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# In Vitro-expanded Antigen-specific Regulatory T Cells Suppress Autoimmune Diabetes

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## Abstract

The low number of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (T<sub>regs</sub>), their anergic phenotype, and diverse antigen specificity present major challenges to harnessing this potent tolerogenic population to treat autoimmunity and transplant rejection. In this study, we describe a robust method to expand antigen-specific T<sub>regs</sub> from autoimmune-prone nonobese diabetic mice. Purified CD4<sup>+</sup> CD25<sup>+</sup> T<sub>regs</sub> were expanded up to 200-fold in less than 2 wk in vitro using a combination of anti-CD3, anti-CD28, and interleukin 2. The expanded T<sub>regs</sub> express a classical cell surface phenotype and function both in vitro and in vivo to suppress effector T cell functions. Most significantly, small numbers of antigen-specific T<sub>regs</sub> can reverse diabetes after disease onset, suggesting a novel approach to cellular immunotherapy for autoimmunity.

Key words: autoimmunity • tolerance • CD4<sup>+</sup>CD25<sup>+</sup> T cells • NOD mice • immunoregulation

## Introduction

It has become increasingly clear that the balance of pathogenic and immune regulatory pathways underlies disease progression in many autoimmune settings. The loss of regulatory pathways such as CTLA-4, TGF- $\beta$ , and FoxP3 leads to lethal autoimmunity (1–7). This is best exemplified in type 1 diabetes (T1D) in nonobese diabetic (NOD) mice and humans, where genetic or biologic loss of function of these immunoregulatory pathways exacerbates disease development (8–10). Increasingly, these pathways have pointed to a novel CD4<sup>+</sup> regulatory T cell (T<sub>reg</sub>) lymphocyte subset as the central controller of autoimmunity in a variety of experimental animal models as well as an intrinsic regulator of spontaneous autoimmunity (for review see references 11–15). Although the most widely used markers for T<sub>regs</sub> are the expression of CD4 and CD25, other molecules such as CD62L, CTLA-4, glucocorticoid-induced TNF receptor (GITR), and FoxP3 have emerged as additional markers of this unique T cell lineage (5–7, 16–20). Importantly, T<sub>reg</sub> therapy can effectively delay and cure mice of a variety of immunological diseases including diabetes, colitis, gastritis, and graft-versus-host disease (8, 21–24). Several studies

have suggested that the T<sub>regs</sub> are antigen specific, relying on TCR engagement to fully acquire suppressive activity in vivo. The regulatory cells appear to function preferentially at the site of inflammation to effect proliferation and/or cytokine production by the pathogenic T cells (17, 25, 26).

Recent studies have suggested that T<sub>regs</sub> function via the production of immunosuppressive cytokines, particularly TGF- $\beta$  and IL-10 (21, 27–29), whereas other studies indicate that suppressive function requires cell–cell contact and cannot be attributed to soluble inhibitors (30–36). Barthlott et al. (37) and Stockinger et al. (38) suggested that T<sub>regs</sub> function to “take up space,” thus blocking the pathogenic cells from filling up their appropriate niche. The T<sub>reg</sub> population is reduced in autoimmune-prone animals and patients (8, 39). It appears that T<sub>regs</sub> may be defective in NOD mice (8, 39). For instance, T<sub>regs</sub> constitute only ~5% of the circulating CD4<sup>+</sup> T cells in NOD mice, significantly lower than that observed in other strains (8). Moreover, a large number of T<sub>regs</sub> (1:1 T<sub>reg</sub>/T<sub>eff</sub> ratio) are required to suppress ongoing disease in this model and other autoimmune models (8, 16,

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Abbreviations used in this paper: APC, allophycocyanin; CFSE, carboxyfluorescein diacetate succinimidyl ester; Ct, threshold cycle(s); GITR, glucocorticoid-induced TNF receptor; GAD, glutamic acid decarboxylase; NOD, nonobese diabetic; T1D, type 1 diabetes; T<sub>eff</sub>, T effector cell; Tg, transgenic; T<sub>reg</sub>, regulatory T cell.

17). Finally, recent studies have suggested that it might be impossible to reverse ongoing autoimmune diabetes due to the autoreactive T cells becoming resistant to suppression during the active phase of the disease. However, the studies were limited to *in vitro* analyses presumably due to limited cell numbers (40). In spite of this complexity, the potential for  $T_{\text{regs}}$  to actively regulate autoimmunity and induce long-term tolerance has great potential applications both for understanding immune homeostasis and as a strategy for inducing long-lived tolerance.

It has been reported that  $T_{\text{regs}}$  preferentially respond to dendritic cells to proliferate *in vitro* and *in vivo*, but the *in vitro*  $T_{\text{reg}}$  expansion induced by dendritic cells was still very limited (41). In fact, taking advantage of  $T_{\text{regs}}$  has been complicated by the difficulty in expanding and characterizing this minor T cell subset. In this study, we developed a robust technique for expanding antigen-specific  $T_{\text{regs}}$  from autoimmune NOD mice. The expanded  $T_{\text{regs}}$  retained all the quintessential characteristics of this subset including expression of CD25, CD62L, FoxP3, and GITR. The ability of expanded NOD  $T_{\text{regs}}$  to suppress diabetes in prediabetic and diabetic mice *in vivo* was significantly enhanced using the autoantigen-specific T cells when compared with polyclonal  $T_{\text{regs}}$ . Antigen-specific  $T_{\text{regs}}$  effectively suppressed the development of diabetes in  $T_{\text{reg}}$ -deficient CD28<sup>-/-</sup> mice, blocked syngeneic islet graft rejection in chronically diabetic animals, and in contrast to previous reports (40),  $T_{\text{regs}}$  are shown to reverse diabetes in mice with new onset disease.

## Materials and Methods

**Mice.** NOD mice (Taconic), BALB/c mice (Charles River Laboratories), BDC2.5 TCR transgenic (Tg) mice, glutamic acid decarboxylase (GAD)286 TCR Tg mice (42), NOD.CD28<sup>-/-</sup> mice, NOD.RAG<sup>-/-</sup> mice, and NOD.TCR- $\alpha$ <sup>-/-</sup> mice were housed and bred under specific pathogen-free conditions at the University of California San Francisco Animal Barrier Facility.

**Antibodies and Other Reagents.** FITC-labeled mAbs against CD4 (GK1.5) and GITR (DTA-1; reference 19) were purified from hybridoma culture supernatant and conjugated in our lab. R-PE-conjugated anti-CD25 (7D4) mAbs were purchased from Southern Biotechnology Associates, Inc. Allophycocyanin (APC)-labeled mAbs against CD4 (RM4-5) and CD62L were purchased from BD Biosciences or eBioscience. The p31/I-A<sup>g7</sup>mIlgG2a was generated in our lab (43). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes.

**Cell Sorting and Flow Cytometry.** CD4<sup>+</sup> T cells were enriched from pooled LNs and spleens by negative selection using an AutoMACS (Miltenyi Biotec). The cells were then stained with anti-CD4-FITC, anti-CD25-PE, and anti-CD62L-APC, and the  $T_{\text{regs}}$  and CD4<sup>+</sup> CD62L<sup>+</sup> CD25<sup>-</sup> T effector cells ( $T_{\text{eff}}$ ) were sorted on a Mo-Flo cytometer<sup>TM</sup> (DakoCytomation) based on the expression of CD4, CD25, and CD62L to >98% purity. Flow cytometric analyses were performed on a FACScalibur<sup>TM</sup> flow cytometer with CELLQuest<sup>TM</sup> software (Becton Dickinson).

**In Vitro Expansion of T Cells.** FACS<sup>®</sup>-purified T cells were stimulated with anti-CD3 and anti-CD28 coupled to 4.5- $\mu$ m paramagnetic beads (provided by Xcyte Therapeutics Inc.) supplemented with 2,000 IU/ml rIL-2 (Chiron Corp.) in complete medium, which consisted of 10% heat-inactivated fetal bovine se-

rum (Biosource International), nonessential amino acids, 0.5 mM sodium pyruvate, 5 mM HEPES, 1 mM glutamax I (all from Invitrogen), and 55  $\mu$ M  $\beta$ -mercaptoethanol (Sigma-Aldrich) in DMEM base. The cultures were monitored daily and maintained at  $0.7\text{--}1 \times 10^6$ /ml by diluting with IL-2-supplemented complete medium for 8–12 d. At the end of the culture, the anti-CD3 and anti-CD28 beads were removed using AutoMACS, and the cells were routinely assayed for CD4, CD62L, and CD25 expression by flow cytometry and for suppressive activity *in vitro*. It should be pointed out that conventional anti-CD3 plus anti-CD28-coated 6- $\mu$ m polystyrene beads can be adapted for use in this procedure.

**In Vitro Suppression Assays.** Graded numbers of expanded or fresh sorted  $T_{\text{regs}}$  were added to 50,000 CD4<sup>+</sup> T cells stimulated with 50,000 irradiated splenic APC (2,000 rads) and 1  $\mu$ g/ml anti-CD3 in a U-bottomed 96-well plate. CD4<sup>+</sup> T cell cultures without  $T_{\text{regs}}$  were stimulated in the same manner as positive controls. For some experiments, CD4<sup>+</sup> T cells from DO11.10 TCR Tg mice were used as responders and the cocultures were stimulated with anti-CD3 as described above or with 0.1  $\mu$ g/ml OVA peptide. The cultures were maintained at 37°C for a total of 64 or 72 h and pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine for the last 14 or 8 h, respectively. For some experiments, the responder CD4<sup>+</sup> cells were labeled with 2.5  $\mu$ M CFSE before the suppression assay, and the level of proliferation was assessed by determining the dilution of CFSE using flow cytometry 72 h after the initiation of the culture.

**Real Time PCR Analysis.** Total RNA was extracted using Trizol reagent (Invitrogen) or RNeasy (QIAGEN) from expanded T cells. cDNA was synthesized from 50 ng–2.5  $\mu$ g of each RNA sample using SuperScript II RNase H reverse transcriptase and oligo dT as primer (Invitrogen), and 0.6–31.25 ng of the cDNA was used in each quantitative real time PCR reaction. The same amount of RNA and cDNA was used for each  $T_{\text{reg}}$  and CD25<sup>-</sup> sample pair. Primers and probes for FoxP3, CTLA-4, neuropilin, PD-1, TRAIL, and HPRT were purchased as reagent kits from Applied Biosystems. Primer sequences for SOCS-2 were 5'-GCGTCTGGCGAAAGCCCT (forward) and 5'-CTTCATTAACAGTCATACTTCC (reverse), and they were ordered from Integrated DNA Technologies Inc. The probe sequence for SOCS-2 was 5'-FAM-CGCGAGCTCAGTCAAACAGGATGGT-TAMRA-3', which was ordered from Applied Biosystems. The real time PCR was performed on an ABI prism 7700 using Taqman Universal PCR master mix (Applied Biosystems) in duplicates and the average threshold cycles (Ct) of the duplicates were used to calculate the fold change between expanded  $T_{\text{regs}}$  and CD4<sup>+</sup> CD62L<sup>+</sup> CD25<sup>-</sup> cells. Ct for HPRT was used to normalize the samples. Expression ratios between  $T_{\text{regs}}$  and CD25<sup>-</sup> cells were calculated using the following formula: Expression Ratio ( $T_{\text{reg}}/CD25^{-}$ )<sub>Gene</sub>  $\times$  =  $2^n$ ,  $n$  = (CD25<sup>-</sup> Ct<sub>Gene</sub>  $\times$  -CD25<sup>-</sup> Ct<sub>HPRT</sub>) - ( $T_{\text{reg}}$  Ct<sub>Gene</sub>  $\times$  - $T_{\text{reg}}$  Ct<sub>HPRT</sub>).

**Western Blot.**  $5 \times 10^5$  of each cell type were lysed in sample buffer (6.25 mM Tris, pH 6.8, 12.5% glycerol, 2% SDS, 30 ng/ml bromophenolblue), sonicated, and passed through 28-gauge needles. The lysates were clarified by centrifugation and boiled for 5 min before separating on a 10% SDS PAGE gel. The samples were transferred to PVDF membrane after electrophoresis and incubated with rabbit anti-FoxP3 antisera (provided by S. Ziegler, Benaroya Research Institute at Virginia Mason, Seattle, WA) followed by horseradish peroxidase-conjugated anti-rabbit Ig. The blot was developed with SuperSignal<sup>®</sup> Chemiluminescent Substrate (Pierce, Rockford, IL) and visualized on a Kodak Image Station 440CF (Eastman Kodak Co.) and quantified using Kodak Digital Science 1D Image Analysis software 3.0.

**Cytokine ELISA.** The level of IL-2, IFN- $\gamma$ , IL-10, and TGF- $\beta$  in the culture supernatant was determined by ELISA using antibody pairs purchased from BD Biosciences. For TGF- $\beta$  ELISA, the culture supernatant was first treated with acid to lower the pH to 2.0 to denature latency-associated peptide to allow the detection of active TGF- $\beta$ . The supernatant was neutralized to pH 7.0 before ELISA.

**Adoptive Transfer.** Expanded T cells were labeled with 2.5  $\mu$ M CFSE, and  $1-3 \times 10^6$  cells were transferred via retro-orbital injection. The recipient mice were killed on day 7 after cell transfer and the dilution of CFSE in splenic, peripheral LN, and pancreatic LN cell preparations was determined by flow cytometry. For adoptive transfer of diabetes to NOD.RAG $^{-/-}$  recipients, CD4 $^+$  CD62L $^+$  CD25 $^-$  cells from BDC2.5 TCR Tg mice were purified by cell sorting using a DakoCytomation Mo-Flo and  $0.5-1 \times 10^6$  cells were transferred to each recipient mouse. For some experiments, the purified T $_{\text{eff}}$ s were activated with anti-CD3 and anti-CD28 for 8–10 d before transfer. To transfer diabetes with polyclonal T $_{\text{eff}}$ s,  $25 \times 10^6$  pooled spleen and LN cells from diabetic NOD mice were injected into each recipient. When indicated, expanded T $_{\text{regs}}$  were depleted of anti-CD3 and anti-CD28 beads and washed extensively before mixing with effector cells for injection. The expanded T $_{\text{regs}}$  were similarly processed before transferring into NOD.CD28 $^{-/-}$ , chronically diabetic NOD syngeneic islet transplant recipients, and NOD mice with new onset diabetes. Nonfasting blood glucose levels in recipient mice were monitored using an Accu-Check glucometer (Roche Diagnostics Corp.)

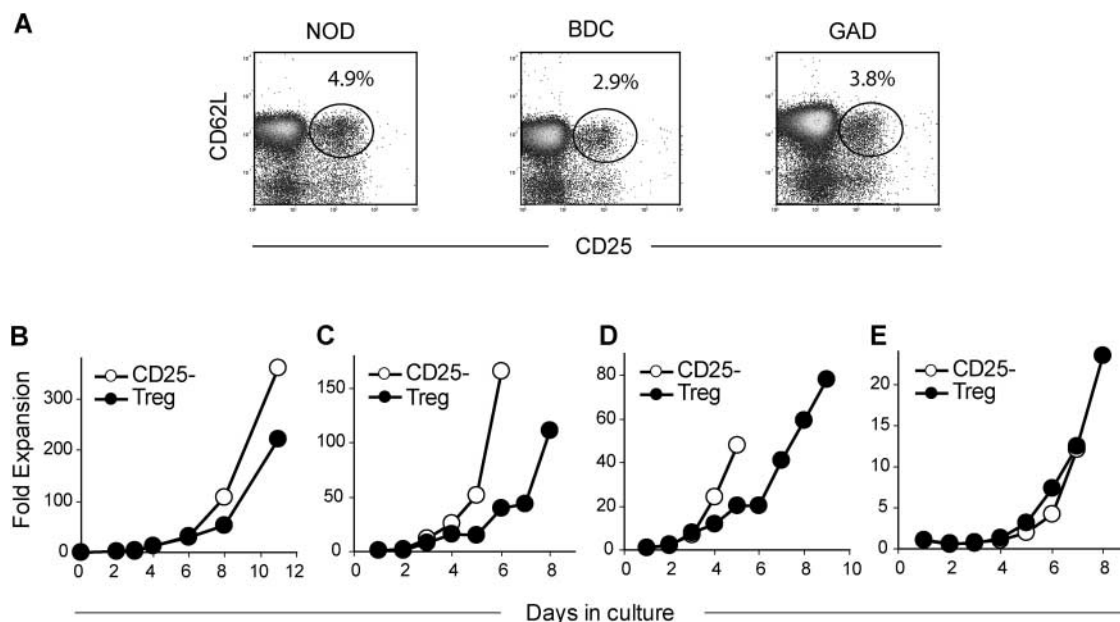
**Murine Pancreatic Islet Isolation.** Murine islets were isolated using a modified previously published protocol (44). In brief, a 3-ml collagenase P (Roche Molecular Biochemicals) solution (0.75 mg/ml) was injected into the pancreatic duct of 4-wk-old NOD mice. The distended pancreases were removed and incubated at 37°C for 17 min. The liberated free islets were purified

by centrifugation on Eurocollin-Ficoll gradients that comprised four different densities (1.108, 1.096, 1.069, and 1.037). After centrifugation, the islet-containing layers between densities 1.069 and 1.096 were collected and washed. Islets were then handpicked for transplantation.

**Murine Islet Transplantation.** Naturally diabetic NOD mice were used as recipients. The mice were diabetic for at least 2 wk before transplantation. The recipient mice were maintained with subcutaneous insulin pellets (Lin-Shin Canada, Inc.). 1 d before transplantation, the insulin pellets were removed and hyperglycemia was confirmed on the day of transplantation. 500 isolated and handpicked islets were transplanted beneath the left renal capsule of each recipient. Nonfasting blood glucose levels were determined in all animals daily after transplantation. Return to normoglycemia within 24 h after transplant was indicative of successful surgery. Rejection of the islets grafts was considered to have occurred when nonfasting blood glucose concentration exceeded 250 mg/dl for 3 consecutive days.

## Results

**Expansion of T $_{\text{regs}}$  from Autoantigen-specific TCR Tg NOD Mice.** Previous studies have shown that the number and function of T $_{\text{regs}}$  in NOD mice decrease over time correlating with clinical disease onset between 16 and 24 wk of age (9, 40). These observations support the use of T $_{\text{regs}}$  to prevent or treat diabetes even after disease onset. However, the ability to use these cells therapeutically is severely limited by the small numbers of cells resident in the circulation or lymphoid organs (<5% of CD4 $^+$  T cells in NOD mice and <2% of CD4 $^+$  T cells in humans with T1D; references 8 and 39). Moreover, a large number of cells are required for therapeutic efficacy due to an inability at present to se-



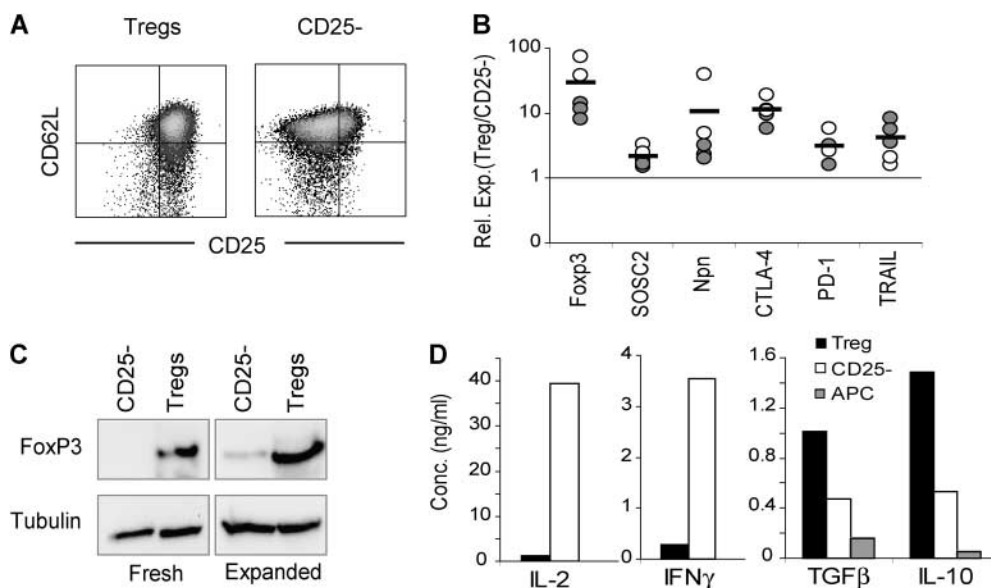
**Figure 1.** In vitro expansion of T $_{\text{regs}}$ . (A) Representative flow cytometry plots of CD25 and CD62L expression on CD4 cells from NOD (left), BDC2.5 (middle), and GAD286 (right) mice. FACS $^{\text{R}}$ -purified T $_{\text{regs}}$  (●) and CD4 $^+$  CD62L $^+$  CD25 $^-$  cells (○) from NOD (B), BDC2.5 TCR Tg (C), or GAD286 TCR Tg (D) mice were stimulated in vitro with anti-CD3- and anti-CD28-coated beads along with IL-2. (E) T cells from BDC2.5 TCR Tg mice were expanded as described above with p31-linked IA $^{\text{g7}}$ -mIgG2a immobilized on latex beads. All cultures were quantitated by viable cell counting.

lect the cells based on antigen specificity. Therefore, we developed a technique for rapid and efficient expansion of autoantigen-specific  $T_{\text{regs}}$  based on observations that these cells, present in TCR Tg mice, can be driven into cell cycle with coimmobilized anti-CD3 and anti-CD28 antibodies plus exogenous IL-2. As shown in Fig. 1, A and B, FACS<sup>®</sup>-purified NOD  $T_{\text{regs}}$  cultured with anti-CD3/anti-CD28-coated beads in the presence of IL-2 expanded 150–225-fold in 11 d (Fig. 1 B). In general, the  $CD4^+ CD25^-$  T cells expanded more vigorously (ranging from 300–800-fold in multiple experiments). A purity of  $>98\%$   $CD4^+ CD25^+ CD62^+$  T cells was essential to enable successful  $T_{\text{reg}}$  expansion as a small contamination of either  $CD25^- CD4^+$  or  $CD8^+$  T cells significantly impacted the ability to specifically expand the  $T_{\text{regs}}$  (unpublished data). It should be noted that the  $T_{\text{reg}}$  expansion is dependent on the high level of IL-2 (2,000 IU/ml). No  $T_{\text{reg}}$  expansion was observed when 200 IU/ml IL-2 was used (unpublished data).

Previous studies have shown that  $CD4^+ CD25^+ T_{\text{regs}}$  isolated from young NOD mice suppressed the ability of  $T_{\text{eff}}$ s from diabetic NOD mice to transfer disease in immunodeficient NOD mice (8, 16, 17). However, the process was highly inefficient and the suppressive effects of  $T_{\text{regs}}$  in this setting required a 0.5:1 or 1:1 ratio of  $T_{\text{reg}}/T_{\text{eff}}$ . This is likely due to the low precursor frequency of antigen-specific  $T_{\text{regs}}$ . Thus, we examined whether  $T_{\text{regs}}$  from two different antigen-specific TCR Tg mice (Fig. 1 A) could be expanded in vitro using the same methodology as with the polyclonal NOD  $T_{\text{regs}}$ . BDC2.5 TCR Tg mice express a TCR specific for an islet antigen expressed in the granules of  $\beta$  cells, whereas the GAD286 TCR Tg recognizes a peptide derived from the islet antigen GAD.  $T_{\text{regs}}$  were purified from BDC2.5 and GAD286 mice and expanded using

the anti-CD3/anti-CD28 plus IL-2 cocktail (Fig. 1, C and D). The BDC2.5 cells expressed the Tg TCR  $\alpha\beta$  based on efficient staining with an MHC peptide tetramer previously shown to react with this TCR (43), and the expanded GAD286  $T_{\text{regs}}$  expressed the Tg TCR- $\beta$  chain (unpublished data). The  $CD4^+ CD62L^+ CD25^-$  and  $T_{\text{regs}}$  from BDC2.5 TCR Tg mice can also be expanded using immobilized MHC peptide dimers (Fig. 1 E). These results suggest that a population of  $CD4^+ CD25^+ CD62L^+$  exists in both wild-type and TCR Tg mice that can be expanded using this protocol.

Next, we examined the phenotype of the expanded  $T_{\text{regs}}$  by flow cytometry, Western blot, and real time PCR. As can be seen in Fig. 2 A, the expanded  $T_{\text{regs}}$  maintained high levels of expression of CD25 as compared with expanded  $CD25^-$  T cells, whereas the expression of CD62L remained high in both cell types. In addition, quantitative PCR showed that all of the  $T_{\text{regs}}$  expressed high levels of SOCS2, PD-1, and CTLA-4 as compared with similarly expanded  $CD25^-$  T cells. Moreover, the recently identified markers neuropilin and TRAIL (20, 45) were also highly expressed on the expanded  $T_{\text{regs}}$  (Fig. 2 B). A high level of cell surface GITR expression was observed on the expanded  $T_{\text{regs}}$ . However, this previously identified  $T_{\text{reg}}$  marker was also induced on the expanded  $CD25^-$  T cells (19, 20, and unpublished data). It should be noted that the quantitative PCR studies were performed on five separate expanded  $T_{\text{reg}}$  populations (including both polyclonal and BDC2.5 TCR Tg  $T_{\text{regs}}$ ) and the relative expression of the  $T_{\text{reg}}$ -specific genes was highly reproducible. Finally, we examined the recently identified lineage/differentiation marker for  $T_{\text{regs}}$ , FoxP3 (Fig. 2, B and C). As noted by both real time PCR and Western blot analyses, the ex-



**Figure 2.** Phenotype of in vitro-expanded  $T_{\text{regs}}$ . (A) Expression of CD25 and CD62L on expanded  $T_{\text{regs}}$  and  $CD4^+ CD62L^+ CD25^-$  cells was determined by flow cytometry on day 8 after the culture initiation. Results are representative of more than 20 independent experiments. (B) Levels of mRNA for the indicated genes in expanded NOD (filled symbols) or BDC2.5 TCR Tg T cells (open symbols) were determined by real time PCR analysis on day 10 after the initiation of the cultures. The relative expression ratio ( $T_{\text{reg}}/T_{\text{CD25}^-}$ ) for each pair of cultures was calculated from Ct values as described in Materials and Methods. The dashed line represents the ratio of 1 (i.e., identical level of gene expression in  $T_{\text{reg}}$  and  $CD4^+ CD62L^+ CD25^-$  cultures). (C) Western blot analysis of FoxP3

protein expression in fresh and expanded T cells. The level of tubulin expression was included as a loading control. Results are representative of three independent experiments. (D) Cytokine secretion by expanded BDC2.5 T cells 48 h after restimulation with antigenic peptide and splenic APC. Results are representative of two independent experiments.

expanded  $T_{\text{regs}}$  expressed levels of FoxP3 similar to those observed in fresh  $T_{\text{regs}}$  and significantly higher than those in fresh or expanded  $CD25^{-}$  T cells. The RNA expression (10-fold) and protein amounts (20-fold) were consistent with previous studies of fresh  $T_{\text{regs}}$ , although there was clearly some increase in FoxP3 in  $CD25^{-}$   $T_{\text{effs}}$ , suggesting that the culture conditions may induce some  $T_{\text{regs}}$  within the  $CD25^{-}$  subset, or FoxP3 is expressed at a low level in activated  $T_{\text{effs}}$ .

We also examined the ability of the expanded  $T_{\text{regs}}$  to secrete cytokines. Unlike activated  $CD25^{-}$  T cells, the  $T_{\text{regs}}$  did not produce IL-2 or IFN- $\gamma$ , but rather expressed the immunosuppressive cytokines IL-10 and TGF- $\beta$  (Fig. 2 D). Thus, the extensive activation and proliferation of the  $T_{\text{regs}}$  does not alter the phenotype of the  $T_{\text{regs}}$ , which remained distinct from the  $CD25^{-}$  T cell subset.

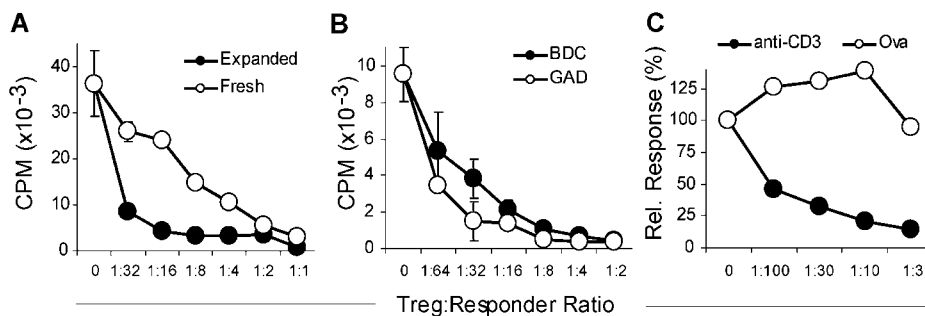
**Functional Activity of In Vitro-expanded  $T_{\text{regs}}$ .** Previous studies have shown that  $T_{\text{regs}}$  can effectively suppress proliferative responses of  $CD25^{-}$  T cells stimulated with anti-CD3 and splenic APC. The expanded NOD  $T_{\text{regs}}$  efficiently suppressed proliferative responses (Fig. 3 A) and cytokine production including IL-2 and IFN- $\gamma$  (unpublished data). In fact, in multiple experiments, the expanded  $T_{\text{regs}}$  suppressed significantly better than fresh NOD  $T_{\text{regs}}$ . The suppression was routinely observed at  $T_{\text{reg}}/T_{\text{eff}}$  ratios of <1:10. Similar results were observed using the expanded  $T_{\text{regs}}$  from the TCR Tg mice, as the expanded BDC2.5  $T_{\text{regs}}$  were effective in suppressing the proliferative response of BDC2.5 (unpublished data) as well as polyclonal NOD T cells (Fig. 3 B). Although the expanded  $T_{\text{regs}}$  expressed significant levels of IL-10 and TGF- $\beta$ , suppressor activity was unaffected by the addition of anti-IL-10, anti-TGF- $\beta$ , or a combination of both antibodies to the in vitro cultures (unpublished data). These results are consistent with numerous models of  $T_{\text{reg}}$  suppression where cell-cell contact is the primary means of immunosuppression in the in vitro setting (30–36).

To further assess the antigen specificity of the expanded  $T_{\text{regs}}$  and determine whether the expanded  $T_{\text{regs}}$  were constitutively suppressive, expanded  $T_{\text{regs}}$  from normal BALB/c mice were examined for their ability to suppress T cells from the OVA-specific DO11.10 TCR Tg mouse.  $T_{\text{regs}}$  and DO11.10 Tg  $T_{\text{effs}}$  were cocultured in the presence of OVA antigen (to activate only the  $T_{\text{effs}}$ ) or anti-CD3 (to

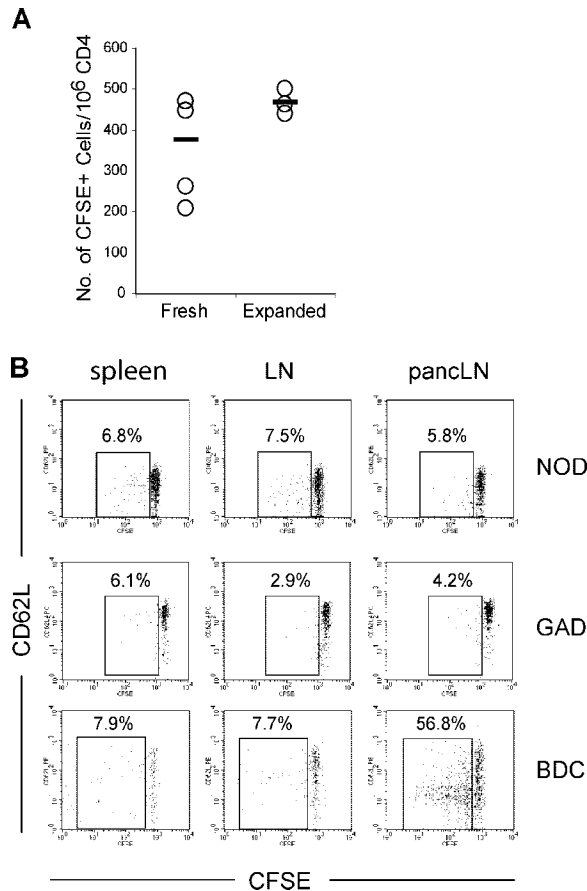
activate both the  $T_{\text{eff}}$  and  $T_{\text{regs}}$ ). Expanded BALB/c  $T_{\text{regs}}$  did not inhibit the proliferative response of the DO11.10 T cells stimulated by the OVA peptide. However, the anti-CD3 response was fully inhibited at low  $T_{\text{reg}}/T_{\text{eff}}$  ratios. These results suggest that the expanded  $T_{\text{regs}}$  lack constitutive suppressive activity but require TCR-specific activation for effective suppression. This result also ruled out the trivial possibility that the cells were inhibiting the cultures by consuming available IL-2 through the high level of CD25 expression.

**In Vivo Survival and Activation of Expanded  $T_{\text{regs}}$ .** Effective suppression of immune responses in vivo by  $T_{\text{regs}}$  requires that the cells migrate to appropriate sites, respond to antigen, and survive long-term. We have observed recently that blockade of the CD28/B7 pathway resulted in rapid loss of  $T_{\text{regs}}$  in vivo and subsequent loss of critical immune regulation (8, 46). Thus, we examined the ability of expanded  $T_{\text{regs}}$  to survive and proliferate in vivo. Expanded  $T_{\text{regs}}$  were labeled with CFSE and transferred into normal nonlymphopenic syngeneic mice. At 30 d after transfer, the mice were killed and examined for the number of CFSE $^{+}$  cells as an indication of cell survival. As seen in Fig. 4 A, a significant number of CFSE $^{+}$  cells were recovered from mice transferred with expanded  $T_{\text{regs}}$ . The number of CFSE $^{+}$   $T_{\text{regs}}$  was equal to that observed with fresh  $T_{\text{regs}}$  transferred in the same manner (Fig. 4 A). In fact, Thy1.1-marked expanded  $T_{\text{regs}}$  were observed at least 50 d after transfer (unpublished data).

Next, we analyzed the ability of the adoptively transferred  $T_{\text{regs}}$  to respond to antigen and proliferate in vivo. Expanded  $T_{\text{regs}}$  from NOD, BDC2.5, and GAD286 mice were labeled with CFSE and transferred into normal nonlymphopenic NOD recipients. At 7 d after transfer, the mice were killed and examined for the dilution of CFSE to assess in vivo proliferation. As seen in Fig. 4 B, top, a small but significant number of  $T_{\text{regs}}$  proliferated, as indicated by CFSE dilution. However, there was no selective proliferation of the NOD  $T_{\text{regs}}$  in the pancreatic LNs (Fig. 4 B, pancLN), suggesting that a detectable number of islet autoantigen-specific cells did not exist within the NOD  $T_{\text{reg}}$  repertoire. In contrast to the NOD  $T_{\text{regs}}$ ,  $T_{\text{regs}}$  from BDC2.5 Tg mice proliferated extensively and selectively in the pancreatic LNs, dividing at least three to four times during the 7-d period (Fig. 4 B, bottom). Interestingly, the



**Figure 3.** In vitro suppression by expanded  $T_{\text{regs}}$ . (A) Fresh and expanded  $T_{\text{regs}}$  were compared for their ability to suppress the proliferation of  $CD4^{+}$  responder T cells stimulated with anti-CD3 and T cell-depleted splenocytes. (B) Suppression by  $T_{\text{regs}}$  expanded from BDC2.5 TCR Tg or GAD286 TCR Tg mice was assayed as described in A. (C) Suppressive activity of BALB/c-expanded  $T_{\text{regs}}$  on  $CD4^{+}$  responder T cells from DO11.10 TCR Tg mice stimulated with anti-CD3 or an OVA peptide. Results are representative of three independent experiments.



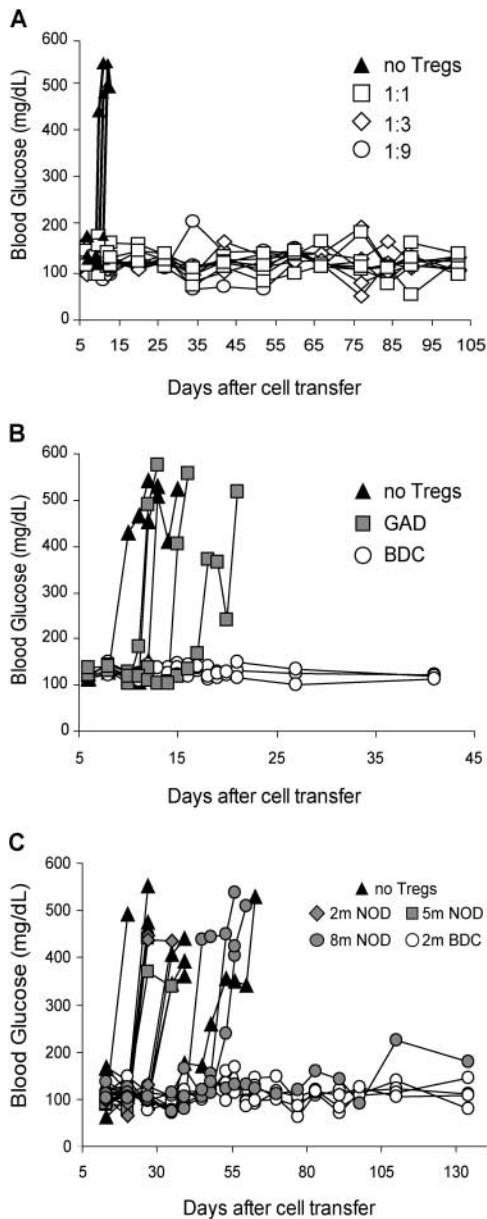
**Figure 4.** In vivo survival and activation of expanded  $T_{reg}$ s. (A) Freshly isolated and expanded BALB/c  $T_{reg}$ s were labeled with CFSE and then injected into normal BALB/c mice ( $10^6$ /mouse). All recipient mice were killed on day 30 after injection and the numbers of CFSE<sup>+</sup> cells in the peripheral LN and spleen (not shown) were determined by flow cytometry. The data are presented as the number of CD4<sup>+</sup> CFSE<sup>+</sup> cells/ $10^6$  endogenous CD4<sup>+</sup> T cells. Each circle represents the value from one mouse and the black bar represents the mean of the group. (B) Expanded  $T_{reg}$ s from NOD.Thy1.2 (top) and GAD286 TCR Tg Thy1.2 (middle) were labeled with CFSE and  $3 \times 10^6$  were transferred to normal 8–12-wk-old NOD.Thy1.1 recipients. Expanded BDC2.5 TCR Tg Thy1.1 were labeled with CFSE and  $3 \times 10^6$  were transferred to normal 8–12-wk-old NOD.Thy1.2 recipients. The presence of transferred cells and their activation status in spleens, LNs, and pancreatic LNs were determined by flow cytometry on day 7 after cell transfer. The dot plots shown are gated on the Thy1.2 for NOD and GAD286 cells and Thy1.1 for BDC2.5 cells. The percentages of cells with CFSE dilution are shown on the plots. Results are representative of at least three recipient mice in two separate experiments.

expression of the CD62L molecule was down-regulated on the surface of expanded  $T_{reg}$ s. This is surprising because the cells had undergone multiple proliferative cycles in vitro before transfer and had maintained high levels of CD62L expression. In contrast to the BDC2.5  $T_{reg}$ s, the GAD286  $T_{reg}$ s did not proliferate in vivo (Fig. 4 B, middle). The results of previous studies suggest that T cells in these two TCR Tg mice differ significantly in their thymic development. The BDC2.5 Tg mice do not negatively select the islet-specific T cells in the thymus, but rather develop a small, reproducible number of  $T_{reg}$ s. These cells have been

shown to block disease manifested by potential pathogenic CD4<sup>+</sup> CD25<sup>-</sup> T cells resident in these animals (47). By comparison, the majority of GAD286 TCR Tg T cells are deleted in the NOD thymus by negative selection. In fact, the minor population of cells that escape use alternative TCR- $\alpha$  chains. Thus, although the peripheral GAD286 TCR Tg cells respond to GAD peptide in vitro, the reactivity is weak and, in contrast to the BDC2.5, they are unable to induce diabetes upon adoptive transfer, suggesting the “absence” of an autoreactive repertoire (42). These results support the conclusion that the two Tg mice are resistant to diabetes for distinct reasons. The GAD286 TCR Tg mice are protected from the development of diabetes due to the potent central tolerance mechanism of clonal deletion and receptor editing. By comparison, the BDC2.5 TCR Tg have circulating autoreactive  $T_{reg}$ s that home to peripheral target tissues where they are activated and expand after encountering autoantigen, resulting in immune suppression and homeostasis.

*In Vitro-expanded  $T_{reg}$ s Suppress Adoptive Transfer of Diabetes In Vivo.* Next, we examined the ability of the expanded BDC2.5  $T_{reg}$ s to suppress diabetes after in vivo cotransfer of activated BDC2.5 T cells into NOD.RAG mice. The  $T_{reg}$ s were effective in blocking the transfer of diabetes, functioning at as low as a 1:9 ratio of  $T_{reg}/T_{eff}$  (Fig. 5 A), whereas the GAD286  $T_{reg}$ s did not protect even at a  $T_{reg}/T_{eff}$  ratio of 1:1 (Fig. 5 B). In fact, the expanded BDC2.5  $T_{reg}$ s suppressed polyclonal T cell-mediated disease. As few as  $2 \times 10^6$  expanded BDC2.5  $T_{reg}$ s blocked the ability of  $25 \times 10^6$  diabetogenic NOD spleen and LN cells to transfer disease (Fig. 5 C). The expanded antigen-specific  $T_{reg}$ s from the BDC2.5 mice were far more efficient than expanded polyclonal NOD  $T_{reg}$ s in preventing the onset of diabetes. The same number ( $2 \times 10^6$ ) as well as  $5 \times 10^6$  expanded NOD  $T_{reg}$ s did not confer any protection under the same conditions (Fig. 5 C). In fact, even the transfer of four times as many expanded NOD  $T_{reg}$ s ( $8 \times 10^6$ ) only slightly delayed diabetes onset and prevented diabetes in only one diabetogenic cell recipient (Fig. 5 C). This result is consistent with previous findings suggesting that a high ratio of polyclonal  $T_{reg}$ s to  $T_{eff}$ s is necessary to efficiently suppress disease transfer in this setting (8, 16, 17). Importantly, these data suggest that in vitro activity of the  $T_{reg}$ s does not predict in vivo function in this disease setting.

*Expanded  $T_{reg}$ s Prevent Diabetes In Vivo in a Nonlymphopenic Setting.* Although there are multiple models demonstrating the immunoregulatory activity of  $T_{reg}$ s, many of the systems are based on adoptive transfer models that take advantage of lymphopenic mice to enhance  $T_{reg}$  proliferation (8, 21–24). Questions have been raised whether disease suppression observed in lymphopenic settings after  $T_{reg}$  transfer is due to active regulation or a side effect of competition for “space” (37, 38). Therefore, we examined the ability of the expanded  $T_{reg}$ s to prevent diabetes in a nonlymphopenic animal model. Previous studies have shown that CD28<sup>-/-</sup> NOD mice have normal numbers of T cells and Th1 responses. In fact, these mice develop exacerbated

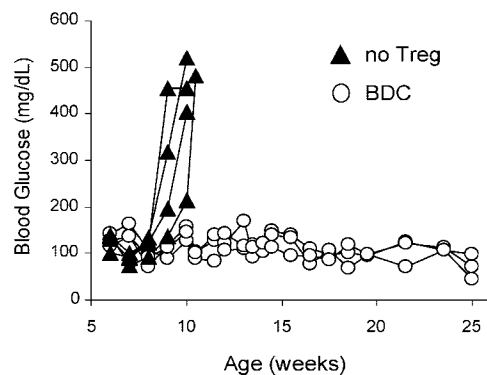


**Figure 5.** Prevention of diabetes transfer by expanded  $T_{\text{regs}}$ . (A) Activated diabetogenic BDC2.5  $CD4^+ CD62L^+ CD25^-$  cells ( $3.5 \times 10^5$ ) were cotransferred with BDC2.5-expanded  $T_{\text{regs}}$  to 8-wk-old NOD.RAG $^{-/-}$  recipients at the indicated ratio. The blood glucose for individual recipient mouse was monitored and plotted to access diabetes.  $n = 3$  for no  $T_{\text{regs}}$  and 1:9 groups;  $n = 4$  for 1:1 and 1:3 groups. Results are representative of three independent experiments. (B) Diabetes was induced in 6-wk-old NOD.RAG $^{-/-}$  mice in the same manner as described in A, except that the number of transferred expanded  $T_{\text{regs}}$  from GAD286 TCR Tg mice and BDC2.5 TCR Tg mice equaled the number of transferred  $T_{\text{effs}}$  ( $n = 3$  mice/group). (C) Diabetes was induced in 5–8-wk-old NOD.RAG $^{-/-}$  or NOD.TCR- $\alpha^{-/-}$  recipients by injection of  $25 \times 10^6$  pooled spleen and LN cells from diabetic donors ( $n = 8$ ). Some recipient mice were coinjected with expanded  $T_{\text{regs}}$  from NOD ( $2 \times 10^6$ ,  $n = 3$ ;  $5 \times 10^6$ ,  $n = 3$ ;  $8 \times 10^6$ ,  $n = 4$ ) or BDC2.5 TCR Tg mice ( $2 \times 10^6$ ,  $n = 4$ ). Results represent two independent experiments.

autoimmunity due to a deficiency in Th2 and  $T_{\text{regs}}$ , which were shown to be exquisitely CD28 dependent (8, 21–24).

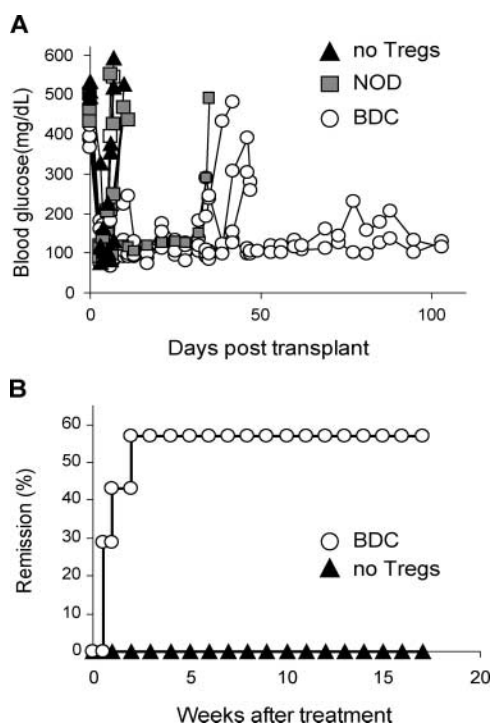
Thus, we examined whether wild-type expanded BDC2.5  $T_{\text{regs}}$  transferred into CD28 $^{-/-}$  NOD mice could delay or prevent onset of disease.  $5 \times 10^5$   $T_{\text{regs}}$  were transferred into 5-wk-old CD28 $^{-/-}$  NOD mice and monitored for diabetes (Fig. 6). The transfer of expanded BDC2.5  $T_{\text{regs}}$  prevented the development of diabetes in 100% of mice followed for as long as 20 wk after transfer. In contrast, the transfer of similar numbers of expanded NOD  $T_{\text{regs}}$  had no effect on disease incidence (unpublished data). These results suggest that the antigen-specific expanded  $T_{\text{regs}}$  functioned *in vivo* in the face of a fully functional pathogenic T cell response.

**Expanded  $T_{\text{regs}}$  Reverse Diabetes *In Vivo*.** The ultimate utility of  $T_{\text{reg}}$  therapy depends on an ability to treat individuals with ongoing disease. Thus, we examined the regulatory effects of expanded BDC2.5  $T_{\text{regs}}$  in NOD mice that had been diabetic for at least 2 wk to ensure total endogenous islet cell destruction. Expanded BDC2.5  $T_{\text{regs}}$  were transferred into diabetic NOD mice in conjunction with 500 syngeneic NOD islet transplant. Mice reverted to normoglycemia within 24–48 h after transplantation. However, unlike the control mice that rejected the transplanted islets within 2 wk, the transfer of  $2 \times 10^6$  BDC2.5-expanded  $T_{\text{regs}}$  blocked rejection of the syngeneic islets, consistent with an ability of the suppressor cells to block ongoing autoimmunity in this setting. The transfer of  $5 \times 10^6$  polyclonal NOD  $T_{\text{regs}}$  had no effect in this model. More significantly, the adoptive transfer of expanded BDC2.5  $T_{\text{regs}}$  reversed diabetes in new-onset diabetic NOD mice (Fig. 7 B). Previous studies have shown that NOD mice diagnosed within the first week of hyperglycemia retain sufficient insulin-producing  $\beta$  cell activity, such that effective immunosuppression introduced at that time can reverse diabetes. To test the efficacy of expanded BDC2.5  $T_{\text{regs}}$  in this setting,  $10^7$   $T_{\text{regs}}$  were transferred into NOD mice diagnosed with disease based on recently elevated blood glucose levels ( $>300$  mg/dL). The transferred  $T_{\text{regs}}$  reversed diabetes in 60% of the mice. Thus, the expanded  $T_{\text{regs}}$  were



**Figure 6.** Prevention of autoimmune diabetes in NOD.CD28 $^{-/-}$  mice with BDC2.5-expanded  $T_{\text{regs}}$ . 5-wk-old prediabetic NOD.CD28 $^{-/-}$  mice were injected with  $5 \times 10^5$  BDC2.5-expanded  $T_{\text{regs}}$  ( $n = 3$ ) or left untreated ( $n = 4$ ). The development of diabetes was monitored and blood glucose levels of individual mice were plotted. Results are representative of at least five independent experiments.





**Figure 7.** Reversal of diabetes with expanded  $T_{regs}$ . (A) NOD mice with chronic diabetes were transplanted with syngeneic islets under the kidney capsule. On the day of transplantation, some recipient mice received  $5 \times 10^6$  NOD-expanded  $T_{regs}$  ( $n = 4$ ) or  $2 \times 10^6$  BDC2.5-expanded  $T_{regs}$  ( $n = 5$ ), and the remaining mice ( $n = 3$ ) were left untreated. Blood glucose level was monitored. All islet recipients normalized blood glucose within the first day after transplantation. Results are representative of two independent experiments. (B) NOD mice with new onset diabetes (blood glucose  $> 300$  mg/dL,  $n = 7$ ) were injected with  $10^7$  BDC2.5-expanded  $T_{regs}$  and blood glucose was monitored. Two consecutive readings of blood glucose of  $< 250$  mg/dL was considered remission of diabetes.

extremely effective in blocking and reversing diabetes in an ongoing autoimmune setting.

## Discussion

The past few years have seen an increased interest in and understanding of the role of  $T_{regs}$  in immune homeostasis. As an example, we have recently shown that anti-CD3 therapy in new-onset diabetes leads to the production and expansion of TGF- $\beta$ -dependent  $T_{regs}$  that reverse diabetes and promote long-term tolerance (9). Moreover, Edinger et al. (24) have shown that the adoptive transfer of  $T_{regs}$  in mice can block graft-versus-host disease without affecting graft-versus-leukemia responses. These studies and others have led investigators to conclude that these cells might be involved in human autoimmune diseases. The results have also prompted investigators to consider this cell type for immunotherapy. However, successful application of adoptive cellular immune therapy with these cells will depend on a large, reliable source of well-characterized  $T_{regs}$ .

In this study, we describe a robust and effective method for expanding  $T_{regs}$  while retaining their phenotype and suppressive activities. We demonstrate that expanded anti-

gen-specific  $T_{regs}$  prevent the development of diabetes and even restore an immune regulatory state that reverses diabetes and allows the mice to maintain long-term immune homeostasis. The expanded antigen-specific  $T_{regs}$  survived long-term in vivo, were less dependent on CD28 costimulation (unpublished data), but required antigen exposure for functional activity. To our knowledge, this is the first example of  $T_{reg}$  activity in a lymphocyte-sufficient diabetic animal. These results are especially important in light of a recent publication by Gregori et al. (40), suggesting that  $T_{regs}$  do not effectively suppress  $T_{eff}$  in the setting of diabetes. The difference between the two studies may reflect either of the different assay systems: in vivo versus in vitro or that the expanded  $T_{regs}$  might be more efficient. In this regard, we have noted that the expanded  $T_{regs}$  do indeed survive better in vivo than the fresh  $T_{regs}$ .

Among the more interesting and perhaps unexpected observations in this study was the differential dependency of antigen specificity for in vivo versus in vitro  $T_{reg}$  functions. Studies in multiple models have shown that polyclonal  $T_{regs}$  are effective in blocking autoimmunity (8, 21–24). In fact, in most settings (with a few notable exceptions such as allogeneic organ transplantation; reference 28), the ability to functionally suppress in vitro has been highly predictive of in vivo efficacy and presumed to be antigen non-specific. Yet, in this study, the islet autoantigen-specific BDC2.5  $T_{regs}$  were significantly more efficient than polyclonal NOD  $T_{regs}$  in regulating autoimmune responses in vivo. This discriminating activity was not predicted by the in vitro studies that demonstrated equal efficacy in blocking anti-CD3 responses among the various expanded  $T_{regs}$ . There are several potential explanations for this observation. First, it is possible that the expansion method causes selective depletion of the autoantigen-specific  $T_{regs}$  in the polyclonal NOD populations. This seems unlikely as the BDC2.5  $T_{regs}$  grew in vitro equally well when compared with the polyclonal NOD  $T_{regs}$ . Moreover, cursory analysis of the TCR usage in the expanded  $T_{regs}$  showed no preferential changes in the repertoire of the cells. Second, it is possible that the different expanded polyclonal  $T_{reg}$  populations homed or functioned differently in vivo. However, analysis of a panel of cell surface markers, intracellular proteins, and soluble cytokines suggested that the various cell populations all retained the essential properties of  $T_{regs}$  including the high expression of CD25 and FoxP3. Most likely, the differences observed reflected the model in which we have tested the  $T_{regs}$ . In many models,  $T_{regs}$  are cotransferred with effector cells into lymphoid cell-deficient animals, in which the  $T_{regs}$  home to the site of inflammation and inhibit  $T_{eff}$  response indirectly by competing for the “niche.” In fact, in one model of inflammatory bowel disease (37), CD25 $^-$  T cells are as effective as CD25 $^+$   $T_{regs}$  with little evidence for antigen specificity. Similarly, there has been little evidence supporting an essential role for antigen-specific cells for the treatment of graft-versus-host disease. In sharp contrast,  $T_{reg}$ -mediated immune regulation is routinely found to be antigen specific in the non-

lymphopenic setting (28, 48–50). Thus, the greater functional activity of the antigen-specific  $T_{\text{regs}}$  in the NOD models described herein is most likely due to the fully functional immune system in these animals and the requirement for effective antigen-mediated reactivation of  $T_{\text{regs}}$  at the inflammatory site.

It is important to note that in the absence of antigen-specific activation, the  $T_{\text{regs}}$  have no effect on regulating disease. This is most evident in the adoptive transfer studies using the  $T_{\text{regs}}$  isolated from Tg mice on the NOD background expressing a TCR specific for peptide epitope 286–300 (p286) of GAD65. Although lymphocytes from these TCR Tg mice proliferated and produced cytokines when stimulated *in vitro* with GAD65 peptide 286–300 (42), the response was weak and the T cells escaping deletion expressed alternative TCR- $\alpha$  chains that were unable to transfer diabetes. In fact, the only model where the GAD Tg T cells were functional was in an adoptive transfer system where the p286-tetramer<sup>+</sup> CD4<sup>+</sup> T cells from TCR Tg mice delayed diabetes induced in NOD.SCID mice by diabetic NOD spleen cells independent of CD25 expression, not unlike other systems where the transferred T cells suppress independent of CD25 expression or antigen-specific mechanisms (42).

Finally, the observation that the  $T_{\text{regs}}$  were able to reverse diabetes has important implications for clinical therapy. We imagine a scenario in a number of autoimmune settings where  $T_{\text{regs}}$  are isolated from patients either during remission (as would be the case for systemic lupus erythematosus or multiple sclerosis) or soon after disease onset (as would be the case for T1D). The cells would be expanded and reintroduced at the time of maximal disease activity to moderate the inflammatory response. In some cases this could be combined with rapamycin, anti-CD3, or other drugs that cause deletion of the pathogenic cells without affecting the  $T_{\text{regs}}$  (unpublished data). Together, these therapies could both reduce the short-term pathogenic responses while reinstating a homeostatic balance for long-term tolerance induction. Findings in this study suggest that the efficacy of  $T_{\text{reg}}$ -based immune therapy is critically dependent on the antigen specificity of the  $T_{\text{regs}}$ , at least in the autoimmune diabetes setting. Therefore, it is important to develop a procedure to selectively and reproducibly expand antigen-specific  $T_{\text{regs}}$  from polyclonal populations for therapeutic use. Many islet-specific T cell antigens have been identified to contribute to diabetes development in both mice and humans and MHC multimer coupled with these antigenic epitopes has been developed (43, 51–53). It is possible that these MHC multimer reagents can be adapted for expanding islet-specific  $T_{\text{regs}}$ . Current efforts are underway to identify antigen-specific  $T_{\text{regs}}$  in the polyclonal population and develop a protocol to selectively expand islet antigen-specific  $T_{\text{regs}}$  from normal NOD mice using immobilized antigenic peptide-linked MHC multimers. In addition, we are developing similar techniques for purifying and expanding human  $T_{\text{regs}}$  for clinical use in these settings.

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