Foxp3+, strongly suggesting that effector T cells do not convert into Foxp3+ cells under the condition of combined treatment in this model system. Therefore, we think that the increase of Tregs led to an increased percentage of CD44+CD62Lhi central memory cells in the expanded Tregs compared to the nTreg group. Treg transfer group than the group that received T effector cells, suggesting that the combined treatment preferentially leads to substantial expansion of Tregs rather than the conversion of CD4+CD25− conventional T cells into Tregs.

The expanded Tregs express classical Treg markers

Next, we investigated the phenotype of Tregs expanded by the combined treatment 2 d after the last injections (day 5) using flow cytometry analysis. As shown in Fig. 3, the expanded Tregs were found to express molecules that are crucial to Treg function such as Foxp3 (Fig. 3A–F), CTLA-4 (Fig. 3G), GITR (Fig. 3H), and CD2L (Fig 3I). After treatment, the expression level of Foxp3 is increased in Tregs (Fig. 3B, 3E). Taken together, these data indicate that the expanded Tregs expressed classical markers associated with nTreg phenotype and function.

Effect of treatment on other immune cell subsets

It has been reported that injection of low doses of IL-2 in complex with the anti–IL-2 neutralizing mAb JES6-1 leads to selective expansion of Tregs and minimal or no change in other cell subsets that short-term treatment with IL-2/anti-IL-2 mAb (JES6-1) complex increases Tregs up to 6-fold (29). The increase in Tregs is specific to this IL-2/anti-IL-2 mAb complex, as complexing IL-2 with other anti-IL-2 neutralizing Ab clones, for example, S4B6, drives expansion of CD8+ T cells and NK cells (36). We therefore tested whether our combined treatment has similar effects on different immune cell subsets. As shown in Table I, the combined treatment did not affect the activation status of either CD4+ or CD8+ T cells. With regard to T cell memory pool, the combined treatment increased the production of both CD4+ and CD8+ T cells with memory phenotype (CD44+). Also, the combined treatment led to an increased percentage of CD4+CD25+ central memory CD4+ T cells but had no effect on CD8+ T cells with central memory phenotype (Table I), suggesting a differential responsiveness to cytokine stimulation between CD4+ and CD8+ T cells.

In agreement with previous reports (29), the combined treatment had minimal or no effect on the percentage of non–T cell immune subsets, namely NK cells (TCRαβ DX5+), B cells (TCRαβ CD19+), dendritic cells (TCRαβ CD11c+), and macrophages (TCRαβ CD11b+) (Table II).

The suppressive function of the expanded Tregs is enhanced both in vitro and in vivo

nTregs suppress the proliferation of CD4+CD25+ T cells in response to CD3 ligation (41). In the present study, we tested the ability of Tregs expanded by the combined treatment to inhibit the proliferation of the diabetogenic CD8+ T cells activated by CD3/CD28 ligation for 3 d. The suppressive function of the expanded Tregs in inhibiting 8.3 T cell proliferation (as measured by

### Table I. Effect of treatment on the activation of conventional CD4+ T cells and CD8+ T cells

<table>
<thead>
<tr>
<th>Activation/Memory Markers among CD4+ T Cells</th>
<th>Activation/Memory Markers among CD8+ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>% CD62Lhigh cells</td>
<td>58.98 ± 5.65</td>
</tr>
<tr>
<td>% CD69+ cells</td>
<td>5.21 ± 0.08</td>
</tr>
<tr>
<td>% CD44+ cells</td>
<td>15.12 ± 2.42</td>
</tr>
<tr>
<td>% CD44+CD62Lhigh cells</td>
<td>5.19 ± 0.67</td>
</tr>
</tbody>
</table>

Spleen cells were isolated from NOD mice untreated or treated with BDC2.5 peptide, IL-2/IL-2 mAb, and rapamycin (n = 3/group). Cells were labeled with anti-CD4, anti-CD8, anti-CD44, anti-CD62L, and anti-CD69 Abs and analyzed by FACS. The results are the representative of three different experiments with similar findings.

### Table II. Effect of treatment on some other immune cell subsets (NK cells, B cells, dendritic cells, and macrophages)

<table>
<thead>
<tr>
<th>Percentage in the Spleen</th>
<th>Absolute Numbers (×10^6) in the Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>NK cells (TCRαβ DX5+)</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>B cells (TCRαβ CD19+)</td>
<td>42.0 ± 6.0</td>
</tr>
<tr>
<td>Dendritic cells (TCRαβ CD11c+)</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Macrophages (TCRαβ CD11b+)</td>
<td>0.75 ± 0.05</td>
</tr>
</tbody>
</table>

Spleen cells were isolated from NOD mice untreated or treated with BDC2.5 peptide, IL-2/IL-2 mAb, and rapamycin (n = 3/group). Cells were labeled with anti-CD3, anti-DX5, anti-CD19, anti-CD11b, and anti-CD11c Abs and analyzed by FACS. The results are representative of three different experiments with similar findings.
Previous studies have shown that Tregs control the progression of diabetes and that diabetes onset in NOD mice may correlate with impaired Treg functions and/or numbers (reviewed in Ref. 42). Daily injections of IL-2/anti–IL-2 mAb complexes into prediabetic NOD mice for 5 d followed by twice a week maintenance treatment for a total duration of 10 wk prevented the onset of diabetes in these mice (43). We tested the effect of a very short-term (3 d) treatment of NOD mice with the combined treatment on the prevention of spontaneous diabetes development. As expected, 60% control mice progressively developed diabetes starting at 10 wk of age whereas ~80% treated mice remained disease-free for >30 wk of age (Fig. 5A). Next, we tested the effect of the treatment on the development of diabetes induced by adoptive transfer of activated islet Ag-specific CD8 T cells (in this study the islet Ag IGRP-specific 8.3 T cells). NOD mice were treated for 3 d or left untreated with the combined treatment. At day 5 all the mice from both groups were injected with 2 × 10⁶ in vitro–activated CD8⁺ 8.3 T cells and the mice were monitored daily for blood glucose. As shown in Fig. 5B, >80% of treated mice remained disease-free up to 42 d after treatment whereas all control mice became severely sick (blood glucose > 600 mg/dl) and were sacrificed for humane reasons by day 7 after treatment.

To investigate whether the observed protection in treated mice was due to the expanded Tregs and was therefore transferable, NOD mice were injected with Tregs isolated from untreated or treated NOD BDC2.5 mice, or with Tregs from treated or untreated NOD mice, or effector T cells from untreated NOD mice. By 30 wk of age, we observed that only 10% of both the control mice and the mice that received untreated effector T cells from NOD mice remained diabetes-free (Fig. 5C). Polyclonal Tregs from untreated NOD mice provided temporary marginal protection, but Tregs from treated NOD mice provided significantly greater protection from T1D for a longer period (p < 0.05); treated and untreated Tregs from NOD BDC2.5 mice (clonal islet Ag-specific Tregs) were most potent at preventing spontaneous diabetes development compared with polyclonal Tregs from NOD mice (Fig. 5C). These data corroborate previous findings that ex vivo–expanded islet Ag-specific Tregs provide better protection of NOD mice from T1D than polyclonal natural Tregs (17, 18). Importantly, our data extend the previous findings in that the islet Ag-specific Tregs are more potent at blocking homeostatic proliferation of naive CD4⁺ CD25⁺ T cells in lymphopenic NOD SCID mice (Fig. 4B, 4C) compared with Tregs from untreated mice. Taken together, these data demonstrated that the combined treatment significantly enhanced the immunosuppressive function of Tregs.

Combined treatment significantly inhibits the development of spontaneous and adoptively transferred diabetes in NOD mice

FIGURE 4. Suppressive function of the expanded Tregs in vitro and in vivo. (A) In vitro suppression of CD8⁺ 8.3 T cell proliferation by treated and untreated Tregs: CFSE-labeled Thy1.1⁺CD8⁺8.3⁺ cells (1 × 10⁶/well in 96-well round-bottom plates) were cultured for 3 d with or without treated CD4⁺CD25⁺ cells or with untreated CD4⁺CD25⁺ cells at a 1:1 ratio of Tregs/8.3 cells. The number of 8.3 T cells was fixed, and the number of CD4⁺CD25⁺ Tregs was titrated down to cover a ratio of Tregs/8.3 T cells from 1:1 to 1:32. After 3 d, cells were harvested then labeled with anti-CD4, anti-CD8, anti-Thy1.1, and anti-Thy1.2 Abs. The percentage divided cells (CFSElo cells) was analyzed by FACS among the gated CD8⁺Thy1.1⁺ cells. The differences between the untreated Treg and treated Treg groups are statistically significant (p < 0.05, paired t test). (B) Homeostatic expansion of conventional CD4⁺CD25⁻ T cells: 1 × 10⁶ Thy1.1⁺CD4⁺CD25⁻ cells (Tconvs) were purified from untreated Thy1.1⁺ NOD mice and the Tconvs were injected into female NOD SCID mice (n = 3 mice/group). At day 7, cells were isolated from spleens and labeled with anti-CD4, anti-CD8, and anti-Thy1.1 Abs and analyzed by FACS to quantitate the transferred Thy1.1⁺CD4⁺ Tconvs. (C) Inhibition of homeostatic expansion of conventional CD4⁺ T cells by treated Tregs: 1 × 10⁶ CD4⁺CD25⁻ Tregs were purified from untreated NOD mice or NOD mice treated with the combined treatment for 3 d, and 1 × 10⁶ Thy1.1⁺CD4⁺CD25⁻ cells (Tconvs) were purified from untreated Thy1.1⁺ NOD mice; the Tregs and Tconvs CFSE dilution) was higher compared with Tregs from untreated mice (Fig. 4A). Similarly, the expanded Tregs were significantly more potent at blocking homeostatic proliferation of naive CD4⁺ CD25⁺ T cells in lymphopenic NOD SCID mice (Fig. 4B, 4C) compared with Tregs from untreated mice. Taken together, these data demonstrated that the combined treatment significantly enhanced the immunosuppressive function of Tregs.

At 30 wk of age, <20% of the mice that received untreated Tregs from NOD mice remained disease-free compared with 70% of the mice that received untreated Tregs from NOD BDC2.5 mice provided the best protection; at 32 wk of age, NOD BDC2.5 mice provided temporary marginal protection, but Tregs from treated NOD mice provided significantly greater protection from T1D for a longer period (p < 0.05); treated and untreated Tregs from NOD BDC2.5 mice (clonal islet Ag-specific Tregs) were most potent at preventing spontaneous diabetes development compared with polyclonal Tregs from NOD mice (Fig. 5C). These data corroborate previous findings that ex vivo–expanded islet Ag-specific Tregs provide better protection of NOD mice from T1D than polyclonal natural Tregs (17, 18). Importantly, our data extend the previous findings in that the islet Ag-specific Tregs are expanded in vivo.

were coinjected at 1:1 ratio into female NOD SCID mice (n = 3 mice/group). At day 7, cells were isolated from spleens and labeled with anti-CD4, anti-CD8, and anti-Thy1.1 Abs and analyzed by FACS to quantitate the transferred Thy1.1⁺CD4⁺ Tconvs. Shown are representative results of two independent experiments with similar results. The groups were compared using ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.
Tconv cells were isolated from untreated NOD mice, and CD4+CD25+ Tregs were purified from untreated NOD, treated NOD, and untreated and treated BDC2.5 peptide, IL-2/IL-2 mAb, and rapamycin. At day 5 the mice were injected with $2 \times 10^6$ CD8+ T cells activated in vitro for 3 d with anti-CD3/CD28 beads. Urine/blood glucose was assessed daily. The data shown are pooled from two independent experiments. (C) CD4+CD25- Tconv cells (Tconvs) were isolated from untreated NOD mice, and CD4+CD25+ Tregs were purified from untreated NOD, treated NOD, and untreated and treated NOD BDC2.5 mice. One million Tregs or Tconvs were injected (i.v.) into female NOD mice at 6 wk of age. A group of untreated NOD mice ($n = 22$) was used as control (no cell transfer). Below are the total numbers of recipient NOD mice per group: $n = 22$ for treated BDC Treg group, $n = 20$ for treated NOD Treg group, $n = 35$ for untreated NOD Treg group, and $n = 15$ for untreated NOD Tconv group. Urine/blood glucose was assessed weekly starting at 10 wk. The data shown are pooled from three independent experiments. The percentage diabetes-free mice were plotted using a Kaplan–Meier curve and log rank statistical analysis was performed. (D) NOD mice were left untreated ($n = 10$) or treated ($n = 10$) with BDC2.5 peptide (or insulin B9–23 or GAD65524–543 peptide as indicated), IL-2/IL-2 mAb, and rapamycin for 3 d. At day 5 the mice were injected with $2 \times 10^6$ CD8+ T cells activated in vitro for 3 d with anti-CD3/CD28 beads. Urine/blood glucose was assessed daily. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

90% of the mice that received treated Tregs from NOD BDC2.5 mice remained disease-free (Fig. 5C). Taken together, these data indicate that the observed protection is due to Tregs, and that Ag-specific–treated Tregs (whether from treated NOD mice or treated NOD BDC2.5 mice) are more potent in vivo compared with untreated polyclonal Tregs.

Because in this study we used a mimotope peptide (BDC2.5) instead of a bona fide islet Ag peptide such as insulin B9–23 (39) or GAD65524–543 (38), we tested whether, in our setting, natural islet Ag peptides would also be efficient at preventing the development of induced T1D. NOD mice were left untreated or treated for 3 d with either one of the following treatments: 1) rapamycin, IL-2/anti–IL-2 mAb complexes; 2) rapamycin, IL-2/anti–IL-2 mAb complexes, BDC2.5 peptide; 3) rapamycin, IL-2/anti–IL-2 mAb complexes, insulin B9–23 peptide; and 4) rapamycin, IL-2/anti–IL-2 mAb complexes, GAD65524–543 peptide.

At day 5 all of the mice from all five groups ($n = 10$/group) were injected with $2 \times 10^6$ CD8+ T cells activated in vitro for 3 d and the mice were monitored daily for blood glucose. As shown in Fig. 5D, the mimotope peptide (BDC2.5) and natural peptides (insulin B9–23 and GAD65524–543) provided similar levels of protection ($70–80\%$ disease-free) that were significantly higher ($p < 0.01$) compared with the level of protection in untreated mice (10%). However, no significant difference was found between the mimotope peptide and natural islet Ag peptides or between these three peptides and the group comprising rapamycin and IL-2/anti–IL-2 mAb complexes alone. Taken together, these data indicate that in our setting the combined treatment comprising either a mimotope peptide (BDC2.5) or a natural islet Ag peptide (insulin or GAD65) equally protects against induced T1D development in NOD mice.

**Treatment with IL-2/IL-2 mAb complexes or rapamycin alone does not provide optimal protection from T1D in NOD mice**

We further evaluated whether the single component of the treatment can achieve similar protection. As shown in Fig. 6A, rapamycin alone did not protect NOD mice from spontaneous T1D development; similarly, IL-2/anti–IL-2 mAb complexes alone provided some protection, but it was not as efficient and long-lasting as the one provided by the combined treatment (Fig. 6B). Combination of rapamycin and IL-2/anti–IL-2 mAb complexes provided efficient and long-lasting protection (Fig. 6C). Consistent with the previous report (43), continuous, 10-wk-long treatment with IL-2/anti–IL-2 mAb complexes alone provided 80% protection from T1D development in female NOD mice (Fig. 6D).

**Effects of treatment on histology of pancreas and cytokine profiles**

To understand the mechanism of protection in our treated groups, we tested the effect of the combined treatment on cytokine profiles in both CD4+ and CD8+ T cells. As shown in Fig. 7A and 7B, the treatment skewed the immune profile toward a Th2 response, mostly owing to an increased percentage of CD4+ T cells producing IL-4 and IL-10, suggesting that the lower ratio of IFN-$\gamma$/IL-10 and IFN-$\gamma$/IL-4 production in T cells (Table III) may play a role in the observed protection from T1D mediated by treated Tregs.

Histology of pancreata from different groups was evaluated as shown in representative results in Fig. 7C, demonstrating that the mice that received treated Tregs had less severe islet infiltrates, compared with the mice in untreated groups.

We also examined the expression of Foxp3 in the Tregs transferred from treated NOD mice to other NOD mice. As shown in Fig. 7D, even after 3 wk, the Tregs from the treated group still...
stably express Foxp3, even at a slightly higher percentage than the Tregs from untreated group, indicating that the in vivo–expanded Tregs are relatively stable phenotypically. In agreement with this, following the combined treatment, on days 21 and 69 the expanded Tregs could still exert their function and protect the treated NOD mice from induced T1D (Supplemental Fig. 2).

Discussion
In this study, we show that Tregs expanded in vivo using a combined treatment of islet Ag (BDC2.5 mimetope peptide), IL-2/IL-2 mAb complexes, and mammalian target of rapamycin inhibitor (rapamycin) displayed an enhanced suppressive function and protected NOD mice from diabetes.

The expanded CD4+Foxp3+ T cells expressed normal levels of molecules associated with Treg development and regulation, including CTLA-4 and GITR. The expanded Tregs suppressed the in vivo proliferation of responder CD4+CD25+ T cells and the in vitro proliferation of responder CD8+ 8.3 T cells more efficiently than did control Tregs. These data suggest that our combined treatment, on days 21 and 69 the expanded Tregs could still exert their function and protect the treated NOD mice from induced T1D (Supplemental Fig. 2).

Some previous reports raised concerns about the stability of expanded Tregs upon their transfer in vivo, due to a loss of Foxp3 expression and suppressive function, especially in the context of autoimmune settings (46, 47). Therefore, we evaluated the in vivo stability of the expanded Tregs in this study and found that the treated Tregs did not lose their expression of Foxp3 (Fig. 7D). Moreover, the short-term combined treatment resulted in protection against both spontaneous and induced T1D development in NOD mice, suggesting that expanded Foxp3 expressing Tregs were stable (for >30 wk in the spontaneous T1D model and for at least 7 wk in the induced T1D model). Previous studies reported that IL-2/anti–IL-2 mAb complexes provided some protection against autoimmune diseases (experimental autoimmune encephalomyelitis) in mice. However, such protection required either continuous treatment for several days (48), weeks (43), or a combined treatment of rapamycin and IL-2/anti–IL-2 mAb complexes (29). The dose of rapamycin and the length of treatment should be considered carefully because higher doses and/or repeated treatments of rapamycin have been shown to have detrimental effects in vivo (49–52).

Because administration of peptide Ag together with immunomodulatory reagents favors the induction of Ag-specific Tregs in vivo (32, 53, 54), we assume it should be desirable to include islet Ag peptide in the combination, and indeed such a combination promoted significant Ag-specific Treg expansion in the spleens and pancreatic lymph nodes (Fig. 2B, 2C). Among the expanded Tregs, a small proportion are Ag specific (~0.4%), mostly due to the low frequency of BDC2.5 peptide–specific Tregs in the endogenous
Treg pool before treatment. This also indicates that some of the cells expanded with the combined treatment are not Ag specific, which explains why even the 3-d treatment with IL-2/IL-2 mAb alone provided some protection (Fig. 6B), and the 10-wk-long IL-2 complexes treatment achieved similar protection (Fig. 6D). The IL-2 complexes with rapamycin (but without islet Ag peptide) achieved significant protection from T1D as well (Fig. 6C). It seems that the Ag nonspecific Tregs expanded can provide a good level of protection, and for expansion of protective Tregs in vivo, the minimal requirement is the combination of IL-2 complexes plus rapamycin, because IL-2 complexes alone or rapamycin alone did not provide optimal protection against T1D (Fig. 6A, 6B).

Notably, our short-term combined treatment provided long-lasting protection against diabetes; mice can be protected for >1 y without developing T1D (data not shown). Repetitive, multiple treatments were not needed for the protection.

In conclusion, the combination of IL-2/IL-2 mAb complexes, rapamycin, and islet Ag peptide can efficiently expand islet Ag-specific Tregs in vivo. The expanded Tregs exhibit a classical Treg phenotype and are suppressive both in vitro and in vivo. Importantly, this combination therapy prevented both spontaneous and induced diabetes development in NOD mice. This protection was transferrable by adoptive transfer of the expanded Tregs, and it could be attributed to reduced infiltration of immune cells into the islets.

Table III. Combined treatment leads to a decreased Th1/Th2 cytokine ratio

<table>
<thead>
<tr>
<th>Cytokine Ratios for CD4⁺ Cells</th>
<th>Cytokine Ratios for CD8⁺ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>IFN-γ/IL-10</td>
<td>22.34 ± 0.59</td>
</tr>
<tr>
<td>IFN-γ/IL-4</td>
<td>85.64 ± 16.29</td>
</tr>
</tbody>
</table>

*Spleen cells were isolated from treated or untreated 6- to 8-wk-old NOD mice (n = 3/group) and stimulated with PMA/ionomycin with GolgiPlug for 6 h. Cells were labeled with anti-CD4 and anti-CD8 Abs first and then permeabilized and stained with different anti-cytokine Abs and analyzed by FACS. The ratios of IFN-γ/IL-10 and IFN-γ/IL-4 within the CD4 and CD8 T cell subsets were calculated. The results are the representative of two independent experiments with similar findings.
pancreatic islets as well as polarization of the immune response toward a Th2 cytokine profile.

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Disclosures
The authors have no financial conflicts of interest.

References


