Cyclophosphamide-Induced Type-1 Diabetes in the NOD Mouse Is Associated with a Reduction of CD4+CD25+Foxp3+ Regulatory T Cells

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Regulatory T cells (Tregs) have been implicated as key players in immune tolerance as well as suppression of antitumor responses. The chemotherapeutic alkylating agent cyclophosphamide (CY) is widely used in the treatment of tumors and some autoimmune conditions. Although previous data has demonstrated that Tregs may be preferentially affected by CY, its relevance in promoting autoimmune conditions has not been addressed. The nonobese diabetic mouse spontaneously develops type-1 diabetes (T1D). We demonstrate in this study that CY targets CD4⁺CD25⁺Foxp3⁺ Tregs in vivo. CD4⁺CD25⁺ T cells isolated from CY-treated mice display reduced suppressive activity in vitro and increased expression of apoptotic markers. Although Treg numbers rapidly recovered to pretreatment levels in the peripheral lymphoid tissues, Tregs failed to recover proportionally within pancreatic infiltrates. T1D progression was effectively prevented by adoptive transfer of a small number of islet Ag-specific CD4⁺CD25⁺ Tregs to CY-treated recipients. Prevention of T1D was associated with reduced T cell activation and higher Treg proportions in the pancreas. We conclude that acceleration of T1D by CY is associated with a reduction in CD4⁺CD25⁺Foxp3⁺ Tregs and can be prevented by transfer of CD4⁺CD25⁺ Tregs. The Journal of Immunology, 2006, 177: 6603–6612.

Cyclophosphamide (CY) is a nitrogen mustard compound cytotoxic chemotherapeutic agent well-established in the treatment of chronic lymphocytic leukemias, lymphomas, and solid tumors, as well as an immunomodulatory agent. CY exhibits greatest cytotoxicity against cells actively replicating their DNA, as unpairing of DNA strands at this stage makes the nucleotide residues more susceptible to alkylation.

At high doses, CY prevents graft-vs-host disease during bone marrow transplantation and has sometimes been used as a disease-modifying agent in advanced, refractory rheumatoid diseases (1, 2), whereas, paradoxically, lower doses can lead to enhanced immune responses (3, 4) including those to tumors (5–7) and autoantigens (8–10).

Until recently, little was known about the molecular mechanism of CY-mediated immunomodulation. Studies in the 1980s showed that mice or rats given low-dose CY 2–3 days before sensitization displayed enhanced contact sensitivity or delayed-type hypersensitivity (11–13). Increased delayed-type hypersensitivity could be overridden by restoring the lymphoid compartment by administration of thymocytes after CY treatment (14). Administration of high-dose CY to prediabetic NOD mice leads to rapid synchronous onset of type-1 diabetes (TID) (8), and transfer of mononuclear cells from syngeneic nondiabetic donors after CY treatment protects from T1D onset (15). These observations led to the proposal that CY causes a breakdown of regulatory networks by removal of a suppressor population (3, 10, 14, 16), although the nature of this cell type remained elusive until recently. Even though CY is cytotoxic to a number of lymphoid populations, in particular B cells (15, 17, 18), recent data has focused on its selective effect on CD4⁺CD25⁺ Tregs (19–22).

CD4⁺CD25⁺ Tregs arise naturally from the thymus and represent a developmentally distinct T cell lineage that is characterized by a combination of surface markers including CD25, CD103, CTLA-4, CD62L, glucocorticoid-induced TNFR family related protein, as well as the expression of the X-linked forkhead/winged helix transcription factor (Foxp3) (23). Although several different kinds of regulatory T cells (Tregs) have been described, Foxp3⁺ Tregs have emerged as key players in the control of self-tolerance (24).

Selective effects of CY on CD4⁺CD25⁺ Tregs have been described in studies of tumor-bearing rats (22), mice (20), and humans (19) and can result in augmentation of antitumor responses. CD4⁺CD25⁺ Tregs display higher basal in vivo proliferation than CD4⁺CD25⁺ T cells and result in a greater sensitivity to the cytotoxic effects of CY (20). In addition, Tregs isolated from CY-treated mice exhibited increased levels of apoptosis and their ability to suppress CD8⁺ T cell proliferation in vitro was abrogated. These recent findings have pointed toward a role of CY in ablating CD4⁺CD25⁺ Tregs in vivo, although the relevance of this to precipitating autoimmune conditions remains unknown.

Although CY may affect adaptive Treg function, for example, Tr1- and Th3-type cells, we have focused in this study on its effect on naturally arising Tregs. In this study, we demonstrate that CY results in the synchronous onset of T1D in prediabetic NOD mice accompanied by a reduction in naturally occurring CD4⁺CD25⁺Foxp3⁺ Tregs. In addition, CD4⁺CD25⁺ Tregs isolated shortly after CY treatment were found to be functionally impaired in their in vitro suppressive activity and displayed higher levels of apoptosis. We find that whereas cell numbers recover in

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4 Abbreviations used in this paper: CY, cyclophosphamide; TID, type-1 diabetes; Foxp3, forkhead/winged helix transcription factor; Treg, regulatory T cell; PLN, pancreatic lymph node; 7-AAD, 7-aminoactinomycin D.

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lymphoid tissues immediately before onset of T1D, frequencies of Tregs in the pancreas remain low. The importance of Tregs in this model was further demonstrated by adoptive transfer of islet Ag-specific CD4\(^+\)CD25\(^+\) Tregs from TCR-transgenic BDC2.5NOD mice which increased the frequency of pancreatic Tregs and suppressed T1D.

**Materials and Methods**

**Mice**

NOD mice, transgenic GFP.NOD mice, BDC2.5.NOD TCR transgenic mice, and GFP.BDC2.5NOD TCR transgenic mice were housed and bred under specific pathogen-free conditions in the Pathology Department, University of Cambridge animal facilities (Cambridge, U.K.). GFP.BDC2.5.NOD mice were generated by crossing BDC2.5NOD mice and GFP.NOD mice, which was a gift from Dr. D. Kioussis (National Institute of Medical Research, London, U.K.). GFP protein expression is under the control of the CD2 promoter. C57BL/6 mice were purchased from Harlan, U.K. All animal work was conducted under U.K. Home Office project license regulations after approval by the Ethical Review Committee of the University of Cambridge.

**Cyclophosphamide**

CY (Endoxana) was obtained from Baxter Healthcare and prepared in 0.9% normal saline at 20 mg/ml immediately before i.p. administration.

**T cell purification**

For adoptive transfer and in vitro suppression assays, CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\) T cells were extracted from mice using the mouse CD4\(^+\)CD25\(^+\) magnetic cell separation kit (Miltenyi Biotec) according to the manufacturer’s instructions. Cells were checked for purity by flow cytometry. Approximately 80% of CD4\(^+\)CD25\(^+\) T cells stayed positive for Foxp3, whereas purified CD4\(^+\)CD25\(^-\) cells contained <1% Foxp3\(^+\) cells.

**Isolation of pancreatic-infiltrating leukocytes**

Whole pancreas samples were harvested and processed from individual mice. Briefly, pancreases were torn into smaller pieces in cold PBS containing 5% FCS, 56 mM glucose, and Complete Mini Protease inhibitors (Roche). The tissues were washed twice in cold PBS, before incubation in 2 ml of prewarmed PBS containing 15% FCS and Liberase CI (Boehringer Mannheim). After digestion, tissues were washed and cell suspensions were prepared by forcing through a cell strainer. Suspensions were twice left to settle and the supernatants were decanted to remove stromal debris, before the cells were washed ready for use.

**In vitro suppression assays**

For analysis of the effects of CY on Treg cell function, CD4\(^+\)CD25\(^+\) T cells were isolated from the pooled spleens of 10-wk-old male NOD mice treated as described above. CD4\(^+\)CD25\(^-\) cells were labeled by incubation in PBS with 5 \(\mu\)M CFSE (Molecular Probes) for 20 min at 37°C. Cells were then incubated in triplicate wells on a 96-well plate at 1 \(\times\) 10\(^4\) wells with the indicated ratios of unlabeled CD4\(^+\)CD25\(^+\) cells in the presence of 1 \(\mu\)g/ml anti-CD3 mAb and 5 \(\times 10^5\) irradiated splenocytes from male NOD mice. Proliferation of CD4\(^+\)CD25\(^+\) cells was assessed after 72 h by CFSE dilution. Alternatively, proliferation was assessed by incorporation of \(^3\)HThymidine. A total of 1 \(\mu\)Ci \(^3\)HThymidine (Amersham Pharmacia Biotech) was added during the last 12 h of culture and incorporation detected using a beta-scinillation counter (Wallac).

**Adoptive T cell transfers**

Purified populations of CD4\(^+\)CD25\(^+\) and CD4\(^+\)CD25\(^-\) T cells were prepared as described using magnetic sorting. For the analysis of the effects of polyclonal NOD Tregs in vivo, 2 \(\times 10^6\) purified CD4\(^+\)CD25\(^+\) cells, or CD4\(^+\)CD25\(^-\) cells were transferred i.v. into 10-wk-old male NOD mice given 200 mg/kg 24 h previously. For the analysis of the effects of islet Ag-specific BDC2.5NOD or GFP.BDC2.5NOD Tregs, CY-treated NOD mice received from 1 \(\times 10^5\) to 2 \(\times 10^6\) purified T cells i.v. at the indicated time point after CY administration. All recipient mice were then studied for T1D incidence, or sacrificed for analysis of the distribution of GFP cells.

**Flow cytometric analysis**

Single-cell suspensions were prepared from various organs as described above. Cells were washed and resuspended in staining buffer (PBS, 1% FCS, and 0.05% NaN\(_3\)) before staining with appropriate combinations of FITC, PE, PerCP, or allophycocyanin conjugates (all obtained from BD Pharmingen) of Abs to the following cell surface markers (hybridoma clone indicated in parentheses): CD3 (2C11), CD4 (L3T4), CD8a (53–6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD25 (PC61), CD62L (MEL-14), and CD44 (IM7). Appropriate isotype control Abs were included.

Intracellular Foxp3 staining was performed according to the manufacturer’s instructions (Foxp3 anti-mouse/rat Foxp3 staining set; eBioscience). For visualization of intracellular GFP together with Foxp3, the fix/perm step was shortened to 1 h.

Cells were acquired using a BD LSR I flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star) software.

**Apoptosis**

Staining for apoptosis was conducted on single-cell suspensions of spleen and pancreatic lymph node (PLN) cells. Briefly, single-cell suspensions were prepared in complete medium and cells were washed in binding buffer (10 mM Hepes/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl\(_2\)). A total of 1 \(\times 10^6\) cells were incubated for 15 min at room temperature with Annexin V\(^\text{FITC}\) (1/20 dilution) (BD Pharmingen), anti-CD4-allophycocyanin mAb, anti-CD25-PE mAb. Cells were washed and resuspended in 100 \(\mu\)l of binding buffer in the presence of 1 \(\mu\)l of 7-aminoactinomycin D (7-AAD) to exclude dead cells.

**Statistics**

Appropriate statistical tests were performed on all data as described in the figure legends using GraphPad Prism version 4.0 software (GraphPad).

**Results**

**Differential effects of CY on lymphoid populations in the NOD mouse**

T1D is an autoimmune disease characterized by specific destruction of the insulin-producing \(\beta\) cells of the pancreatic islets of Langerhans. The NOD mouse provides a spontaneous model for T1D, in which \(\sim 80\%\) of females and \(20\%\) of males develop hyperglycemia by 30 wk of age. Previous studies have established that 40–60% of 10-wk-old male NOD mice, given a single dose of CY, synchronously progress to T1D within 14 days (8). To examine the effects of CY during this critical time, 10-wk-old male NOD mice were given CY (200 mg/kg) and sacrificed 2, 6, and 9 days later for comparison with untreated littermate controls. Lymphocytes were extracted from spleen and PLN as well as the pancreas and analyzed by flow cytometry.

In keeping with previous studies (15, 20, 21), CY treatment caused a significant drop in the number of splenocytes (50%) on day 2 (Fig. 1A). Splenic cellularity remained low on day 6 and then returned or exceeded pretreatment levels on day 9. Similar changes were observed in the PLN (data not shown). CY treatment also resulted in changes in specific cell populations, sharply decreasing CD19\(^+\) B cells and, to a lesser extent, CD3\(^+\)CD4\(^-\) T cells and CD3\(^+\)CD8\(^-\) T cells. As a consequence of these differential effects, the percentage of CD4\(^+\) and CD8\(^-\) T cells was increased (Fig. 1B), whereas the CD4:CD8 ratio remained largely unaltered in all tissues (Fig. 1C). Comparable relative changes were observed in the PLN and pancreas (Fig. 1D).

**CY treatment causes loss of CD4\(^+\)CD25\(^-\)Foxp3\(^+\) Tregs**

Further analysis of the CD4\(^+\) T cell population indicated a sharp drop in the number of CD4\(^+\)CD25\(^+\) T cells 48 h after CY administration (Fig. 2A). This decline was most marked in the pancreas, where levels of CD4\(^+\)CD25\(^+\) T cells dropped from 22.5 (\(\pm 1.16\%\)) to 3.3% (\(\pm 0.1\%\)) corresponding to an overall loss of 85%. However, CD25 is not a unique marker for Tregs (23), but is also found on activated CD4\(^+\) T cells. Thus, although the ablation of CD4\(^+\)CD25\(^+\) T cells suggests a loss of Treg cells, it might equally represent an initial loss of activated T cells that also express CD25.

To confirm the true nature of the affected CD4\(^+\)CD25\(^+\) T cell population, we performed intracellular staining with an Ab against the natural Treg-specific marker Foxp3 (Fig. 2B). Treatment with
FIGURE 1. CY induced changes in cellular composition of peripheral lymphoid organs. Nondiabetic 10-wk-old male NOD mice were given CY (200 mg/kg) i.p. and sacrificed 2, 6, and 9 days after administration and their cellular composition was compared with untreated controls using flow cytometry. A. Total cell counts of splenic lymphocytes for individual mice. Splenocytes from controls (n = 3/day) were analyzed on days 2, 6, and 9 and are represented as a pooled result. B. Percentages of splenocytes staining for CD19+, CD3+CD4+, and CD3+CD8+. Data points represent arithmetic mean and SEM for combined analysis of individual mice (n = 3). C. Analysis of the CD8+:CD4+ ratio and the CD19+:CD4+ ratio (D) was performed in spleen, PLN, and pancreas and are represented as arithmetic means and SEM derived from individual mice (n = 4). Significant differences (p ≤ 0.03, two-sided Mann-Whitney U) from controls are highlighted (+).

CY was found markedly to decrease the numbers of CD4+Foxp3+ T cells on day 2 in the spleen, PLN, and most prominently in the pancreas. Foxp3+ T cells were found to be mostly, but not exclusively, CD25+ (data not shown) and recovered to above pretreatment levels in lymphoid organs by day 9. In contrast to CD4+CD25+ cells, CD4+Foxp3+ T cells failed to recover in the pancreas. Taken together, this indicates the loss of Tregs and simultaneous emergence of primed autoreactive CD4+CD25− T cells within the pancreas, at a time point immediately before T1D onset.

CD4−CD25+Foxp3+ Tregs from NOD mice are not inherently more susceptible to CY-mediated cytotoxicity

The high incidence of T1D in NOD mice after administration of CY (8) is in stark contrast to other mouse strains (20, 21). Therefore, we investigated whether Foxp3+ Tregs from NOD mice are inherently more susceptible to depletion by CY. Age-matched NOD mice and nonautoimmune prone C57BL/6 mice were given CY and analyzed for changes in the percentage of CD4+Foxp3+ T cells. Only lymphoid tissues were compared due to the absence of lymphocytic infiltrates in the pancreas of C57BL/6 mice. Percentages of CD4+Foxp3+ T cells in NOD mice were lower than in the C57BL/6 strain, but both strains displayed a significant drop of the CD4+Foxp3+ T cell subset after administration of CY (Fig. 2C). Normalization of the data showed that both T cell populations are equally affected by CY (Fig. 2D) with more pronounced depletion of CD4+Foxp3+ T cells in the lymph nodes.

CD4−CD25+ Tregs from CY-treated NOD mice display reduced in vitro suppressive activity and increased apoptosis

Previous studies in C57BL/6 (20) and BALB/c mice (21) have shown that CY not only ablates CD4+CD25+ T cells in vivo but can also abrogate their suppressive function in vitro. To verify whether NOD Tregs from CY-treated animals showed a comparable functional impairment, CD4+CD25+ T cells were isolated 48 h after CY dosing and compared with CD4+CD25+ Treg cells from untreated littermate controls. Analysis of the proportions of apoptotic cells stained by annexin V (Fig. 3A) before and after CY treatment showed a significantly greater percentage (11.19 ± 0.83 vs 5.5 ± 0.66%) of apoptotic CD4+CD25+ T cells than CD4+CD25− T cells in the CY-treated group, indicating their greater susceptibility to the drug. CD4+ T cells from untreated controls did not show any significant apoptotic staining (1.04 ± 0.34 vs 1.56 ± 0.54%). Similar findings were made for CD4+CD25− T cells isolated from PLN (6.06 ± 2.92 vs 1.82 ± 1.17%). When used in an in vitro suppression assay, splenic NOD CD4+CD25+ T cells from CY-treated mice were impaired in their ability to suppress the proliferation of CD4+CD25+ T cells from untreated control mice (Fig. 3B). The lack of in vitro suppression was not directly associated with a reduction in the protein expression levels of Foxp3 on a per-cell basis (Fig. 3C).

Adaptive transfer of NOD CD4+CD25+ Tregs suppresses CY-induced T1D

The above results show that onset of T1D in mice given CY is accompanied by a significant drop in the CD4+Foxp3+ Treg population that fails to recover proportionally in the pancreas. Moreover, CD4+CD25+ T cells from CY-treated NOD mice are diminished in their in vitro suppressive activity. We argue that the proportional lack and functional impairment of Tregs after CY treatment might permit progression to T1D. A previous study showed that transfer of a large number of mononuclear cells (5 × 10^7 over five consecutive days) from nondiabetic syngeneic donors protected CY-treated recipients from T1D onset (15). However, these studies did not identify the cell population responsible for this protection. Therefore, we assessed the ability of purified CD4+CD25− Treg cells from age-matched nondiabetic NOD donors to protect CY-treated recipients when given 24 h after CY administration. NOD Tregs were purified based on their expression of CD25 and subsequent analysis showed that ~70% of the cells expressed Foxp3 (data not shown). A 24-h time point for adoptive transfers was chosen based on the biological half-life of CY (~30 min) to avoid its toxic effects on the transferred Tregs (25). Fig. 4A illustrates that transfer of 2 × 10^7 NOD CD4+CD25− Treg cells reduced the T1D incidence in CY-treated recipient mice, whereas the incidence of T1D in littermates receiving the equivalent number of NOD CD4+CD25− T cells was not altered. Although NOD
CD4+CD25+ Tregs consistently lowered T1D incidence in repeat experiments, we noted considerable interexperiment variability, possibly as a result of a low frequency of islet-specific Tregs within the transferred cell population.

Islet Ag-specific CD4+CD25+ Treg cells are highly effective in suppressing CY-accelerated T1D

We next examined whether islet Ag-specific Tregs were more effective in suppressing T1D than polyclonal NOD Tregs. CD4+CD25+ Tregs from TCR transgenic BDC2.5.NOD mice express a TCR specific for an as yet unknown pancreatic islet Ag (26, 27). These cells have been shown to be highly effective in prevention, or even reversal of spontaneous T1D onset in NOD mice (28, 29).

Fig. 4B indicates that adoptive transfer of 1 × 10^6 BDC2.5, NOD CD4+CD25+ T cells completely prevented the onset of CY-induced T1D. Administration of as few as 1 × 10^5 Tregs reduced T1D incidence to 8.3% compared with 91% (p = 0.0001) in control recipients (Fig. 4C). In contrast, transfer of purified naive BDC2.5 NOD CD4+CD25− T cells from the same donor mice
FIGURE 3. Effects of CY on the in vitro suppressor function and apoptosis of NOD CD4\(^+\)CD25\(^+\) Tregs. A, Staining for apoptosis in CD4\(^+\) T cells. NOD mice were treated with 200 mg/kg CY i.p. and lymphocytes prepared from spleen and PLN after 48 h for comparison with untreated littermate controls. Whole lymphocytes were stained with Annexin V\(^{FITC}\) as described together with CD4 and CD25 surface markers and analyzed by flow cytometry. Cells were initially gated on forward scatter (FSC)/side scatter (SSC) and CD4 expression, excluding all cells staining positive for 7-AAD. A representative FACS dot plot shows the individual gates selecting CD4\(^+\)CD25\(^+\) and CD4\(^+\)CD25\(^-\) cells. The percentage of annexin V\(^+\) cells was determined as shown in the histogram overlays. Graphs represent the arithmetic means and values for each individual mouse for the percentage of annexin V\(^+\) cells. Indicated \(p\) values for significance were calculated by two-sided Mann-Whitney \(U\) test. B, In vitro CD4\(^+\)CD25\(^+\) Treg suppression assay. CD4\(^+\)CD25\(^-\) T cells were prepared from the pooled spleens of 10-wk-old male NOD mice previously (48 h) treated with 200 mg/kg CY i.p. (\(n=6\)) or their untreated littermate controls (\(n=3\)). These cells were cultured in the presence of 1 \(\times\) 10\(^5\) CFSE-labeled CD4\(^+\)CD25\(^-\) T cells (Teff) prepared from the spleens of the untreated control mice, at the indicated ratios. A total of 5 \(\times\) 10\(^5\) T cell depleted irradiated male NOD splenocytes were added to the cultures, along with 1 \(\mu\)g/ml anti-CD3 mAb. Proliferation of CD4\(^+\)CD25\(^-\) T cells was assessed 72 h later by CFSE dilution and by incorporation of \(^{3}\)H\)thymidine over the last 12 h of culture. Data points show mean percentage of cells showing CFSE dilution or thymidine incorporation counts \(\pm\) SEM for triplicate wells. Controls included Teff cells cultured without irradiated splenocytes (No APC) and Teff cells cultured without Tregs (0:1 ratio). *, Significant differences. Data are representative of two repeat experiments. C, Per-cell Foxp3 protein expression in CD4\(^+\)CD25\(^+\) T cells from CY-treated and untreated NOD mice. NOD mice were treated with 200 mg/kg CY i.p. and splenocytes were isolated after 48 h for comparison with untreated littermate controls. Cells were analyzed by flow cytometry gating on the CD4\(^+\)Foxp3\(^+\) population. Data shows representative FACS dot plots from both groups and the graph with the geometric mean fluorescence intensity (GMFI) representing individual data points for each mouse and their arithmetic mean.
augmented T1D incidence without significantly affecting the kinetics of T1D development.

Effectiveness of T1D suppression depends on the time between CY administration and transfer of Tregs

To establish the time frame in which effective protection after CY treatment is obtained, we assessed suppression of T1D by BDC2.5.NOD CD4\(^+\)CD25\(^+\) T cells administered at 1, 5, or 8 days after CY dosing. Fig. 5 demonstrates that 2.5 \times 10^5 BDC2.5.NOD Tregs completely suppressed T1D (p = 0.0002) when given 24 h after CY dosing. Adoptive transfer 5 days after CY treatment (Fig. 5B) was still effective (p = 0.0013) in reducing T1D incidence (91 vs 41%) without affecting the kinetics of disease onset. Later transfers of Tregs 8 days after CY dosing failed to significantly affect progression to T1D (Fig. 5C).

Adoptive transfer of islet Ag-specific BDC2.5.NOD Tregs results in their accumulation and long-term survival within pancreatic islet infiltrates

The above data suggested that CY treatment induces an imbalance between resident CD4\(^+\)CD25\(^+\) T cells and naturally occurring CD4\(^+\)CD25\(^+\) Tregs, which ultimately leads to priming of autoreactive T cells and progression to T1D. To further clarify how BDC2.5.NOD Tregs control progression of CY-mediated T1D, we studied the distribution of transferred CD4\(^+\)Foxp3\(^+\) T cells and changes in the activation status of endogenous CD4\(^+\) T cells by using GFP-labeled CD4\(^+\)CD25\(^+\) Tregs prepared from GFP.BDC2.5.NOD donor mice. Spleen, PLN, and pancreases were analyzed on days 2, 6, and 9 after CY dosing. One group of CY-treated mice was given 2 \times 10^6 GFP-labeled CD4\(^+\)CD25\(^+\) Treg cells (CY plus Treg), whereas the other group served as controls receiving CY only (CY). Interestingly, there were no differences seen between the two groups at any time point in the spleen and PLN in terms of the proportions of CD4\(^+\)Foxp3\(^+\) T cells and CD4\(^+\)CD62L\(^{hi}\) cells (data not shown). In contrast, lymphocytes isolated from the pancreas showed that, in keeping with earlier data (Fig. 2B), the diminished proportion of pancreatic CD4\(^+\)Foxp3\(^+\) T cells failed to recover by day 9 in the CY group (Fig. 6A). In the CY plus Treg group, there was a significant increase in the proportion of CD4\(^+\)Foxp3\(^+\) T cells (Fig. 6A; 34.5 ± 3.9 vs 16.1 ± 1.4%). This selective increase of Tregs was mirrored by a lower proportion of activated CD4\(^+\) T cells on day 9 (Fig. 6B) and directly correlated with a high proportion of GFP\(^+\)CD4\(^+\) T cells in the pancreas (Fig. 6C).

To evaluate the long-term fate of adoptively transferred Tregs in protected NOD mice, we analyzed the proportion of CD4\(^+\)Foxp3\(^+\) T cells in the subset of control mice remaining nondiabetic 1 mo after CY treatment and compared these to CY-treated mice which had received 2 \times 10^6 GFP-labeled CD4\(^+\)CD25\(^+\) Tregs from GFP.BDC2.5.NOD donor mice. Mice treated with CY alone
showed recovered levels of CD4+Foxp3+ T cells equivalent to those seen in untreated control mice (Fig. 7A). However, the CY-Treg group continued to display a significant increase (p = 0.002) in the percentage of CD4+Foxp3+ Treg in the pancreas. Indeed, adoptively transferred Foxp3+GFP+ Tregs could still be detected in spleen, PLN, and the pancreas, but only in the pancreas did they significantly (19 ± 4.1%) contribute to the overall CD4+Foxp3+ Treg population (Fig. 7B). This data supports the idea that Ag-specific Tregs can survive long-term.

Discussion
The paradoxical ability of CY to suppress and enhance immune responses is a long-standing observation. Administration of high-dose CY is usually immunosuppressive and has been used therapeutically in a wide variety of clinical situations. In contrast, administration of a lower dose has been associated with enhanced immune responses which were thought to arise by selective targeting of a suppressor cell population (14). Initial work linking CY with dysregulated T cell responses demonstrated an increase in the immune response in a mouse model of delayed-type hypersensitivity if CY was given before the Ag challenge. Transfer of thymocytes after CY treatment reversed the increase indicating that the anti-suppressor effects of CY are mediated through T cells.

Following on from these reports, Harada and Makino (8) proposed that the CY-mediated acceleration of T1D in the NOD mouse is mediated by targeted effects on a suppressor T cell population. The hypothesis that CY selectively ablates suppressor cell activity was also consistent with data from adoptive transfer models where splenocytes from CY-treated NOD mice transferred T1D to irradiated recipients, whereas splenocytes from untreated control mice failed to transfer disease (10). Charlton et al. (15) subsequently demonstrated that acceleration of T1D in NOD mice could be prevented by administration of large numbers of mononuclear cells, although these studies did not identify the protective cell population.

The recent renaissance of suppressor T cells as the more clearly defined CD4+CD25+ or CD4+Foxp3+ Treg population has helped to unravel the role CY in enhancing immune responses.
However, no study to date has examined the role of this Treg population in controlling CY-induced autoimmune responses. Previous work has suggested that onset of T1D in NOD mice is due to a general deficiency of Tregs (30, 31). There are also indications that NOD Tregs are functionally impaired, requiring higher cell numbers to suppress ongoing disease than in other autoimmune models (32, 33). Functional evidence for the role of Tregs in preventing T1D has been provided by cell transfer models. Adoptive transfer of isolated islet-infiltrating cells into NOD.scid recipient mice rapidly precipitates T1D, whereas cotransfer with NOD CD4$^{+}$CD25$^{+}$ T cells is protective (31). Treatment of NOD mice with neutralizing anti-IL-2 mAb triggers early onset of T1D and a wide spectrum of other T cell-mediated autoimmune diseases (34). Similar to our observations with CY, this neutralization of IL-2 selectively reduces the number of Foxp3-expressing CD4$^{+}$CD25$^{+}$ T cells, but not CD4$^{+}$CD25$^{-}$ T cells.

In rats, mice, and humans, CY treatment is associated with enhanced immune responses suggestive of a direct effect on CD4$^{+}$CD25$^{+}$ Tregs (19, 35, 36). Our initial studies in NOD mice confirmed these findings showing that CY preferentially depletes CD4$^{+}$CD25$^{+}$ T cells. By using Foxp3 as a more selective marker for naturally occurring Tregs, we showed that the effects of CY on CD4$^{+}$Foxp3$^{+}$ cells mirror those seen on CD4$^{+}$CD25$^{+}$ T cells providing further support for the argument that CY preferentially affects Tregs.

To establish whether acceleration of T1D was associated with a mouse strain-specific increase in the sensitivity to CY-mediated cytotoxicity, we compared the effects of CY in nonautoimmune C57BL/6 mice. Our data indicate that NOD mice are inherently no more susceptible to CY. As previously shown in nonautoimmune-prone mouse strains (20, 21), we found that CD4$^{+}$CD25$^{+}$ Tregs from CY-treated NOD mice displayed reduced in vitro suppressive activity and showed higher proportions of apoptotic cells compared with CD4$^{+}$CD25$^{-}$ T cells from the same mice. Lutsiak et al. (20) proposed that this greater susceptibility to apoptosis may arise from the higher level of physiological proliferation in response to normal self-Ags. A link between increased apoptosis and T1D induction has also been shown for the CD62L$^{high}$ T cell compartment of NOD mice which is preferentially affected by CY. It is likely that the CD62L$^{high}$ compartment corresponds to CD4$^{+}$Foxp3$^{+}$ Tregs. Blockade of the FasL-Fas interaction by administration of Fc-Fas fusion protein to neutralize FasL has been shown to be sufficient to prevent T1D onset by CY and is linked to reduced apoptosis of CD62L$^{high}$ cells (37). Furthermore, the loss of CD62L$^{high}$ or CD4$^{+}$CD25$^{+}$ T cells from PLN can be prevented by administration of G-CSF and has been associated with
The effectiveness of Ag-specific Tregs has been recently highlighted and shown to provide a means for an effective therapeutic approach (28, 29). We show here that CD4⁺CD25⁺ T cells from TCR-transgenic BDC2.5.NOD mice were highly effective in suppression of T1D when given 24 h after CY treatment. As few as 1 × 10⁴ BDC2.5.NOD Treg cells were sufficient to prevent CY-mediated T1D. Tang et al. (47) have argued that BDC2.5.NOD Tregs exert their regulatory function by interaction with dendritic cells in the PLN which subsequently prevents the priming of naive autoreactive T cells. To assess in vivo changes of CY-treated mice that accompanied injection of BDC2.5.NOD Tregs, we examined lymphoid tissues and pancreases of NOD recipients for T cell activation markers in the presence or absence of GFP-labeled BDC2.5.NOD CD4⁺CD25⁺ Tregs. Analysis of lymphoid tissues 6 days after CY treatment suggests that all affected lymphocyte activation marker levels remain low. Priming of autoreactive T cells may not have occurred to any significant extent in the PLN before this time point, which would explain why the transfer of BDC2.5.NOD Tregs 5 days after CY treatment is still able to suppress T1D. The lack of protection by adoptive transfer of BDC2.5.NOD Tregs 8 days after CY administration supports the notion that suppression of priming of autoreactive T cells is necessary to prevent infiltration of pancreatic islets by activated autoreactive T cells. In contrast, spontaneous T1D onset can be reversed when BDC2.5.NOD Tregs are given up to 1 wk after the first signs of glycosuria (28). Our studies indicate that in the CY-accelerated model of T1D BDC2.5.NOD Tregs are only able to control disease if given before diabetes onset. Interestingly, analysis of pancreases from protected mice suggests that although autoreactive T cells are still present within pancreatic infiltrates they may be held in check by the greater proportion of CD4⁺CD25⁺CD45RA⁻Foxp3⁺ T cells. The substantial proportion of GFP⁺ Tregs within the pancreatic Treg pool 1 mo after CY administration also implies their long-term survival. It remains to be established how resident Tregs mediate their inhibitory function in situ. In this context, it is interesting to note that in vitro-conditioned BDC2.5 Tregs control pancreatic autoreactive T cells by deletion through Fas-FasL interaction (48).

In conclusion, we have shown that CY-induced T1D is associated with a selective apoptotic loss of CD4⁺CD25⁺ Treg cells from peripheral lymphoid tissues and the pancreas. Similarly, it has recently been reported that fludarabine, a cytotoxic purine analog, used in treatment of chronic B cell lymphocytic leukemia, also reduces the number and functional activity of circulating Tregs in human patients (19). This raises interesting parallels to the effects reported here with CY. Furthermore, we have shown that the subsequently emerging autoreactive polyclonal T cell response associated with CY administration is effectively controlled by transfer of a low number of islet Ag-specific BDC2.5.NOD CD4⁺CD25⁺ T cells. This control may be exerted not only in the PLN as proposed by Tang et al. (47) but also in the pancreas where we could observe the predominant localization of the transferred Tregs as suggested by the studies of Chen et al. (49). We believe that the effectiveness of T1D suppression after CY treatment by a very small number of islet Ag-specific Tregs depends on the additional ablation of activated autoreactive T cells. These studies further highlight the potential of Tregs as a therapeutic means in controlling autoimmune disease.

**FIGURE 7.** Transfer of GFP⁺CD4⁺CD25⁺ Tregs in CY-treated NOD mice results in long-term survival and restoration of the pancreatic CD4⁺Foxp3⁺ Treg proportions to pretreatment conditions. Nondiabetic 10-wk-old male NOD mice were injected i.p. with CY (250 mg/kg). After 24 h, one group (CY + Treg) received 1 × 10⁶ purified CD4⁺CD25⁺ T cells from spleens of age-matched GFP.BDC2.5.NOD donors. A control group (CY) did not receive any cells. Incidence of diabetes was determined by testing for glycosuria. After 30 days, single-cell suspensions were prepared from spleen, PLN, and pancreas of all nondiabetic animals, CY-treated mice (CY), and protected mice (CY + Treg) that were transferred with GFP.BDC2.5.NOD Treg cells. Graphs show the proportion of CD4⁺Foxp3⁺ T cells within the pancreatic T cell population for IL-2 (41– 43). It seems plausible that the rapid loss of islet-specific Tregs may result from a lack of survival factors (40). Although Tregs isolated from nondiabetic NOD mice could inhibit CY-induced T1D, protection was more significant using Ag-specific Tregs. In support of this, others have shown that transfer of polyclonal CD4⁺CD25⁺ Tregs is inefficient and requires infusion of high cell numbers (30, 46). It is now thought that effective suppression of immune responses in vivo by Tregs requires that cells migrate to the appropriate sites, respond to Ag, and survive long term. The effectiveness of Ag-specific Tregs has been recently highlighted and shown to provide a means for an effective therapeutic approach (28, 29). We show here that CD4⁺CD25⁺ T cells from TCR-transgenic BDC2.5.NOD mice were highly effective in suppression of T1D when given 24 h after CY treatment. As few as 1 × 10⁴ BDC2.5.NOD Treg cells were sufficient to prevent CY-mediated T1D. Tang et al. (47) have argued that BDC2.5.NOD Tregs exert their regulatory function by interaction with dendritic cells in the PLN which subsequently prevents the priming of naive autoreactive T cells. To assess in vivo changes of CY-treated mice that accompanied injection of BDC2.5.NOD Tregs, we examined lymphoid tissues and pancreases of NOD recipients for T cell activation markers in the presence or absence of GFP-labeled BDC2.5.NOD CD4⁺CD25⁺ Tregs. Analysis of lymphoid tissues 6 days after CY treatment suggests that all affected lymphocyte activation marker levels remain low. Priming of autoreactive T cells may not have occurred to any significant extent in the PLN before this time point, which would explain why the transfer of BDC2.5.NOD Tregs 5 days after CY treatment is still able to suppress T1D. The lack of protection by adoptive transfer of BDC2.5.NOD Tregs 8 days after CY administration supports the notion that suppression of priming of autoreactive T cells is necessary to prevent infiltration of pancreatic islets by activated autoreactive T cells. In contrast, spontaneous T1D onset can be reversed when BDC2.5.NOD Tregs are given up to 1 wk after the first signs of glycosuria (28). Our studies indicate that in the CY-accelerated model of T1D BDC2.5.NOD Tregs are only able to control disease if given before diabetes onset. Interestingly, analysis of pancreases from protected mice suggests that although autoreactive T cells are still present within pancreatic infiltrates they may be held in check by the greater proportion of CD4⁺Foxp3⁺ T cells. The substantial proportion of GFP⁺ Tregs within the pancreatic Treg pool 1 mo after CY administration also implies their long-term survival. It remains to be established how resident Tregs mediate their inhibitory function in situ. In this context, it is interesting to note that in vitro-conditioned BDC2.5 Tregs control pancreatic autoreactive T cells by deletion through Fas-FasL interaction (48).

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Disclosures

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