Regulatory T Cells Prevent Transfer of Type 1 Diabetes in NOD Mice Only When Their Antigen Is Present In Vivo

Daniel R. Tonkin, Jing He, Gene Barbour and Kathryn Haskins

J Immunol 2008; 181:4516-4522; doi: 10.4049/jimmunol.181.7.4516
http://www.jimmunol.org/content/181/7/4516
Regulatory T Cells Prevent Transfer of Type 1 Diabetes in NOD Mice Only When Their Antigen Is Present In Vivo

Daniel R. Tonkin, Jing He, Gene Barbour, and Kathryn Haskins

Regulatory T cells (Tregs) have become a major research focus in autoimmune diseases like type 1 diabetes due to their ability to suppress pathogenic T cell responses, and they are being hotly pursued as tools for providing or augmenting immunosuppression in transplantation and autoimmunity in human patients. However, control of Treg function and persistence in sites of inflammation is complex and remains poorly understood. Of particular importance is the question of Ag dependence. For therapy in humans, a likely approach is to derive Tregs from the patient’s own T cells, a population that contains a broad range of Ag specificities with only small percentages specific for any given tissue. It is essential, therefore, to determine whether polyclonal Tregs will be effective in providing suppression of autoimmune responses or if it will be necessary to isolate Ag-specific Tregs. In this study, we assessed whether the presence of Ag is necessary for effective protection against adoptive transfer of diabetes in the NOD mouse model.

Originally identified as nonactivated CD4\(^{+}\)CD25\(^{+}\) T cells in the periphery (1, 2), “natural” Tregs are generally identified by expression of the Treg-associated transcription factor Foxp3 (3–5). Naturally occurring Tregs can be found in the periphery at levels of \(\sim 5\%–10\%\) of CD4 T cells (6), but it has also been shown that Foxp3-negative CD4 T cells in the periphery can acquire a Foxp3-positive, immunosuppressive Treg phenotype. These induced (or adaptive) Tregs can be generated in vitro by activating Foxp3-negative T cells in the presence of TGF-\(\beta\) (7, 8). A protective role for Tregs has been demonstrated in the NOD mouse model of autoimmune diabetes, in which both spontaneous disease and diabetes induced by adoptive transfer of pathogenic T cells can be suppressed by injection of either natural Tregs (9–12) or by TGF-\(\beta\)-induced Tregs (13).

Because Tregs were initially observed to be anergic to in vitro stimulation through the TCR, there was some question as to whether the TCR was even involved in Treg function. Subsequent studies revealed that Tregs were not completely anergic and could proliferate in vitro (14), and a requirement for Treg Ag in vitro was indicated in reports showing that Tregs obtained from TCR transgenic (TCR-Tg) mice suppressed T effector cells only in the presence of the Treg Ag (15, 16). In contrast, a recent report by Andersson et al. (17) demonstrated Ag-independent activation of Tregs in response to IL-2 alone, although this activation may be dependent on recent in vivo encounter with self-Ag.

The Treg requirement for Ag in vivo has been difficult to demonstrate conclusively. In the NOD mouse, Treg Ag dependence in vivo has been investigated largely through comparison of TCR-Tg Tregs with a dominant Ag specificity to polyclonal Tregs, obtained from the peripheral T cells of NOD mice, with a wide range of Ag specificities. In three separate studies comparing suppression of diabetes by islet Ag-specific Tregs derived from the BDC-2.5 TCR-Tg NOD mouse to that provided by polyclonal Tregs, the findings indicated that protection in vivo occurs only in the presence of the islet-specific BDC-2.5 TCR-Tg Tregs and not with polyclonal Tregs (12, 18, 19). In contrast to these reports, however, it has been demonstrated by others, and is also illustrated in our results, that transfer of polyclonal NOD Tregs can suppress diabetes (9, 11, 20).

We have used TGF-\(\beta\)-induced Tregs to investigate the suppression of diabetes with particular focus on the role of Treg Ag. By taking advantage of the difference in Ag specificity of a second pathogenic T cell clone in our panel, BDC-6.9, we were able to develop an adoptive transfer model to test in vivo the activity of Tregs either in the presence or the absence of Ag. Ag-specific Tregs were generated from the BDC-6.9 TCR-Tg/NOD mouse through nonspecific activation of the CD4 T cells with anti-CD3 in the presence of TGF-\(\beta\). In the BDC-6.9 TCR-Tg mouse, the T cells bear the TCR for an Ag present only in NOD islets and not
in islets from other mouse strains (21). Based on the restriction of the BDC-6.9 TCR reactivity to NOD islets, a gene controlling the Ag was mapped to a locus on chromosome 6 (22). A congenic mouse, the NOD.C6, was bred such that this locus was replaced with BALB/c DNA (21), and as a result T cells expressing the BDC-6.9 TCR cannot respond to NOD.C6 islets and cannot induce diabetes in the NOD.C6 mouse, which otherwise develops diabetes at a similar rate as NOD mice. By using the NOD.C6 mouse, we could test the ability of 6.9 TCR-Tg/NOD Tregs to protect against adoptive transfer of diabetes in the absence of their islet Ag. This study is the first to evaluate tissue-specific suppression by the same TCR-Tg Tregs in the presence and absence of Ag in vivo, and our results provide a clear demonstration of the in vivo Ag dependence of Tregs. We also report on Ag-dependent differences in Treg trafficking in vivo and on the suppression of Th1 effector cell cytokines in an Ag-deficient pancreas.

Materials and Methods

Mice

NOD and NOD-scid breeding mice were initially acquired from The Jackson Laboratory or the Barbara Davis Center for Childhood Diabetes (Denver, CO) and were housed in specific pathogen-free conditions at the University of Colorado Denver (UCD) Center for Laboratory Animal Care (CLAC). Experimental animals were monitored for development of disease by urine glucose (Diastix, Bayer) and hyperglycemia confirmed by One-Touch Ultra glucometer (LifeScan). Mice were considered diabetic when blood glucose levels were >15 mmol/l (270 mg/dl). The 2.5 TCR-Tg/NOD and 6.9 TCR-Tg/NOD mice were produced using TCR genes from diabetogenic T cell clones BDC-2.5 and BDC-6.9, respectively (21, 23). NOD congenic mice lacking the BDC-6.9 Ag (NOD.C6) were produced as previously described (21) and were crossed with 6.9 TCR-Tg/NOD mice to produce 6.9 TCR-Tg/NOD.C6 mice.

The 2.5 TCR-Tg/NOD mouse (hereafter referred to as 2.5 TCR-Tg) was used as a source of Th1 effector T cells. The T cells from the 2.5 TCR-Tg are different in their Ag specificity and can be distinguished from 6.9 TCR-Tg T cells by means of a clonotypic Ab (24) to the BDC-2.5 TCR. Because 6.9 TCR-Tg/NOD.C6 (hereafter referred to as 6.9 TCR-Tg) mice do not become diabetic and are therefore easier to maintain, the 6.9 TCR-Tg on the NOD.C6 background was routinely used as a source of CD4 T cells for Tregs instead of 6.9 TCR-Tg/NOD mice; moreover, Tregs derived from both strains were indistinguishable from one another. All procedures were in accordance with Institutional Animal Care and Use Committee guidelines and approved by the UCHSC Animal Care and Use Committee.

Induction of Th1 cells and Tregs

Th1 cells and induced Tregs were generated by a protocol similar to one previously described (13). CD4 T cells were purified from 2- to 3-mo-old NOD, 2.5 TCR-Tg, or 6.9 TCR-Tg mice by harvesting spleen and lymph nodes, followed by positive selection with magnetic anti-CD4 microbeads (Miltenyi Biotec). CD4 T cells were resuspended at 1 × 10^6 cells/ml in complete medium, which is DMEM supplemented with 44 mM sodium bicarbonate, 0.55 mM t-ariginine, 0.27 mM L-asparagine, 1.5 mM L-glutamine, 0.1 mM sodium pyruvate, 50 mM 2-ME, 10 mM HEPES, and 10% FCS. T cells were activated by placing 5 × 10^5 cells per well in 6-well tissue culture plates coated with 1 µg/ml anti-CD3 (BD Biosciences). For Th1 cells, 100 U/ml of recombinant human IL-2 (National Cancer Institute) was added to the media. For induction of Tregs, medium was supplemented with 100 U/ml IL-2, 3 ng/ml human TGFB-1 (PeproTech), and 2.5% anti-IFN-γ supernatant from the XMG1.2 hybridoma. Cells were incubated at 37°C for 3 days and then harvested. Foxp3 protein expression was evaluated with the anti-mouse Foxp3 staining set (eBioscience).

Analysis of IFN-γ production

For analysis of Treg-mediated suppression of IFN-γ produced by Th1 cells, IL-2-expanded Th1 cells or Tregs (4 × 10^6/ml) were cultured with 2 × 10^5 irradiated (4000 rads) spleen cells as APCs. Islet cells (4 × 10^5/ml) from NOD or NOD.C6 mice were used as Ag. After 24 h, the supernatant was harvested and assayed for IFN-γ by ELISA using paired Abs (BD Biosciences).

Intracellular cytokine staining

For intracellular cytokine staining, cytokine secretion was blocked with brefeldin A, (GoPiPlug, BD Biosciences) for 4–5 h at 37°C. Cells were then harvested, washed, and resuspended in staining buffer (0.5% BSA/0.05% PBST) containing GolgiPlug. For identification of T cells and macrophages, cells were then incubated for 30 min at 4°C with anti-FITC-conjugated anti-mouse CD4 (mAb GD 4517 The Journal of Immunology). Cells were fixed with 4% paraformaldehyde for 10 min and were washed in staining buffer containing 0.5% saponin to permeabilize the cells. All antibodies were used at the recommended concentration. CD4 T cells were labeled with CFSE before transfer into NOD or NOD.C6 male mice, age 6–10 wk.

Ex vivo analysis of pancreas-infiltrating T cells

For ex vivo analysis of Treg migration, cervical lymph nodes, pancreatic lymph nodes, spleens, and pancreata were harvested separately from each mouse. Lymph nodes and spleens were homogenized into single-cell suspensions. Pancreata were digested with 5 mg/ml collagenase (Sigma-Aldrich) at 37°C for 30 min with periodic vortexing, followed by mechanical disruption with glass homogenizers. CFSE-positive cells were then identified by flow cytometry.

For investigation of cytokine production ex vivo, pancreata were pooled for mice in each experimental group. After digestion and homogenization, cells from each group were divided in half and cultured in the presence of GolgiPlug for 4–5 h at 37°C with no stimulation or in wells containing anti-CD3 (5 µg/ml) and anti-CD28 (5 µg/ml). Cells were then harvested and stained for surface markers and intracellular cytokines.

Statistical analysis

Statistical significance of the effect of T cell treatments (Tregs, CD4 T cells) on diabetes transfer was determined by a Wilcoxon test of survival. Statistical significance of the effect of T cell treatments (Tregs, CD4 T cells) on diabetes transfer was determined by a Wilcoxon test of survival. Statistical significance of the effect of T cell treatments (Tregs, CD4 T cells) on diabetes transfer was determined by a Wilcoxon test of survival. Statistical significance of the effect of T cell treatments (Tregs, CD4 T cells) on diabetes transfer was determined by a Wilcoxon test of survival.

Results

Tregs induced from TCR-Tg NOD mice with TGF-β express Fox3 and produce little IFN-γ

It has been previously reported that T cells from the 2.5 TCR-Tg mouse can be induced to express Fox3, either through retroviral transduction (19) or in vitro culture with TGF-β (13). To confirm that the Tregs generated from CD4 T cells of the 6.9 TCR-Tg mouse expressed Fox3, we incubated CD4 T cells isolated from these mice either under Th1- or Treg-promoting conditions, as described in Materials and Methods. After 3 days, cells were harvested and Fox3 protein production was evaluated by intracellular staining. Our results indicated that <10% of T cells in the Th1 population expressed Fox3 (Fig. 1A), a level similar to what is observed with natural Tregs in unmanipulated populations.
Th1 T cells in vitro and to determine whether there was a require-
ment of anti-IFN-γ and TGF-β for induction of Tregs. Intracellular Foxp3 protein production was analyzed by flow cytometry. A. After harvest and a 3-day expansion with IL-2, 6.9 TCR-Tg Th1 cells and Tregs were activated with anti-CD3 (1 μg/ml) and cytokine production was measured by intracellular staining.

In cells that underwent Treg induction, however, Foxp3 was ex-
pressed in 80–95% of the T cells (Fig. 1A). Th1 cells and Tregs were activated with anti-CD3, and intracellular staining showed that IFN-γ production was greatly reduced in the Treg population (Fig. 1B). This robust induction of Foxp3, accompanied by reduc-
tion of IFN-γ, demonstrates that in vitro treatment of 6.9 TCR-Tg T cells with TGF-β leads to the induction of T cells with a Treg phenotype.

We also tested for production of two suppressive cytokines, TGF-β and IL-10. Although TGF-β production was increased in our Treg population (Fig. 1B), this effect was variable and in some assays was difficult to detect. We did observe low levels of IL-10 in the TGF-β-induced Tregs, but these levels were similar to those observed in the Th1 population (data not shown). While others have observed greater TGF-β and IL-10 production by Tregs in-
duced from 2.5 TCR-Tg/NOD mice (13), we have found that T cells from 6.9 TCR-Tg/NOD mice generally produce lower levels of cytokines. Although this difference between the two TCR-Tg mouse strains is not fully understood, it may account for the lower levels of IL-10 and TGF-β production by 6.9 TCR-Tg Tregs.

TGF-β-induced Tregs suppress in vitro IFN-γ production only in the presence of their cognate islet Ag

To demonstrate the ability of TGF-β-induced Tregs to suppress Th1 T cells in vitro and to determine whether there was a require-
ment for Treg Ag during this process, we tested the IFN-γ re-
ponse of Th1 T cells to Ag/APC, in the presence or absence of Tregs, in an assay using islets that were either positive or negative for the Treg Ag. For this experiment and subsequent experiments in this study, 2.5 TCR-Tg T cells were used as a source of Th1 cells. Although the Ags for 2.5 TCR-Tg T cells and 6.9 TCR-Tg T cells are undefined, the two clones respond to different epitopes. The 2.5 TCR-Tg Th1 T cells were placed in culture with islet cells and NOD APC, with or without TGF-β-induced Tregs from 6.9 TCR-Tg mice. Islet cells were prepared from either NOD mice (Ag-positive for 6.9 TCR-Tg) or NOD.C6 mice (Ag-negative for 6.9 TCR-Tg). Under these conditions, the prediction is that 2.5 TCR-Tg Th1 cells should respond to their Ag (which is present in both the NOD and the NOD.C6 islets), whereas the 6.9 TCR-Tg Tregs will recognize only the NOD islet cells as Ag. As shown in Fig. 2, measurements of IFN-γ in the supernatant indicated that 2.5 TCR-Tg Th1 cells were equally activated by both NOD and NOD.C6 islet cells. On the other hand, the 6.9 TCR-Tg Tregs effectively suppressed IFN-γ production when NOD islets were the source of Ag but did not inhibit such production when NOD.C6 islets were used. This result unequivocally establishes that in vitro suppression by TGF-β-induced Tregs is dependent on the presence of the Treg Ag.

TGF-β-induced Tregs suppress diabetes transfer in both immunodeficient and immunocompetent recipients

To demonstrate that TGF-β-induced Tregs obtained from the BDC-6.9 TCR-Tg mouse could effectively protect against adoptive transfer of diabetes in either immunodeficient NOD.scid recipients or in immunocompetent NOD mice, we performed experiments in which 6.9 TCR-Tg Tregs were cotransferred with 2.5 TCR-Tg Th1 T cells. As illustrated in Fig. 3A, the 2.5 TCR-Tg Th1 T cells rapidly transferred diabetes in NOD.scid recipients. In these mice, after cotransfer of 6.9 TCR-Tg Tregs with 2.5 TCR-Tg Th1 cells at a 5:1 ratio, diabetes was completely suppressed for up to 6 mo (p = 0.0008). Protection was also achieved for at least 3 mo when Tregs were used at a 2:1 ratio (data not shown). There was no protection, however, when 6.9 TCR-Tg CD4 T cells without TGF-β induction were used in cotransfers with the Th1 cells, indicat-
ing that coinjection of untreated transgenic T cells was not adequate to suppress disease.

Similar experiments were conducted in immunocompetent NOD mice, a more physiologically relevant model. In these recipients transfer was somewhat less consistent and greater numbers of 2.5 TCR-Tg Th1 cells (1 × 10⁷) were required to achieve rapid in-
duction of diabetes in most recipients (Fig. 3B). In cotransfers with 6.9 TCR-Tg Tregs (3 × 10⁴), diabetes was prevented in most of the young NOD mice (p = 0.004), demonstrating the ability of TGF-β-induced Tregs to effectively suppress diabetes in an immunocompetent animal.

**FIGURE 2.** TGF-β-induced Tregs suppress IFN-γ production by Th1 T cells in vitro only in the presence of Treg Ag. The 2.5 TCR-Tg Th1 cells or 6.9 TCR-Tg Tregs (1 × 10⁴) were activated with NOD or NOD.C6 islets (1 × 10⁴) as a source of Ag, and thioglycolate-elicited peritoneal exudate cells (5 × 10⁴) as APC, in 96-well plates. For co-cultures of Th1 cells and Tregs, 1 × 10⁵ cells of each T cell type were cultured together. After 24 h, IFN-γ in the supernatant was measured by ELISA. Error bars indicate SD from the mean of triplicate cultures, and data are representative of three experiments.
Tregs suppress cytokine production by pancreas-infiltrating effector T cells

In previous studies, we have demonstrated that upon adoptive transfer of diabetogenic Th1 T cell clones to NOD.scid mice, substantial amounts of inflammatory cytokines such as IFN-γ and TNF can be detected ex vivo in T cells isolated from the pancreas (25). To explore the mechanism by which Tregs protect against pathogenic T cells infiltrating the pancreas, we performed ex vivo analysis of Th1 T cells retrieved from the pancreas of NOD.scid recipients following adoptive transfers of 2.5 TCR-Tg Th1 T cells either alone or in the presence of 6.9 TCR-Tg Tregs. Six to 9 days following transfers, pancreata were harvested, digested with collagenase, and mechanically homogenized to isolate infiltrating leukocytes. Both Th1 cells and Tregs were observed within the pancreas, and we evaluated cytokine production of pancreas-infiltrating T cells by stimulating the cells immediately after harvest with plate-bound anti-CD3 Ab in the presence of GolgiPlug (brefeldin A) to prevent cytokine secretion. Intracellular cytokines were detected with fluorescent Abs by flow cytometry and the Th1 cell population was identified using a clonotypic Ab to the BDC-2.5 TCR. As illustrated in Fig. 4, we observed that the percentages of Th1 cells producing IFN-γ and TNF were reduced at all time points in mice that received cotransfers of Th1 T cells and Tregs. These results indicate that one effect of Tregs in the pancreas is a reduction in the percentage of Th1 cells producing inflammatory cytokines. In data not shown here, there was no decrease in numbers of pancreas-infiltrating Th1 cells until later time points, suggesting that the reduction in cytokine production is not due to Tregs preventing Th1 survival or migration to the pancreas. Rather, it appears that the Tregs suppressed the cytokine production of Th1 cells recruited to the pancreas, but it is not yet clear if this effect requires Treg activity in the pancreas, the draining lymph node, or both.

Protection by polyclonal Tregs is effective only with large numbers of Tregs

Because human Tregs must be derived or isolated from a polyclonal source, the efficacy of polyclonal Tregs is of particular therapeutic relevance, but reports of the suppressive capacity of polyclonal Tregs in NOD mice have been variable (9, 11–12, 18–20). We assessed whether Tregs with diverse TCR Ag specificities could suppress adoptive transfer of diabetes by carrying out cotransfers of TCR-Tg Th1 T cells with TGF-β-induced Tregs obtained from polyclonal populations of NOD CD4 T cells. We injected NOD.scid mice with 2:1 and 5:1 ratios of polyclonal NOD Tregs to 2.5 TCR-Tg Th1 T cells. Fig. 5 shows that at a 2:1 ratio, the polyclonal Tregs appeared completely unable to suppress, whereas at a 5:1 ratio polyclonal Tregs provided protection in about half of the recipients. Our results are consistent with the conclusion that polyclonal Tregs are protective only if sufficient numbers of Ag-specific Tregs are contained within the Treg population, and they may help to explain the variable results on efficacy of polyclonal Tregs reported in the literature.

TGF-β-induced Tregs fail to protect in vivo in the absence of their Ag

To evaluate the in vivo Ag dependence of TGF-β-induced Tregs during suppression of diabetes, we compared protection of Tregs obtained from 2.5 TCR-Tg or 6.9 TCR-Tg mice in NOD.C6 recipients in which the islet Ag for the BDC-2.5 TCR is present but is lacking for the BDC-6.9 TCR. As presented in Fig. 6, 2.5 TCR-Tg Th1 cells induced diabetes in most of the recipients, and in cotransfers with 2.5 TCR-Tg Tregs, transfer of disease was
completely suppressed ($p = 0.007$). In contrast, in the cotransfers of 2.5 TCR-Tg Th1 effectors and 6.9 TCR-Tg Tregs, there was no significant delay of diabetes ($p = 0.12$). As 6.9 TCR-Tg Tregs prevent diabetes induction in NOD mice but not in Ag-negative NOD.C6 mice, these results provide clear evidence that TGF-β-induced Tregs require in vivo antigenic stimulation to suppress diabetes.

Numbers of TGF-β-induced Tregs in the pancreas are greater in the presence of Treg Ag

It is not known how the in vivo behavior of Tregs is altered in the absence of Ag. To address this question, we labeled 6.9 TCR-Tg Tregs with CFSE before transfer into NOD and NOD.C6 recipients to compare Treg migration in the presence and absence of Ag. Four days after transfer, the pancreas, pancreatic lymph nodes, and distal lymph nodes were harvested from each mouse strain. Fig. 7 indicates that similar numbers of 6.9 TCR-Tg Tregs were present in distal lymph nodes and pancreatic lymph nodes of both NOD and NOD.C6 mice. Transferred cells in these lymphoid organs had undergone minimal proliferation. Similar results were observed in the spleen (data not shown). In contrast, in the pancreata of NOD.C6 mice the numbers of CFSE-positive 6.9 TCR-Tg Tregs were considerably smaller than those found in NOD pancreata.

Despite the lack of Ag in the NOD.C6 mice, the 6.9 TCR-Tg Tregs did undergo several rounds of proliferation in the pancreas of both NOD and NOD.C6 mice, possibly due to the presence of IL-2 produced by preexisting inflammation. It is clear, however, that in the pancreas the numbers of 6.9 TCR-Tg Tregs are markedly affected by the presence of Ag, whether due to reduced migration of Tregs or to decreased retention of Tregs in the Ag-deficient pancreas.

Suppression of effector Th1 T cells by TGF-β-induced Tregs occurs only in the presence of Treg Ag

In our results shown in Fig. 4, we observed that the percentages of Th1 cells producing IFN-γ and TNF were consistently reduced in NOD.scid mice that received cotransfers of Th1 T cells and Tregs, indicating that one mechanism by which Tregs protect is through suppression of cytokine production by pathogenic Th1 T cells in the pancreas. To identify Ag-dependent effects of in vivo suppression, we compared the suppression of cytokine production in 2.5 TCR-Tg Th1 cells by 6.9 TCR-Tg Tregs in the pancreas of Ag-positive NOD and Ag-negative NOD.C6 mice. The 2.5 TCR-Tg Th1 cells were injected into NOD and NOD.C6 mice alone or in the presence of 6.9 TCR-Tg Tregs. We harvested the pancreata on day 5 or 6 and measured cytokine production after in vitro stimulation with anti-CD3 and anti-CD28 Abs. As illustrated in Fig. 8, 6.9 TCR-Tg Tregs caused a modest decrease in TNF production by Th1 effectors in the NOD recipients. In the NOD.C6 recipients, however, there was little to no suppression of TNF, indicating that
Tregs require their Ag to inhibit TNF production in the pancreas. A similar effect was observed for IFN-γ, but these results were somewhat more variable (data not shown). Taken together, these results provide strong evidence that Tregs suppress cytokines by Th1 effectors only if the Treg Ag is present.

Discussion

We have reported herein on the protective activity of Tregs generated through in vitro culture of CD4 T cells from the BDC-6.9 TCR-Tg mouse, the second transgenic mouse produced from a diabetogenic TCR of a CD4 T cell clone from our BDC panel (26). TGF-β-induced Tregs from the BDC-2.5 TCR-Tg mouse were shown by Weber et al. to protect both against adoptive transfer by BDC-2.5 TCR-Tg Th1 effector T cells and development of spontaneous disease in NOD mice (13). In this study, we exploited the restricted islet Ag specificity of the BDC-6.9 T cells to investigate the Treg requirement for the presence of Ag in vivo. Our results demonstrate conclusively that suppression of pathogenic Th1 T cell responses by TGF-β-induced Tregs occurs in an Ag-dependent manner. While our studies have taken advantage of the largely clonal populations of T cells provided by TCR-Tg mice, our results indicate that through induction of Tregs with TGF-β, polyclonal populations of T cells from nontransgenic NOD mice can also protect when used in sufficiently high numbers. Of greater note, our data provide the first demonstration of how Tregs function in vivo in the presence or absence of the Treg Ag.

For suppression of diabetes by Tregs specific for islet cell Ag, the requirement for Ag was clearly shown in NOD.C6 mice that lack the Ag for the BDC-6.9 T cell clone. TGF-β-induced Tregs from the 6.9 TCR-Tg mouse could not suppress diabetes in these animals, were reduced in number in the pancreas after adoptive transfer, and did not alter cytokine production by transferred Th1 T cells. Although the mechanism by which TGF-β-induced Tregs function is not well understood, it is clear that the suppressive effects of Tregs are Ag-dependent. Additionally, our experiments with TGF-β-induced Tregs obtained from polyclonal NOD CD4 T cells provide some explanation for the inconsistent results obtained with polyclonal Tregs in other reports. We showed that polyclonal Tregs transferred into NOD.scid mice could protect at sufficiently high Treg/Th1 ratios (5:1), but were not effective at lower numbers (e.g., 2:1), suggesting that the proportion of Ag-specific Tregs in a polyclonal population dictates effective suppression of Th1 activity.

Our data also provide some explanation of how Tregs may operate in vivo, depending on whether their Ag is present in the inflammatory site. For example, our results demonstrate that accumulation of TGF-β-induced Tregs in the pancreas is largely dependent on the presence of Treg Ag in the pancreas. Although 6.9 TCR-Tg Tregs could be found in the Ag-negative NOD.C6 pancreas, their numbers were only about one-fourth of what was observed in an Ag-positive NOD pancreas, due either to decreased migration to the pancreas or to decreased retention of cells within the pancreas. The Tregs were transferred into recipients that were ~6 wk old, and pancreatic infiltration by host cells could already be observed in uninjected mice of this age. We hypothesize that Tregs migrated to the pancreas in response to chemokines produced by the infiltrating host cells. This scenario could also help to explain why Tregs were dividing only in the pancreas, and not in other tissues of both NOD and NOD.C6 recipients; we speculate that IL-2 produced by host T cells in the pancreas could be driving Ag-independent proliferation of Tregs at this site. Our results furthermore demonstrate that merely the presence of TGF-β-induced Tregs in an inflammatory site is not enough to trigger suppressive activity. Only through activation of the TCR in response to Ag can these Tregs suppress inflammation.

The in vivo requirement for Ag by TGF-β-induced Tregs indicates that use of Tregs for human therapy will likely require use of Ag-specific Tregs. Transfer of polyclonal Tregs would be the simplest approach, but a polyclonal population is likely to have limited effect on a specific organ. Additionally, it is possible that transfer of large numbers of polyclonal Tregs into a patient might result in undesired suppression of immunity toward pathogens and tumor Ags. It might be possible to isolate Ag-specific Tregs in humans by
a protocol similar to that demonstrated in wild-type NOD mice in which a tetramer specific for the BDC-2.5 TCR was used to isolate and expand islet-specific Tregs (27). However, production of peptide-MHC complexes that can be used to isolate and stimulate T cells specific for target Ags in every patient is unlikely to be feasible. A more practical approach may be to induce islet Ag-specific Tregs from a polyclonal population through in vitro culture with TGF-β. Although activation of TGF-β-induced Tregs with anti-CD3 Abs has been the most common approach, Tregs have also been generated from TCR-Tg mice in response to peptide Ag (8, 28). Another report has shown that allogeneic dendritic cells can be generated from TCR-Tg mice in response to peptide Ag (8, 28). CD3 Abs has been the most common approach, Tregs have also been generated from TCR-Tg mice in response to peptide Ag (8, 28). Another report has shown that allogeneic dendritic cells can be generated from TCR-Tg mice in response to peptide Ag (8, 28). This could be advantageous in therapy, because Tregs with specificity for one organ-specific Ag should be adequate to suppress T effector cells with a wide range of Ag specificities within that organ.

Disclosures
The authors have no financial conflicts of interest.

References